Koneman's

COLOR ATLAS & TEXTBOOK OF Diagnostic Microbiology

SEVENTH EDITION

Gary W. Procop
Deirdre L. Church
Geraldine S. Hall
William M. Janda
Elmer W. Koneman
Paul C. Schreckenberger
Gail L. Woods

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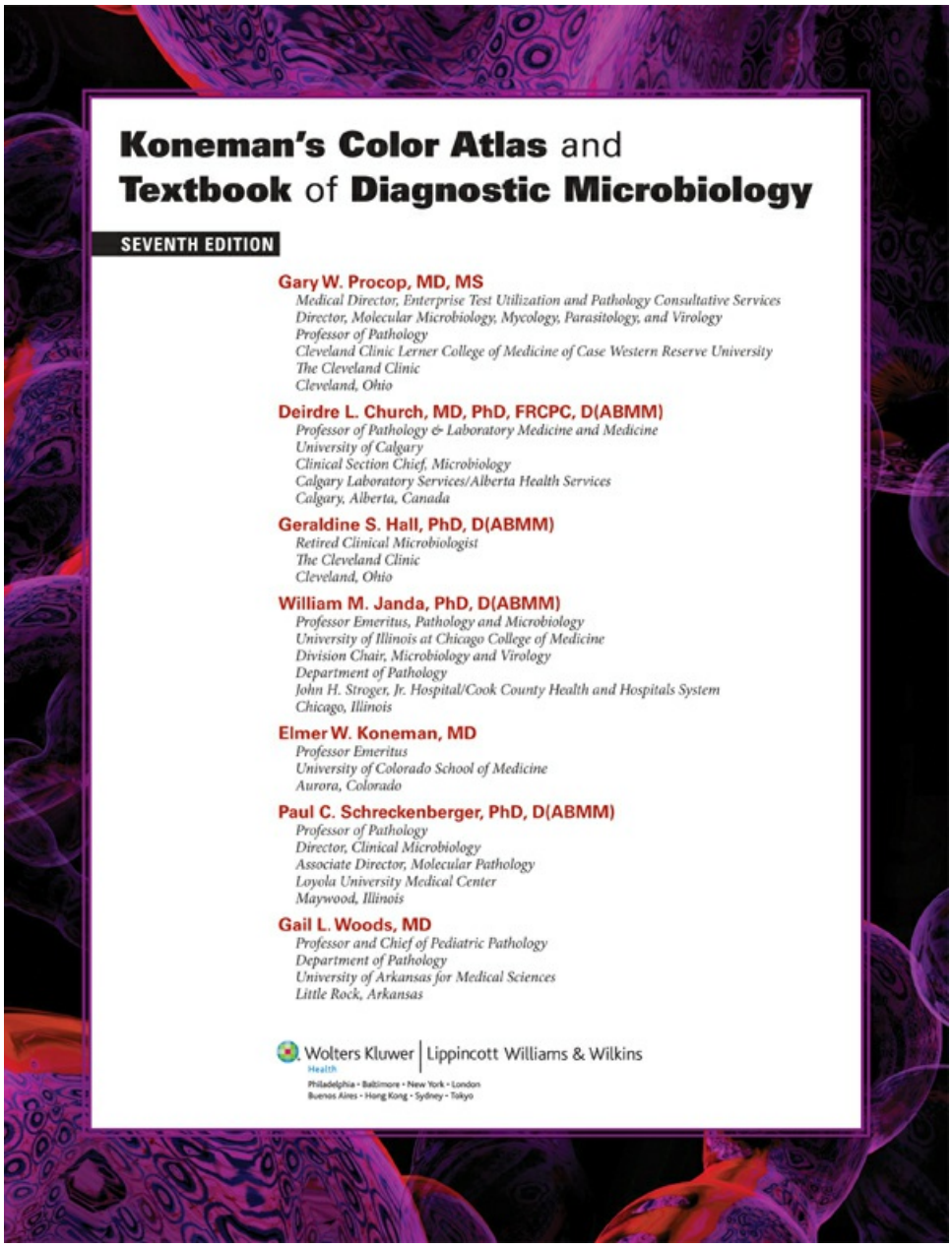
Koneman's Color Atlas and
Textbook of Diagnostic Microbiology
SEVENTH EDITION
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In Remembrance of Our Former Colleagues and Co-Authors

Wash —*We particularly remember your leadership in the College of American Pathology and as the editor-in-chief of the sixth edition of this text. You are sorely missed.*

Steve —*We particularly remember your leadership on the American Board of Pathology and in Infectious Disease Pathology. You are sorely missed.*

Gerri —*We particularly remember you as the consummate educator, whom all the students loved, and for your leadership in the American Society for Microbiology and other national organizations. You, too, are sorely missed, and this edition of this text would not have been possible without you - thanks.*

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To Tamera and London—the best wife and son a guy could have. The Best of the Best!

Gary W. Procop, MD

To my husband Gord and family for all their support

Deirdre L. Church, MD, PhD

Thanks for the support of my husband

Geraldine S. Hall, PhD

To my parents, Robert and Geraldine, my brothers, Robert and Martin, and to Matthew, my life partner.

William M. Janda, PhD

In recognition of the ongoing hard work and dedication of microbiology technologists

Elmer W. Koneman, MD

I thank my wife Ann for her immense support and patience during the prolonged writing phase of this work and for her 45-plus years of steadfast love and encouragement. I also thank my son Adam for his creation of the Web ID Program used for identification of bacteria, which is described in detail in Chapters 6 and 7 of this text.

Paul C. Schreckenberger, PhD

In loving memory of my father

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Foreword

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The diagnostic process for infectious diseases is complex. An astute clinician may be able to make a clinical diagnosis based on the history, presenting features, physical examination, and epidemiologic exposures, including relevant travel. For example, pneumonia is a clinical diagnosis albeit with radiographic confirmation. Establishing an etiologic diagnosis may be much more difficult and is dependent on obtaining a relevant specimen of interpretable quality, swift reliable transport, and the testing repertoire of the supporting clinical microbiology laboratory. Moreover, presenting features of different causative agents often overlap and an infectious process may involve multiple organ systems, thereby adding further complexity to the task of establishing an etiologic diagnosis on which specific antimicrobial therapy depends. Indeed without an accurate and timely etiologic diagnosis, empirical treatment ensues or is prolonged, which may consist of multiple antimicrobial agents, those with unnecessarily broad activity, or both. This increases the risk of adverse drug reactions, alters the patient's microbiome, and promotes selection of resistant mutants that indirectly put other patients at risk. Such collateral damage emphasizes only the need for rapid and accurate tests wisely deployed for the optimal outcome of patient care.

Thus, the founding objective of Koneman's *Color Atlas and Textbook of Diagnostic Microbiology* was to provide a clear exposition of procedures routinely employed in the laboratory identification of microbial agents causing infectious diseases. Since its first edition in 1979, the recognition and emergence of new infectious agents; better tools for their microbiologic diagnosis; more immunocompromised patients due to organ and stem cell transplantation, chemotherapy of malignancies, and immune-modulating agents; and a changing environment both of care and exposures have vastly complicated the quest for an etiologic diagnosis. These transformative developments have only accelerated since the sixth edition in 2006.

Fortunately, the tools available for the challenges at hand have also expanded and improved. Quantitative viral load determinations have become essential for care of

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patients with HIV and hepatitis C virus infections. Many other molecular test procedures are now commonplace in diagnostic microbiology laboratories large and small. The identification of microorganisms has been transformed by the introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Increasing advanced next-generation sequencing strategies are being deployed for microbial agents that have eluded diagnosis in the past or remain difficult to identify. Concurrently, traditional growth and isolation techniques remain important for many, if not most, bacterial, fungal, and mycobacterial agents and are required for reliable antimicrobial susceptibility testing given the growing threat of multidrug-resistant microorganisms. Recovery and testing is also crucial for infection control measures and for public health surveillance. As a result, now more than ever, the invaluable contribution of medical microbiology lies in providing, fostering, and promoting a proper balance between the science of the possible with the art of the appropriate. This is a professional team endeavor incumbent upon all entrusted with the stewardship of finite resources.

Therefore, it is fortuitous that Dr. Gary W. Procop now assumes the role of editor-in-chief for the seventh edition of *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, owing to his many contributions to the field in molecular microbiology, medical mycology, the histopathology of infectious diseases, and his leadership in advancing appropriate testing strategies for improved patient outcomes wherein true value lies. He and his fellow cadre of contributors are admirably positioned by virtue of knowledge, training, and experience in the field to not only maintain but also to augment the usefulness of this edition for all those who might benefit from its content: medical microbiologists, infectious disease specialists, pathologists, and clinical laboratory scientists.

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Preface

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The seventh edition of this text represents a comprehensive update to our audience of the increasingly complex and challenging realm of diagnostic microbiology. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology* is in recognition of Elmer W. Koneman as one of the founding authors of this classic text and a driving force behind publication of the first six editions. Dr. Koneman has continued to provide guidance and editorial support, so his expertise remains an important part of this seventh edition.

The seventh edition welcomes back long-time authors Drs. Paul C. Schreckenberger and William (Bill) M. Janda, who share their in-depth expertise of bacteriology. Dr. Gail Woods also returns to share her world-renowned expertise in mycobacteriology. This edition welcomes Dr. Deirdre Church, a premiere clinical microbiologist and infectious diseases clinician, and Dr. Gerri Hall, who shares her in-depth expertise in anaerobic bacteriology, mycoplasmas, and aerobic actinomycetes. It is a pleasure to join this august company in the production of this next edition of *Koneman's Color Atlas and Textbook of Diagnostic Microbiology.*

There have been substantial advances in clinical microbiology since the last edition of this textbook, with which the authors were challenged to incorporate in clinically meaningful ways. Mass spectrometry, commercially available highly multiplexed syndrome-based PCR assays, and next-generation sequencing were research tools at best when the last edition was published. Some of these, such as MALDI-TOF mass spectrometry and user-friendly nucleic acid amplification tests, are becoming commonplace in even smaller clinical laboratories. It is clear, however, that all laboratories have not adopted these technologies, and even if they desire to do so, the implementation rate is not uniform. Therefore, we were also challenged to retain traditional methods of organisms' detection and identification that are still used in many laboratories, while also including new and advanced methods. This edition attempts to bridge the divide between the traditional and the newer advanced methods that are being employed. It is for this reason that some traditional tests, which will likely become entirely replaced by newer methods, are in part retained. We have

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recommended previous editions for those assays and methods that are more universally considered antiquated.

The general organization of the text remains similar to the previous edition, but all sections have been significantly updated. The first two chapters are an introduction to clinical microbiology. Associated image plates for these chapters include artifacts in Gram-stained smears, emphasizing the importance of knowing what is not real, as well as the morphology of microorganisms. The expanded discussion of management, quality, compliance, and regulatory issues in the diagnostic microbiology laboratory confers necessary knowledge for those in the modern medical practice. Additionally, we have attempted to provide guidance to questions that clinical microbiologists face daily, such as: What do we work up, when do we work it up, and how extensively do we work it up? These challenges have become as important as the more traditional scientific issues confronted in the clinical laboratory, given the ever-dwindling financial and personnel resources.

The traditional techniques that are still important for clinical laboratories receive appropriate attention in this edition, but the increasing importance of immunologic methods (Chapter 3) and particularly molecular techniques (Chapter 4) justifies explicit discussion of principles, as well as expanded coverage in individual chapters as appropriate. Where immunologic and/or molecular approaches have become the norm in diagnostic laboratories, the text has been updated to reflect these changes. Discussions of methods that are obsolete or rapidly becoming archaic have been eliminated or significantly condensed.

The introduction to bacteriology, still the lynchpin of clinical laboratories, follows as Chapter 5 and sets the stage for the subsequent chapters in this vast field. These chapters are followed by those addressing mycobacteria fungi, parasites, and viruses and other intracellular pathogens. The increasing frequency with which ectoparasites are submitted to diagnostic laboratories for identification has been acknowledged with an expanded discussion of tick identification in an appendix, including several image plates to aid in the appropriate identification of these organisms.

In an era of rapid changes in diagnostic methods, the goal of the text remains the same: to provide an extensive but practical discussion of the science and art of diagnostic microbiology. We strongly believe that it is essential to integrate the clinical issues with the practice of laboratory medicine, so as to provide the information that our clinical colleagues need to best serve the patients. This integration expands the role of the microbiologists and removes them from a position in a basement somewhere and from behind a microscope. Although our skills as expert microbiologists must be retained and sharpened, additional value is gained at the health system level when microbiologists and other laboratorians participate on clinical practice committees and help to determine the optimal use of laboratory resources. We are of immense value to our institutions, but too often remain an unrecognized and untapped resource of the healthcare delivery team. Emerge from the laboratory and let your value be known,

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with service to the patient being the driver of all these efforts.

There are two intended audiences for this text. The first group consists of students and instructors of microbiology and medicine, particularly those interested in infectious diseases. The book provides a comprehensive review for graduate students, residents in pathology, and fellows in medical microbiology and infectious diseases. For undergraduate students, the volume of material may appear overwhelming, but this denotes the depth and complexity of our field. It is hoped that this depth and complexity will inspire rather than deter, providing a view into a career option that will remain ever evolving, challenging, and interesting. It is hoped that

the dedicated teachers of our craft will find ample and updated material to guide the beginning student, who will then have a resource to carry on into the graduate environment or the workplace, rather than a superficial and outmoded introduction for the bookshelf. The second, equally important audience, is laboratory professionals for whom the text can represent the initial resource for updating competency or troubleshooting problems in clinical practice. To facilitate understanding of the subject matter, we continue to employ tables, detailed charts, summary text boxes, and extensive color illustrations. Each chapter begins with a detailed outline, which represents the chapter at a glance. Charts can be found online at http://thepoint.lww.com/

We are all greatly indebted to a variety of mentors, colleagues, and students who have challenged and inspired us. We are particularly indebted to Drs. L. Barth Reller and Glenn D. Roberts, who generously collaborated to contribute a Foreword for this edition. We also thank Dr. Glenn D. Roberts for contributing many of the images for the mycology chapter. Two of our former co-authors, the late Dr. Washington C. Winn Jr and the late Dr. Stephen D. Allen, and one of the current authors, the late Dr. Gerri Hall, are very much in our memory as we carry the torch forward that they have borne. We could not have done this without the contributions of Dr. Hall to the current edition and the fundamental work of Drs. Winn and Allen in the previous editions. To recognize their contributions, we dedicate this edition to their memory.

Acknowledgments

We are, first of all, indebted to our colleagues in the microbiology laboratories at our respective institutions for their important roles in our professional life. They have challenged us, inspired us, and educated us. We hope this book serves to repay their

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contributions in some small part. Additionally, we are grateful to the members of our families for their forbearance while we struggled to meet deadlines. Their support and encouragement at home are integral to our activities at work.

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BBL Crystal Enteric/Nonfermenter ID System

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RapID onE System
Biolog GN2 Microplate
Microscan System
Sensititre System
Semiautomated and Automated Identification Systems
MicroScan Walkaway
Vitek System
Sensititre Gram-Negative AutoIdentification System
The Phoenix System
The OmniLog ID System
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrophotometry

CHAPTER 7 The Nonfermentative Gram-Negative Bacilli

Introduction to Nonfermenting Gram-Negative Bacilli
Fermentative and Oxidative Metabolism
The Embden–Meyerhof–Parnas Pathway
The Entner–Doudoroff Pathway
The Warburg–Dickens Hexose Monophosphate Pathway
Initial Clues That an Unknown Isolate Is a Nonfermenter
Lack of Evidence for Glucose Fermentation
Positive Cytochrome Oxidase Reaction
Failure to Grow on MacConkey Agar
Tests Used in the Identification of Nonfermenters
Utilization of Glucose
Motility
Pigment Production
Hydrolysis of Urea
Nitrate Reduction
Denitrification of Nitrates and Nitrites
Indole Production
Decarboxylation
Esculin Hydrolysis
Flagella Stains
Leifson Method
Ryu Method
Wet-Mount Technique
Flagellar Morphology

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Organisms That Are Motile With Polar Flagella

- Pseudomonads
  - Family *Pseudomonadaceae*—rRNA Group I
    - Genus *Pseudomonas*
  - Family *Burkholderiaceae*—rRNA Group II
    - Genus *Burkholderia—Pseudomallei* Group
    - Genus *Ralstonia* and Genus *Cupriavidus*
    - Genus *Lautropia*
    - Genus *Pandoraea*
  - Family *Rhodospirillaceae*
    - Genus *Inquilinus*
  - Family *Comamonadaceae*—rRNA Group III
    - *Acidovorans* Group
    - Genus *Delftia*
    - Genus *Comamonas*
    - *Facilis–Delafieldii* Group
  - Family *Caulobacteraceae*—rRNA Group IV
    - Genus *Brevundimonas*
  - Family *Xanthomonadaceae*—rRNA Group V
    - Genus *Stenotrophomonas*
    - Genus *Wohlfahrtiimonas*
  - Family *Acetobacteraceae*
    - Genus *Acetobacter*
    - Genus *Acidomonas*
    - Genus *Gluconobacter*
    - Genus *Granulibacter*

Yellow-Pigmented Group

- Family *Pseudomonadaceae*
  - Genus *Pseudomonas*
- Family *Sphingomonadaceae*
  - Genus *Sphingomonas*
- Family *Oceanospirillaceae*
  - Genus *Balneatrix*
- Family *Oxalobacteraceae*
  - Genus *Massilia*
- Unnamed Species
  - CDC Groups O-1, O-2
  - *Agrobacterium* Yellow Group

Curved Rod Group

- Family *Caulobacteraceae*
  - Genus *Caulobacter*

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Family *Oxalobacteraceae*
Genus *Herbaspirillum*

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Family *Neisseriaceae*
Genus *Laribacter*
CDC Group O-3
Halophilic and/or Hydrogen Sulfide-Positive Pseudomonads
Family *Shewanellaceae*
Genus *Shewanella*
Family *Alteromonadaceae*—Halophilic Group
Genus *Alishewanella*
Family *Halomonadaceae*
Genus *Halomonas*
Pink-Pigmented Group
Family *Methylobacteriaceae*
Genus *Methylobacterium*
Family *Acetobacteraceae*
Genus *Roseomonas* and Genus *Azospirillum*
Genus *Asaia*
Unnamed Species
Pseudomonas-Like Group 2
CDC Group 1c
CDC Group WO-1
OFBA-1
Organisms That Are Motile With Peritrichous Flagella
Family *Alcaligenaceae*
Genus *Alcaligenes*
Genus *Achromobacter*
Genus *Advenella*
Genus *Bordetella*
Genus *Kerstersia*
Genus *Oligella*
Family *Rhodobacteriaceae*
Genus *Pannonibacter* and *Achromobacter* Groups A–F
Family *Rhizobiaceae*
Genus *Rhizobium* (Formerly *Agrobacterium*)
Family *Brucellaceae*
Genus *Ochrobactrum*
Organisms That Are Nonmotile and Oxidase Positive

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Family *Flavobacteriaceae*
Genus *Chryseobacterium*
Genus *Elizabethkingia*
Genus *Empedobacter*
Genus *Weeksella* and Genus *Bergeyella*
Genus *Myroides*
Unnamed CDC Groups IIc, IIe, IIg, IIh, and IIi
Family *Sphingobacteriaceae*
Genus *Sphingobacterium*
Family *Moraxellaceae*
Genus *Moraxella*
Genus *Psychrobacter*
Family *Neisseriaceae*
Genus *Neisseria*
Unnamed Species
Gilardi Rod Group 1
Family *Rhodobacteriaceae*
Genus *Paracoccus* and CDC Eugonic Oxidizer (EO) Groups
Genus *Haematobacter*
Organisms That Are Nonmotile and Oxidase Negative
Family *Moraxellaceae*
Genus *Acinetobacter*
Family *Alcaligenaceae*
Genus *Bordetella*
Unnamed Species
CDC Group NO-1
CDC Group EO-5
Levels of Service in Identification of Nonfermenters
Guidelines for Recovery of Nonfermenters
Identification of the Most Common Species
Pseudomonas aeruginosa
Acinetobacter baumannii
Stenotrophomonas maltophilia
Methods for Identification Using Conventional Biochemical Tests
CDC Scheme—Weyant and Associates
Practical Approach to Identification of Nonfermenters—Schreckenberger
Computer-Aided Schemes
ASHEX Web ID Program
Methods for Identification Using Commercial Kit Systems
The API 20E System
The API 20NE System

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The Crystal Enteric/Nonfermenter System
The RapID NF Plus System
The Biolog System
Methods for Identification Using Automated Identification Systems
The Vitek Legacy System
The Vitek 2 System
The Microscan Walkaway-96, Walkaway-40, and Autoscan-4 Systems
The Sensititre AP80 System
The Phoenix System
Methods for Identification Using Molecular Systems
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
16S rRNA Gene Sequencing
Resolution of 16S rRNA Gene Sequencing
Selection of a System

CHAPTER 8 Curved Gram-Negative Bacilli and Oxidase-Positive Fermenters

Historical Background
Classification of *Campylobacter* and Related Taxa
Genus *Campylobacter*
Campylobacter jejuni subsp *jejuni*
Other *Campylobacter* Species
Genus *Arcobacter*
Arcobacter butzleri
Arcobacter cryaerophilus
Arcobacter skirrowii
Genus *Helicobacter*

Helicobacter pylori
Culture and Isolation of *H. pylori*
Identification of *H. pylori*
Other Medically Important *Helicobacter* Species
Other Microaerophilic Gram-Negative Bacilli
Sutterella wadsworthensis
Definitive Identification of Campylobacters and Related Bacteria
Rapid Identification of Campylobacters From Colonies Grown in Culture

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Morphologic Identification
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
Commercial Kit Systems
Methods for Direct Detection of Campylobacters From Stool
Direct Immunofluorescence
Enzyme Immunoassay
PCR
Phylogeny of the *Vibrionaceae*
Genus *Vibrio*
Taxonomy
Description and Associated Clinical Syndromes of *Vibrio* Species of Human Importance
Methods for Laboratory Isolation of *Vibrios*
Biochemical Characterization and Laboratory Identification of *Vibrio* Species
Listonella, Photobacterium*, and *Shewanella
Aeromonas* and *Plesiomonas
Genus *Aeromonas*
Taxonomy
Clinical Significance
Aeromonas Species in Medicinal Leeches
Laboratory Recovery of *Aeromonas* Species From Clinical Specimens
Laboratory Identification of *Aeromonas* Species
Genus *Plesiomonas*
Clinical Significance
Laboratory Isolation and Identification
Genus *Chromobacterium*

CHAPTER 9 Miscellaneous Fastidious Gram-Negative Bacilli

Introduction to the Family *Pasteurellaceae*
***Haemophilus* Species**
Taxonomy
Haemophilus influenzae
Haemophilus Influenzae Type b Vaccines and Immunity
Other *Haemophilus* Species
Haemophilus ducreyi
Laboratory Diagnosis of *Haemophilus* Infections
Direct Examination of Clinical Specimens
Recovery of *Haemophilus* Species in Culture

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Identification of *Haemophilus* Species
Haemophilus ducreyi: Laboratory Diagnosis of Chancroid
Specimen Collection and Direct Examination
Culture
Antimicrobial Susceptibility of *Haemophilus* Species
Genus *Aggregatibacter*
Taxonomy
Clinical Significance
Aggregatibacter aphrophilus
Aggregatibacter segnis
Aggregatibacter actinomycetemcomitans
Cultural Characteristics and Identification of *Aggregatibacter* Species
Antimicrobial Susceptibility of *Aggregatibacter* Species
***Cardiobacterium* Species**
Taxonomy
Clinical Significance
Cultural Characteristics and Identification
Antimicrobial Susceptibility
Eikenella corrodens
Taxonomy
Clinical Significance
Cultural Characteristics and Identification
Antimicrobial Susceptibility
***Kingella* Species**
Taxonomy
Clinical Significance
Cultural Characteristics and Identification
Antimicrobial Susceptibility
***Capnocytophaga* Species**
Taxonomy
Clinical Significance
Cultural Characteristics and Identification
Antimicrobial Susceptibility
***Dysgonomonas* Species**
Taxonomy
Clinical Significance
Cultural Characteristics and Identification
Antimicrobial Susceptibility
Streptobacillus moniliformis
Taxonomy
Epidemiology

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Clinical Significance
Cultural Characteristics and Identification
Antimicrobial Susceptibility
***Simonsiella* Species**
***Pasteurella* and *Mannheimia* Species**
Taxonomy and Characteristics of the Genus *Pasteurella*
Reclassification of Other *[Pasteurella]* Species
Pasteurella Species: Clinical Significance, Identification, and Antimicrobial Susceptibility
Pasteurella multocida
Pasteurella dagmatis, Pasteurella canis, and *Pasteurella stomatis*
[Pasteurella] Species Incertae Sedis: Clinical Significance, Identification, and Antimicrobial Susceptibility
[Pasteurella] aerogenes
[*Pasteurella*] *pneumotropica*
[*Pasteurella*] *bettyae*
[*Pasteurella*] *caballi*
Avibacterium ([*Pasteurella*]) *gallinarum*
Mannheimia Species (Formerly the "*Pasteurella haemolytica/Pasteurella granulomatis*" Complex)

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Genus *Actinobacillus*
Taxonomy
Clinical Significance of *Actinobacillus* Species
Actinobacillus ureae
Actinobacillus hominis
Other *Actinobacillus* Species
Cultural Characteristics of *Actinobacillus* Species
***Brucella* Species**
Introduction
Taxonomy and Epidemiology
Virulence of *Brucella* Species
Clinical Spectrum of Brucellosis
Serologic Diagnosis of *Brucella* Infections
Isolation and Cultural Characteristics
Identification of *Brucella* Species
Treatment of Brucellosis
***Francisella* Species**
Epidemiology of Tularemia

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History and Taxonomy
Clinical Spectrum of Tularemia
Detection, Isolation, and Cultural Characteristics
Treatment of Tularemia
Virulence of *F. tularensis*
Afipia* Species, *Bosea* Species, and Other α-*Proteobacteria
***Bartonella* Species**
Taxonomy
Epidemiology of *Bartonella* Species
Human Infections Associated With *Bartonella* Species
Oroya Fever and Verruga Peruana
"Classical" and "Urban" Trench Fever
Bacillary Angiomatosis
Peliosis
Fever and Bacteremia
Endocarditis
Cat Scratch Disease
Miscellaneous Infections
Emerging Pathogens in the Genus *Bartonella*
Detection, Isolation, and Identification of *Bartonella* Species
Specimen Types
Microscopic Examination of Clinical Specimens
Culture
Gram's Stain and Colony Morphology
Identification Methods
Serologic Diagnosis
In Vitro Antimicrobial Susceptibility
***Bordetella* Species**
Background and Taxonomy of *Bordetella* Species
Epidemiology of Pertussis
Clinical Significance of *Bordetella pertussis*
Pertussis Vaccines
Clinical Significance of Other *Bordetella* Species
Bordetella parapertussis
Bordetella bronchiseptica
Bordetella avium
Bordetella hinzii
Bordetella holmesii
Bordetella trematum
Bordetella petrii
Bordetella ansorpii

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Culture and Isolation of *Bordetella pertussis*
Direct FA Test
Cultural Characteristics and Identification of *Bordetella* Species
Molecular Methods for Detection and Identification of *Bordetella* Species
Serologic Tests for Diagnosis of Pertussis
Treatment of *Pertussis*
Antimicrobial Susceptibility Testing of *Bordetella* Species

CHAPTER 10 *Legionella*

Introduction
Taxonomy and Characteristics of the Genus *Legionella*
Clinical and Pathologic Spectrum of Legionellosis
Predisposing Factors
Pathology and Pathogenesis
Epidemiologic and Ecologic Aspects of Legionellosis
Incidence
Legionellaceae in the Environment
Natural Habitats
Aquatic Habitats Made by Humans (Artificial)
Legionellosis in Travelers
Nosocomial Outbreaks of Legionellosis
Laboratory Diagnosis
Selection, Collection, and Transport of Clinical Specimens
Direct Examination of Clinical Specimens
Gross Examination
Microscopic Examination
Legionella PCR and Other Molecular Methods
Direct Fluorescent Antibody Testing
Legionella Antigen Detection
Detection of *Legionella* in Clinical Specimens
Isolation of *Legionella* Species From Clinical Specimens
Tissue Specimens
Pleural Fluid and Transtracheal Aspirates
Acid-Wash Decontamination Procedure
Blood Cultures
Identification of *Legionella* Species
Antimicrobial Susceptibility and Treatment
Serologic Studies for *Legionella*

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Environmental Microbiology Studies
Molecular Detection of *Legionella* in Environmental Samples
Isolation and/or Detection of *Legionella* in Environmental Samples
Typing of *Legionella* Isolates

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CHAPTER 11 *Neisseria* Species and *Moraxella catarrhalis*

Introduction
Taxonomy of the Family *Neisseriaceae* and the Family *Moraxellaceae*
General Characteristics of the Genus *Neisseria*
Clinical Significance of *Neisseria* Species
Neisseria gonorrhoeae
Epidemiology
Infections Caused by *N gonorrhoeae*
Neisseria meningitidis
Meningococcal Epidemiology: Serogroups, Serotypes, and Serosubtypes
Carriage of *N. meningitidis*
Infections Caused by *N. meningitidis*
Meningococcal Prophylaxis and Meningococcal Vaccines
Other *Neisseria* Species
Clinical Significance of *Moraxella catarrhalis*
Isolation of *Neisseria* Species
Neisseria gonorrhoeae
Direct Gram-Stained Smears
Specimen Collection and Transport
Selective Culture Media: Inoculation and Incubation
Neisseria meningitidis
Specimen Collection and Transport
Laboratory Safety
Direct Gram-Stained Smears and Direct Capsular Antigen Tests
Isolation and Incubation
Identification of *Neisseria* Species
Colony Morphology
Gram Stain and Oxidase Test
Superoxol Test
Differentiation of Other Organisms on Selective Media
Presumptive Criteria for Identification of *N. gonorrhoeae*

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Identification Tests for *Neisseria* Species
Carbohydrate Utilization Tests
Conventional CTA Carbohydrates
Rapid Carbohydrate Utilization Test
CarboFerm *Neisseria* Test
Other Carbohydrate Utilization Methods
Chromogenic Enzyme Substrate Tests
Gonochek II
BactiCard *Neisseria*
Immunologic Methods for Culture Confirmation of *N. gonorrhoeae*
Coagglutination Test
GonoGen II Test
Multitest Identification Systems
DNA Probe Test for Culture Confirmation of *N. gonorrhoeae*
Nucleic Acid Hybridization Tests for *N. gonorrhoeae*
Nucleic Acid Amplification Tests for *N. gonorrhoeae*
MALDI-TOF for Identification of *Neisseria* Species
Sexual Abuse/Assault and *N. gonorrhoeae*
Typing Methods for *N. gonorrhoeae*
Molecular Methods for Detection of *N. meningitidis*
Serogrouping and Typing of *N. meningitidis*
Cultural Characteristics of Other *Neisseria* Species
N. lactamica
N. cinerea
Neisseria subflava biovars, *N. mucosa*, and *N. sicca*
N. polysaccharea
N. flavescens
N. elongata Subspecies
N. weaveri
N. bacilliformis
N. animaloris and *N. zoodegmatis*
N. wadsworthii
N. shayeganii
Cultural Characteristics and Identification of *Moraxella catarrhalis*
Antimicrobial Susceptibility of *Neisseria* Species
N. gonorrhoeae
N. meningitidis
Antimicrobial Susceptibility of *Moraxella catarrhalis*

CHAPTER 12 Gram-Positive Cocci

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Part I Staphylococci and Related Gram-Positive Cocci

Taxonomy of Staphylococci and Related Gram-Positive Cocci
Clinical Significance of Staphylococci and Related Gram-Positive Cocci
Staphylococcus aureus subsp. *aureus* (Herein Called *S. aureus*)
Staphylococcus aureus subsp. *anaerobius*
Coagulase-Negative Staphylococci
Staphylococcus epidermidis
Staphylococcus saprophyticus subsp. *saprophyticus*
Other Coagulase-Negative Staphylococci
Micrococcus Species and Related Genera
Rothia mucilaginosa
Isolation and Preliminary Differentiation of Staphylococci and Related Gram-Positive Cocci
Direct Gram-Stained Smears
Isolation From Clinical Specimens
Colony Morphology
The Catalase Test
Methods for Differentiating Micrococci and Staphylococci
Fermentation of Glucose
Susceptibility to Lysostaphin
Production of Acid From Glycerol in the Presence of Erythromycin

Susceptibility to Furazolidone
Modified Oxidase Test
Susceptibility to Bacitracin
Coverslip Growth Method
Methods for Direct Detection of MRSA in Clinical Specimens
Chromogenic Media for Surveillance of MRSA Colonization
Molecular Methods for Surveillance of MRSA Colonization
Molecular Methods for Detection of MRSA and MSSA in Blood Cultures and Skin/Soft Tissue Infections
Identification of *Staphylococcus aureus*
Slide Coagulase Test
Tube Coagulase Test
Alternative Coagulase Test Procedures
Agglutination Tests
Additional Confirmatory/Identification Tests for *Staphylococcus aureus*

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Deoxyribonuclease Test
Thermostable Endonuclease Test
Mannitol Fermentation
AccuProbe *Staphylococcus aureus*
Rapid Tests for Detection of Methicillin Resistance
Genotype MRSA
Identification of Coagulase-Negative Staphylococci
Conventional Identification Methods
Pyrrolidonyl Arylamidase Activity
Susceptibility to Polymyxin B
Ornithine Decarboxylase Test
Urease Production
Acetoin Production
Susceptibility to Novobiocin for Presumptive Identification of *Staphylococcus saprophyticus*
Commercial Identification Systems
Automated Identification Systems
Manual Identification Systems
Staphylococcus aureus/Coagulase-Negative Staphylococci PNA FISH Culture Confirmation Kit (AdvanDx, Woburn, MA)
Molecular Identification and Typing Methods for Staphylococci
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
Identification of *Micrococcus* and Related Species
Identification of *Rothia mucilaginosa*
Laboratory Approach to the Identification of Staphylococci

CHAPTER 13 Gram-Positive Cocci

Part II Streptococci, Enterococci, and the "*Streptococcus*-Like" Bacteria

General Characteristics of the Streptococci
Group A β-Hemolytic Streptococci (*Streptococcus pyogenes*)
Virulence Factors
Clinical Spectrum of Group A Streptococcal Disease
Group B β-Hemolytic Streptococci (*Streptococcus agalactiae*)
Virulence Factors
Clinical Spectrum of Group B Streptococcal Disease
Prevention of Group B Streptococcal Disease

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Other Infections Caused by Group B Streptococci
Antimicrobial Susceptibility of Group B Streptococci
Group C and Group G β-Hemolytic Streptococci
Group F β-Hemolytic Streptococci
Emerging Pathogens Among the Streptococci
Streptococcus suis
Streptococcus porcinus and *Streptococcus pseudoporcinus*
Streptococcus iniae
Streptococcus pneumoniae
Virulence Factors
Pneumococcal Vaccines
Clinical Spectrum of *S. pneumoniae*
Antimicrobial Susceptibility of *S. pneumoniae*
Viridans Streptococci
The Anginosus Group: *Streptococcus anginosus, Streptococcus constellatus*, and *Streptococcus intermedius*
Group D Streptococci: The "*Streptococcus bovis*" Group
***Enterococcus* Species**
Taxonomy
Virulence Factors
Clinical Spectrum of Enterococcal Infections
Antimicrobial Susceptibility of Enterococci
Genus *Melissococcus* and Genus *Catellicoccus*
The "*Streptococcus*-Like" Bacteria
Abiotrophia and *Granulicatella* Species
Aerococcus and *Helcococcus* Species
Leuconostoc Species
Pediococcus and *Tetragenococcus* Species
Gemella Species
Vagococcus Species
Alloiococcus Species
Globicatella Species
Facklamia Species
Dolosigranulum, *Ignavigranum*, *Dolosicoccus*, and *Eremococcus* Species
Lactococcus Species
Isolation and Identification of Streptococci and the "*Streptococcus*-Like" Bacteria
Direct Gram-Stained Smears
Culture Media
Hemolysis on Blood Agar
Nonculture, Direct Detection Techniques for Group A *β*-Hemolytic Streptococci in

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Pharyngeal Specimens
Nonculture, Direct Detection Techniques for Group B β-Hemolytic Streptococci

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Nonculture, Direct Detection Techniques for *Streptococcus pneumoniae*
Nonculture Techniques for the Detection of Enterococci in Blood Cultures
Colony Morphology and Catalase Testing
Recognition and Preliminary Characterization of Streptococci and the "*Streptococcus*-Like" Bacteria
Presumptive Identification of Streptococci and Enterococci
Susceptibility to Bacitracin
Susceptibility to Sulfamethoxazole-Trimethoprim (SXT)
CAMP Test and Pigment Production
Hydrolysis of Sodium Hippurate
Bile Esculin Test
Salt Tolerance Test (65% NaCl Broth)
Leucine Aminopeptidase (LAP) Test
Pyrrolidonyl Arylamidase (PYR) Test
Commercial Presumptive Identification Tests
Serologic Identification of β-Hemolytic Streptococci
Capillary Precipitin Test
Coagglutination
Latex Agglutination
Phenotypic Characteristics for Identification of Groupable Streptococci
Identification of *S. pneumoniae*: Susceptibility to Optochin, the Bile Solubility Test, and AccuProbe *Pneumococcus* Test
Serologic Identification of *Streptococcus pneumoniae*
Identification of the Viridans Streptococci
Mitis/Sanguinis Group
Mutans Group
Salivarius Group
Anginosus Group
Bovis Group
Identification of *Streptococcus suis* and Other Streptococci Isolated From Animals
Detection of Vancomycin-Resistant Enterococci
Identification of *Enterococcus* Species
Identification of *Abiotrophia* and *Granulicatella* Species
Identification of *Aerococcus* and *Helcococcus* Species
Identification of *Leuconostoc*, *Pediococcus*, and *Tetragenococcus* Species
Identification of *Gemella* Species

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Identification of *Vagococcus* Species
Identification of *Alloiococcus* Species, *Globicatella* Species, *Facklamia* Species, *Dolosigranulum* Species, *Ignavigranum* Species, and *Dolosicoccus* Species
Identification of *Lactococcus* Species
Commercial Systems for Identification of Streptococci, Enterococci, and Selected "*Streptococcus*-Like" Bacteria
Vitek 2
Phoenix (Becton-Dickinson Diagnostic Systems, Sparks, MD)

CHAPTER 14 Aerobic and Facultative Gram-Positive Bacilli

Listeria* Species and *Listeria monocytogenes
Taxonomy of the Genus *Listeria*
Virulence of *L. monocytogenes*
Epidemiology of *L. monocytogenes*
Clinical Significance of *L. monocytogenes*
Isolation of *L. monocytogenes* From Clinical Specimens
Identification of *Listeria* Species
Antimicrobial Susceptibility and Treatment of *Listeria* Infections
Pathogenicity of Other *Listeria* Species
***Erysipelothrix* Species**
Taxonomy of the Genus *Erysipelothrix*
Clinical Significance of *E. rhusiopathiae*
Isolation and Identification of *E. rhusiopathiae*
Antimicrobial Susceptibility of *E. rhusiopathiae*
***Bacillus* Species and Related Genera**
Taxonomy and the Taxonomic Dissection of Genus *Bacillus*
Bacillus anthracis
Epidemiology of Anthrax
Virulence Factors of *B. anthracis*
Clinical Presentations of Anthrax
Antimicrobial Susceptibility and Treatment of *B. anthracis* Infections
Prevention of Anthrax
Bacillus cereus
Virulence Factors of *B. cereus*
B. cereus Gastroenteritis
Opportunistic Infections Caused by *B. cereus*, Other *Bacillus* Species, and *Paenibacillus* Species
B. cereus Bacteremia and Endocarditis

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B. cereus Infections in Compromised Hosts
B. cereus Ocular Infections
B. cereus Skin, Bone, and Soft Tissue Infections
Nosocomial Infections
Laboratory Safety, Specimen Collection, and Processing
Isolation and Identification of the "*Bacillus cereus* Group"
Antimicrobial Susceptibility Testing of *Bacillus* Species
***Corynebacterium* Species**
Introduction and Taxonomy
Identification of *Corynebacterium* Species and Coryneform Bacteria
API Coryne
RapID CB-Plus
Vitek 2 Anaerobe/*Corynebacterium* Card
MALDI-TOF MS
Antimicrobial Susceptibility Testing of *Corynebacterium* Species and Coryneform Bacteria
Members of the Genus *Corynebacterium* Isolated From Humans
Corynebacterium diphtheriae

Epidemiology
Diphtheria Immunizations
Diphtheria: Pathogenesis and Clinical Presentation
Treatment of Diphtheria
Isolation and Identification of *Corynebacterium diphtheriae*
Antimicrobial Susceptibility of *Corynebacterium diphtheriae*
Reporting of *Corynebacterium diphtheriae*
Corynebacterium Species Associated With Animals and the Environment
Other Coryneform Bacteria
Actinotignum and *Actinobaculum* Species
Actinomyces Species
Arcanobacterium and *Trueperella* Species
Arthrobacter and Related Species
Brevibacterium Species
Cellulomonas, *Cellulosimicrobium*, and *Oerskovia* Species
Dermabacter and *Helcobacillus* Species
Exiguobacterium Species
Leifsonia Species
Microbacterium Species
Turicella Species

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Rothia Species
Gardnerella vaginalis
Taxonomy and Cellular Morphology
Clinical Significance of *Gardnerella vaginalis*
Diagnosis of BV and Cultural Characteristics of *Gardnerella vaginalis*
Antimicrobial Susceptibility of *Gardnerella vaginalis*
***Lactobacillus* Species**
Taxonomy and Epidemiology
Clinical Significance of *Lactobacillus* Species
Isolation and Identification of *Lactobacillus* Species
Antimicrobial Susceptibility of *Lactobacillus* Species
***Weissella* Species**

CHAPTER 15 Aerobic Actinomycetes

Introduction, Classification, and Taxonomy
The Nocardioform Group
Nocardia spp.
Non- *N. asteroides* Complex Species
Epidemiology, Pathology, and Pathogenesis
Clinical Disease
Rhodococcus spp.
Taxonomy and Classification
Epidemiology, Pathology, and Pathogenesis
Clinical Disease
Gordonia spp.
Taxonomy and Classification
Epidemiology, Clinical Disease, and Pathogenesis
Tsukamurella spp.
Taxonomy and Classification
Epidemiology and Clinical Disease
Dietzia spp.
Other Nocardioform Bacteria
The *Streptosporangineae*
Actinomadura spp.
Taxonomy and Classification
Epidemiology, Clinical Disease, and Pathology
Nocardiopsis spp.
The Streptomycetes

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Streptomyces spp.
Thermophilic Actinomycetes
Miscellaneous Actinomycetes
Dermatophilus
Tropheryma whipplei
History and Taxonomy
Ecology
Clinical Disease and Pathology
Laboratory Diagnosis of Infections Caused by Aerobic Actinomycetes
Primary Isolation
Differentiation of *Nocardia* From Other Genera of Aerobic Actinomycetes
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CHAPTER 1

1

Introduction to Microbiology

Part I: The Role of the Microbiology Laboratory in the Diagnosis of Infectious Diseases: Guidelines to Practice and Management

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Introduction

Outline of the Book

There are almost as many ways to look at the world of infectious disease as there are infectious agents. In this book, we will concentrate on the detection and identification of infectious agents in the clinical laboratory, followed by determination of susceptibility to antimicrobial agents if appropriate. Conceptually, the book is divided into three sections. The first section, which consists of two chapters, covers general principles of infectious diseases and laboratory diagnosis. In the second section, immunologic and molecular techniques that have virtually universal applicability are presented. Finally, the third and largest section consists of extensive discussion of groups of infectious agents and the diseases that they produce. The general principles of bacteriology are illuminated in a separate chapter because of the diversity of organisms in this large group of human pathogens.

The World of Infectious Disease

Throughout human existence, infectious diseases have been the predominant cause of illness and death, not only limiting improvements in personal comfort but also thwarting the societal development. The improvements in living conditions, sanitation, and medical intervention of the 20th century significantly diminished many infectious diseases. Unfortunately, these challenges remain in resource-limited countries throughout much of the world. The challenge remains for the world community to extend these achievements around the globe.

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By the 1950s, the successes of modern medicine and public health seemed so impressive that many prominent scientists were prompted to predict the conquest of infectious disease and the eradication of pestilence as a cause of misery from the face of the earth. William H. Stewart, the then Surgeon General of the United States, famously stated in 1969 “It is time to close the book on infectious diseases.” It is the misfortune of us all that those men greatly underestimated the adaptability of the multitudinous life forms that share Earth with us—both infectious agents and

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predatory ectoparasites, such as arthropods. Likewise, they could not foresee the unanticipated consequences of major medical advances that prolonged human life, including the effects on host defense mechanisms. Nor did they appreciate the effects of human incursion farther into the environment or the consequences of free movement of plants and animals, including humans, around the globe. As a result, the list of new or newly rejuvenated infectious diseases that have plagued us since those erroneous predictions is long and still growing. A partial accounting is given in Table 1-1.

The Triad of Infectious Disease

In order to understand infectious diseases, the student must appreciate the interactions among three entities:

1. **The affected host**. From our anthropomorphic point of view, this host will usually be a human. The veterinarian will be concerned with animal hosts, while a botanist will focus on plants. The affected host may even itself be an infectious agent, for example, when a bacterium is infected by a bacteriophage.
2. **An infectious agent**. This designation is the broadest description for a variety of life forms that interact intimately with a host (i.e., another life form), usually to the detriment of the latter. Infectious agents often enter (i.e., infect) the host in some manner.
3. **The environment**. The natural environment, both animate and inanimate, is essential for maintenance of most infectious agents and important for transmission of infectious agents from one host to another.

TABLE 1-1 Newly Recognized Infectious Diseases and Newly Identified Pathogens Since the "Conquest of Infectious Diseases"

Year	Agent	Disease(s)
1977	Ebola virus	Ebola hemorrhagic fever
	Legionella spp.	Legionnaires' disease
	Hantaan virus	Hemorrhagic fever with renal symptoms
	Campylobacter jejuni	Gastroenteritis
	Escherichia coli 0157	Hemorrhagic colitis; hemolytic

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	(verotoxin-producing)	uremic syndrome
	Borrelia burgdorferi	Lyme disease
1983	*Helicobacter pylori*	Gastric/duodenal ulcer
	Human immunodeficiency virus	Acquired immunodeficiency syndrome
1989	Hepatitis C virus	Non-A, non-B hepatitis
1991	*Ehrlichia chaffeensis*	Human monocytic ehrlichiosis
	Guanarito virus	Venezuelan hemorrhagic fever
1991	*Cryptococcus gattii*	Meningitis and pulmonary infection
1993	Sin nombre virus	Hantavirus pulmonary syndrome
	Bartonella henselae	Cat scratch disease; bacillary angiomatosis
1994	Human herpesvirus 8	Kaposi's sarcoma
	Anaplasma (*Ehrlichia*) *phagocytophilum*	Human granulocytic anaplasmosis (ehrlichiosis)
1995	Hendra virus	Respiratory disease; meningoencephalitis
1996	Australian lyssavirus	Human rabies
1997	Influenza virus H5N1	Avian influenza in humans
1999	Nipah virus	Respiratory illness; meningoencephalitis
	Influenza virus H9N2	New variant of avian influenza in humans
2001	Human metapneumovirus	Respiratory illness
2003	SARS coronavirus	Severe acute respiratory syndrome
2009	Influenza A virus H1N1	Influenza in humans
2012	MERS coronavirus	Respiratory illness

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2012	MERS coronavirus	Respiratory illness
2013	Chikungunya virus	Fever, joint and muscle pain, headache

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An amusing and readable but highly educational view of these relationships has been presented as a fantastical assembly of bacteria that have come together to voice their complaints about the "spin" humans have placed on their relationships. *The Other End of the Microscope: The Bacteria Tell Their Story* by E.W. Koneman takes a nontraditional view of the interactions between humans, their microbiome, and infectious agents.[68]

A more traditional, but also eminently readable, elucidation of the complex interconnections between humans and infectious agents was prepared by Cedric Mims, the distinguished British microbiologist. It has been maintained by colleagues and is an outstanding way to explore the fascinating subject of pathogenesis further.[83]

The Infectious Agent

Classes of Infectious Agents. Infectious agents can be divided into a finite number of types. Most are free-living and contain all the machinery necessary for the maintenance and replication of their kind; they are generally referred to as **microbes**. Bacteria, fungi, parasites, and viruses are the traditional groups of infectious agents.

1. **Bacteria** contain the largest number of species that are pathogenic for humans. They are single-celled and contain both DNA and RNA. Their DNA, however, is not contained within a nucleus; they are prokaryotic. Bacteria reproduce by binary fission. Although most are autonomous and free-living, there are a few families of bacteria, most notably the *Rickettsiaceae*, *Anaplasmataceae*, and *Chlamydiaceae* that lack all the necessary biochemical processes for replication and must interact with a host cell to reproduce.
2. **Fungi** are single and multicellular organisms that contain a defined nucleus and cytoplasm; they are eukaryotic microorganisms. **Yeasts** are single-celled fungi most of which reproduce by budding, although some reproduce by binary fission. **Molds** or **moulds** are more complex multicellular organisms that reproduce by both sexual and asexual means. Some fungi have both yeast and mold phases; these are referred to as dimorphic fungi. **Microsporidia** are a unique group of fungi that are intracellular pathogens, which were originally thought to be parasites.

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celled animals, such as the protozoa, and very complex, multi-celled organisms that have well-defined organs and tissues, such as gastrointestinal tracts and genital systems. These organisms, whether macroscopic or microscopic, derive some benefit from the infectious state, often nutrition, at the expense of the host.

4. **Viruses** comprise a large number of infectious agents that do not have the complete biochemical organelles or processes to sustain their own propagation. They must, therefore, infect another life form, including humans, animals, plants, bacteria, and even other viruses. They represent the simplest form of infectious agent. With rare exceptions, viruses contain either DNA or RNA, but not both. In contrast to other microbes, which reproduce by replication and subsequent division of their genetic material, viruses reproduce by extensive replication of their genetic material, which is packaged individually into new virions, all within the confines of an infected cell.

In addition to the traditional infectious agents, more evolved members of the animal kingdom, such as insects, may be considered a kind of parasitic agent (i.e., an ectoparasite) if their existence is intimately related to a host. At the other extreme, the entirely unconventional **prions** contain no nucleic acid and, therefore, cannot replicate in the traditional sense. Yet, their abnormal protein structure results in a process that leads to a replicative cycle.

Interactions Between Hosts and Infectious Agents. If two or more organisms exist together and each is neither rewarded nor damaged, the process is called **commensalism.** Such an agent is referred to as a **commensal**.

If the infectious agent derives benefit from the host, but causes no harm to the host, the microbe is designated a **saprobe**. If both organisms are saprophytic and benefit from one another, then the process is called **symbiosis** or **mutualism**.

Conversely, if the host is damaged by the infectious agent with or without benefit to the infectious agent, the process is called **parasitism** and the infectious agent is designated a **parasite** (a more general use of the term than the class of infectious agents known as parasites). Infectious agents of all types may be parasites.

When microbes exist on body surfaces, either external (skin or hair) or internal (upper respiratory and gastrointestinal tracts), they are described as microbiota or **colonizing flora**. The relationship of the microbiota to the host may be commensal, saprobic, or parasitic. These relationships may change over time, if there is an alteration in the virulence of the microbe or a diminution in the ability of the host to resist infection.

An organism that has demonstrated the ability to cause infections regularly is referred to as a **pathogen**. If the microbe produces disease on occasion, it is often termed a **potential pathogen**. A potential pathogen is described as an **opportunistic**

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termed a **potential pathogen**. A potential pathogen is described as an **opportunistic agent** if it produces infection only in individuals who have compromised defense mechanisms.

Infectious agents also have several types of relationships to their hosts at the cellular level. Free-living microbes (some bacteria, fungi, and parasites) exist extracellularly and can grow in vitro in the absence of cells. Facultatively intracellular microbes can grow in the absence of cells in vitro; in vivo they grow either intracellularly or extracellularly. Obligately intracellular infectious agents lack one or more of the biochemical processes necessary for an extracellular existence; they must utilize a host cell to supply the needed process or substance(s). These relationships are summarized in Table 1-2.

Virulence. Virulence is the sum of characteristics of a microorganism that increase the ability of that organism to cause disease. It is not an all-or-none phenomenon; there are widely varying degrees of virulence. Most discussions of virulence focus on microbial factors, but it is actually the balance of all three members of the triad discussed above that is most important.[12]

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TABLE 1-2 Relationships of Infectious Agents and Their Hosts at the Cellular Level

Relationship	Infectious Agents	Examples
Free-living	Most bacteria, fungi, and parasites	Staphylococcus, Enterobacteriaceae, Candida, Aspergillus, Protozoa, Helminths
Facultatively intracellular	Certain bacteria, mycobacteria, certain fungi	Legionella, Brucella, Mycobacterium tuberculosis, Dimorphic fungi
Obligately intracellular	Certain bacteria, fungi, and protozoa, viruses	Rickettsia, Ehrlichia, Anaplasma, Toxoplasma gondii; influenza virus

The most virulent organism will not cause disease if it does not have access to a susceptible host, for one of two reasons:

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hosts. For example, after the yellow fever virus cycle in urban mosquitoes was interrupted, transmission of disease stopped until humans entered a previously unrecognized cycle of yellow fever in species of mosquitoes that existed in the jungle.

2. All of the available hosts have developed protective immunity. For instance, many viral infections that elicit protective immunity, such as measles virus, "burn out" after all the susceptible persons have been infected. Only after the birth of a new generation of individuals, who are previously uninfected and therefore susceptible, can there be a new epidemic.

In contrast, an organism that produces mild or no disease in immunocompetent individuals (i.e., an organism of intrinsically low virulence) may produce devastating disease in an individual whose immune system has been compromised. For example, the spores of *Aspergillus* species are encountered on a daily basis and immunocompetent individuals, who constitute most of the population, do not become infected. However, patients with severe compromises in their immune system may develop severe to life-threatening disease (i.e., invasive aspergillosis).

The most successful infectious agents are those that are highly adapted to their hosts and cause only minimal damage. As an example, it has been hypothesized that Epstein–Barr virus, which infects long-lived memory lymphocytes, has developed a strategy for minimizing negative effects on its host.[113]

Host factors also play an important role. An organism may be particularly virulent in a human host, but continue to exist in nature because it has evolved a permissive relationship with other hosts. For instance, humans may be lethally infected with *Trypanosoma brucei*, whereas a milder form of the disease is seen in other vertebrate hosts.

Virulence may result from factors that are operative at virtually any stage of the infectious process. Some examples are summarized in Table 1-3. A number of excellent references are available for readers who desire more detailed information.[50,55,67,69,76,100,101]

TABLE 1-3 Selected Virulence Factors

Category	Virulence Factor	Example
Survival in environment	Intracellular replication in free-living amoebae	Legionella spp.
	Resistance to desiccation	Variola virus
Effective	Motile forms that can seek out a	

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Effective transfer/transmission	Motile forms that can seek out a susceptible host	Cercariae of schistosomes
	Adaptation to a vector that can transmit infection	Rickettsia rickettsii in ticks
Evasion of host defenses	Lysis of polymorphonuclear inflammatory cells	Streptococcus pyogenes leukocidins
	Destruction of tissue	Proteases of Pseudomonas aeruginosa
	Destruction of tissue	Invasion of blood vessels by Aspergillus fumigatus
	Destruction of immune lymphocytes	Human immunodeficiency virus
	Antigenic modulation to subvert humoral immunity (antibodies)	Antigenic shift and drift of influenza virus; antigenic variation of Borrelia recurrentis or Trypanosoma brucei
Sequestration in an inaccessible site	Intracellular location in macrophages	*Mycobacterium tuberculosis*
	Intracellular location in neurons of spinal ganglia	Varicella zoster virus or herpes simplex virus
Resistance to antimicrobial agents	Resistance to disinfectants	Pseudomonas aeruginosa and antiseptics
	Resistance to fixatives	Prions and formaldehyde
	Resistance to anti-infective agents	Staphylococcus aureus and methicillin
	Resistance to antiviral agents	Human immunodeficiency virus; cytomegalovirus

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TABLE 1-4 Common Routes of Transmission and Portals of Entry for Infectious Agents

Source of Agent	Mechanism for Entry	Portal of Entry	Example
Infected human	Aerosol	Respiratory	Influenza virus
Infected human	Direct contact	Cutaneous	Herpes simplex in wrestlers
Infected human	Sexual intercourse	Genital	Syphilis; Gonorrhea
Infected human or patient	Oral or nasopharyngeal secretions to eye	Ocular	Bacterial or viral conjunctivitis
Infected human	Transfusion of blood products	Intravascular	Hepatitis viruses
Contaminated environment	Food or water	Gastrointestinal	Enteric bacterial and viral pathogens
Contaminated environment	Cooling tower drift	Respiratory	Legionnaires' disease
Infected animal	Animal bite	Cutaneous	Rabies
Infected tick	Tick bite	Cutaneous	Lyme disease
Patient	Aspiration of endogenous flora	Respiratory	Bacterial pneumonia
Patient	Spillage of intestinal flora through a damaged bowel wall	Gastrointestinal	Bacterial peritonitis
Patient	Migration of bacteria from oropharynx to middle ear through eustachian tube	Ear	Bacterial otitis media

The Environment

The host range of some infectious agents is limited to humans. Maintenance of such organisms requires access to a new susceptible host and the ability to survive environmental factors during the transfer. In some instances, the transmission is quickly achieved, such as through a cough or a sneeze, and environmental factors are of less importance. However, in some instances, obligate human infectious agents, such as the bacteria responsible for typhoid or enteric fever, may spend extended time in the environment. In such instances, transmission may be interrupted. This is how the advances in wastewater treatment and drinking water treatment have led to the substantial reduction of infectious diarrheal illnesses in resource-advantaged countries. The environmental aspect of the cycle was interrupted.

Many infectious agents, however, have a phase wherein they live free in the environment, and commensals or infectious agents in nonhuman hosts, and/or pass through a vector, usually an arthropod, between various vertebrate hosts. Some of the interactions may become extremely complex; for instance, the developmental stages of several parasites involve a series of animal hosts.

The link between the infectious agent and the ultimate host is the environment. The transfer to a new host requires a portal of entry. The most common routes of transmission and portals of entry are summarized in Table 1-4. Some of the terms used to describe infectious diseases in populations are defined in Box 1-1.

The Infected Host

The host is the organism that harbors the infection. It may be human, animal, or even

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another microbe, but this text will concentrate on human hosts. Once an individual has been infected, a variety of host responses are usually elicited. The response may be localized at the site of infection or may be generalized (systemic). The local reaction to infection takes the form of inflammation. *Inflammation* is a general term that refers to the response of the body to tissue damage, which may be secondary to infection or other causes (e.g., trauma). Inflammation has both immunologic and nonimmunologic components. In each case, the defense may be cellular or noncellular (humoral). The suffix "-itis" is used to indicate inflammation; when attached to an anatomic site,

it describes a disease process. For instance, appendicitis is an inflammatory process that affects the appendix, whereas hepatitis is inflammation of the liver (hepar), and endocarditis is inflammation of the endocardium (lining of the chambers of the heart).

BOX 1-1

Categories of Infectious Diseases

Communicable disease: An illness that can be transmitted from an external source, animate or inanimate, to a person.
Contagious disease: An illness that can be transmitted from person to person.
Iatrogenic infection: An infection that is produced by medical interventions
Infectious disease: An illness caused by a replicating or multiplying external agent
Nosocomial infection: An infection that is acquired in a healthcare facility
Opportunistic infection: An infection in a patient with compromised defenses by an agent of low virulence that would not produce infection in a patient with a fully functioning immune system.
Subclinical infection: An infection that produces an immunologic response, but minimal-to-no clinical symptoms.

Innate and adaptive immunity are the two major components of the immune response.[42,77,104] The innate immune response is the more primitive of the two. It provides an immediate response against invading microbes, regardless of the particular nature of the microbe. Some of the molecules (i.e., the receptors) that make contact between the microorganism and the cellular defender are compounds called lectins, which are widely distributed in nature. The innate response is limited and does not directly result in the production of defenses specific for the invading microorganism.

Rather, portions of the innate immune response send signals that activate the second arm, which is called adaptive immunity.

The adaptive immune response is immunologically specific to the invading pathogen. Once protein from the invading microorganisms is "processed" by certain cells and "presented" to other cells in the immune system, then an immune response specific to that particular protein of the microorganisms is generated. This is the basis of vaccination.

Innate, Noncellular Defenses. The physical barriers, such as the skin and the epithelium that lines the respiratory, the gastrointestinal, and the genitourinary tracts, may not initially be considered part of the immune defense system, but these physical barriers are highly effective in keeping microorganisms outside our bodies. The disastrous infectious consequences of burn wounds are testimony to the effectiveness of the skin. In addition, patients undergoing chemotherapy will lose some of the epithelium lining their mouths and other mucous membranes. They may develop mucositis and bacteremia with bacteria that are common microbiota in the mouth.

The most basic defense in this category is the mucus that lines all mucosal surfaces of the body. The mucociliary escalator of the lungs is an innate defense. The mucus produced by goblet cells in the tracheobronchial tree is propelled upward by the uniform beating of the cilia of epithelial cells. Microorganisms become entrapped in the mucus, and are thereby prevented from reaching the critical, distal aspects of the lungs. The gastric acid of the stomach is also an innate defense and serves as a barrier to microorganisms and prevents many potential pathogens from reaching the small intestine. In addition, certain antibacterial compounds, such as lysozyme, are present in extracellular fluids and help to diminish the quantity of bacteria. Finally, some "primitive" or "preimmunologic" humoral defenses play an important role in host defense. These compounds, as a group, are referred to as acute-phase reactants.[50] The first to be identified, in 1930, was C-reactive protein, which was found to react with pneumococcal C-polysaccharide. Components of the hemostasis or clotting system also fall into this category. Importantly, the alternate pathway of the complement system is an early defense against some bacterial infections before specific antibodies develop (see below). Finally, the innate cellular defenses produce a variety of inflammatory modulators that include chemoattractants for other inflammatory cells. These compounds are the means by which inflammatory cells are activated and recruited to the point of infection; they are called **cytokines** (sometimes referred to as chemokines or lymphokines).[40] The first recognized cytokine (Interleukin 1 or IL-1), which is the main mediator responsible for the febrile response, is produced by monocytes and macrophages.[44] Cytokines also play a prominent role in the adaptive immune response, as described below.

Innate, Cellular Defenses. The primary inflammatory cells, which are not part of the adaptive or specific immune response, are the fixed tissue macrophages (histiocytes), their circulating counterparts (monocytes), and polymorphonuclear neutrophils (PMNs). The tissue macrophages are the primary defenses against certain infectious agents. For instance, the alveolar macrophages are very effective in phagocytosis and killing of certain bacteria and fungi. There are, however, some microorganisms that can survive in macrophages (e.g., mycobacteria or *Legionella* spp.), which can overcome this immune response and cause infection. Tissue macrophages are present in high concentrations in the liver, spleen, and lymph nodes, which together make up the reticuloendothelial system; this system is critical for monitoring and removing circulating particles, including infectious agents, from the blood and lymph. Patients whose spleens have been removed or whose livers have been damaged are at increased risk of many serious bacterial infections.

The polymorphonuclear (PMN) leukocyte is one of the most important acute inflammatory cells (see below). This cell, which has a segmented nucleus, is often the first to respond to inflammatory mediators and to arrive at the site of infection. It is able to phagocytose and kill a variety of microorganisms through a number of biochemical actions, including the production of small molecules, such as defensins, and reactive oxygen molecules, among other mechanisms, all of which aid in killing the invading microbe.

Types of Inflammation[121]

Acute Suppurative (or Purulent) Inflammation. An acute suppurative infection is the inflammatory response in which pus is formed. Pus is a liquid material that contains large numbers of inflammatory cells and has a specific gravity exceeding 1.013. The initial and dominant inflammatory cell present is the PMN.[75] Macrophages enter the former site of infection, once the neutrophils have eradicated the invading microorganism, to phagocytose, digest, and remove the debris from the infection. This is followed by the ingrowth of fibroblasts and new epithelium, as the final aspect of the healing process, which may include scar formation.

Cellulitis is often used to describe an infection that involves the loose subcutaneous connective tissue. Microorganisms and the purulent exudate spread between layers of the involved tissues. **Necrosis** refers to cell death or dissolution of tissue, which may be caused by destructive enzymes or by restriction of nutrients from the site, usually by blockage of blood flow. **Abscess** is the term used when segmented neutrophils become localized in a walled-off area of suppurative inflammation, with resultant destruction of tissue.

Purulent inflammation is a hallmark of infections caused by certain bacteria and some fungi. For example, *Staphylococcus* and *Streptococcus* are sometimes referred to as suppurative bacteria, because they elicit a neutrophilic response. In fact, the vast

majority of bacteria encountered in the clinical

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microbiology laboratory will elicit a neutrophilic response, if they are the cause of an infection. Fungi, such as *Candida* and *Aspergillus* species, most commonly elicit a neutrophilic response, although exceptions exist.

Granulomatous Inflammation. Granulomatous infection is a subtype of chronic infection in which granulomas are formed. A **granuloma** can be most simply defined as focal collections of activated macrophages or histiocytes that have an increased capacity for phagocytosis and digestion of foreign particles. These cells are also called "epithelioid" cells because they occasionally line up in a manner similar to that seen with squamous epithelium. Macrophages often aggregate and coalesce to form multinucleated giant cells. The other cellular components of granulomas include lymphocytes and eventually fibroblasts. Activation of the macrophages is produced by products of immunologically specific lymphocytes.

Certain granulomas contain a particular type of necrosis, called caseous necrosis, in which the tissue has a homogeneous, cheese-like consistency. These are also sometimes referred to as necrotizing granulomas. The presence of granulomas with multinucleated giant cells and caseous necrosis are characteristic of tuberculosis, but they may also be seen in certain other infections, particularly dimorphic fungal infections. If the granuloma is solid and the cells are intact and no necrosis is present, then it is described as a nonnecrotizing or noncaseous granuloma. Nonnecrotizing granulomas are found in infections by many of the nontuberculous mycobacteria, although *Mycobacterium kansasii* may produce necrotizing granulomas indistinguishable from *Mycobacterium tuberculosis.* Less commonly encountered bacteria, such as *Brucella,* may produce granulomas. Certain parasitic infections, most commonly those involving helminthic parasites, produce granulomas.

Lymphohistiocytic Inflammation. Some infections, particularly those caused by viruses, elicit an inflammatory response composed of lymphocytes, macrophages, and sometimes plasma cells. Both humoral and cellular immune responses are involved in this type of inflammatory response. *Treponema pallidum,* the agent of syphilis, elicits this type of response, which is rich in plasma cells during certain phases of infection. Lymphohistiocytic inflammation is often the residual inflammation, once an acute or granulomatous response has subsided.

Atopic Inflammation. Allergic reactions are mediated by a different group of cells —eosinophils, basophils, and mast cells (fixed tissue basophils). The inciting allergen may be chemical, such as the familiar grasses and pollens, or microorganisms.[66]Helminthic parasites elicit an eosinophilic inflammatory response. This type of response is usually not seen with protozoal parasites, but has been

reported with *Cystoisospora* (*Isospora*) infections. Allergic mucous (i.e., mucus rich in eosinophils that contains Charcot–Leiden crystals) is seen in patients with allergic fungal sinusitis. Allergic inflammatory cells are also seen in patients with bronchopulmonary aspergillosis. The list of microorganisms to consider is relatively short, when eosinophils are the primary inflammatory cells present.

Adaptive Immunologic Cellular Defenses. The lymphocyte is the principal cell of the adaptive immune system, of which there are two primary classes, B and T lymphocytes.[43] The B lymphocyte and plasma cell, which is derived from B lymphocytes, are responsible for producing immunologically specific antibodies, which comprise the humoral immune system.

The T lymphocyte coordinates much of the activity of the adaptive immune response. It is also responsible for specific cellular immunity. T lymphocytes are divided into helper lymphocytes ($CD4^+$ phenotype) and suppressor lymphocytes ($CD8^+$phenotype). $CD4^+$ cells are responsible for immunologic memory and secretion of cytokines to modulate the immune response. $CD4^+$ lymphocytes are further subdivided into type 1 (T_H1) cells and type 2 (T_H1) cells based on the types of cytokines they secrete. $CD8^+$ cells are cytotoxic and responsible for eliminating foreign cellular material, including cells infected with viruses. A variety of different subtypes of lymphocytes exist, each with particular functions, but further discussion is beyond the scope of this text.

Adaptive Immunologic Noncellular (Humoral) Defenses. Humoral immune defenses are driven and produced by the immunologically specific cells. This sophisticated communication system coordinates activity of a large complement of the host defenses. The signaling molecules produced by lymphocytes that coordinate this response are called cytokines.[40] In addition to coordinating the humoral response, cytokines also function as effector molecules for the cytotoxic lymphocytes.

The end product of the humoral response is the production of antibodies (see Chapter 3). In brief, antibodies, also known as immunoglobulins, are proteins produced by plasma cells that are secreted into the lymph and blood and can penetrate into tissues. These are Y-shaped molecules and may form complexes depending on class. The "top" of the Y consists of two Fab regions that recognize and specifically bind with particular antigens, such as those found on invading microorganisms. The "bottom" portion of the Y is the Fc region and it functions to provide signals to other portions of the immune defense system, such as signals for phagocytosis or complement activation.

The complement system consists of a cascade of enzymes that eventually result in the formation of compounds (C7-8-9) that are known collectively as the attack

complex.[116,117] These are effector molecules that damage certain pathogens, such as meningococci. The complement system functions in a manner similar to the clotting system. Additionally, intermediate components of the system, particularly C3a and C5a, are extremely important as chemoattractants (i.e., they recruit the various inflammatory cells to the site of an infection).

The complement system has two arms, which converge at C3; beyond that point the pathway is the same. The classical arm is immunologically specific; it is triggered by complexes of antigen and corresponding antibodies. The alternate arm (formerly called the properdin system) and the more recently recognized lectin-binding system are not immunologically specific and form part of the innate immune response.

On occasion, the immune response can fail to differentiate "self" from "nonself," which results in an autoimmune disease.[65] Although most autoimmune diseases are not associated with infections, there are some instances wherein the immune response initially directed against a pathogen will subsequently be directed against self.[41,71] Rheumatic

fever is the classic example of such a response, wherein the immune response mistakes myosin of the cardiac muscle fibers for the very similar M protein of *Streptococcus pyogenes*.[93] In addition to immunologic damage caused by specific antibodies reacting with human antigens that are molecularly similar to microbial antigens, there may also be nonspecific damage caused by the inflammatory response. For example, the enzymes and toxic molecules (e.g., oxygen free radicals) that are produced within neutrophils to destroy an invading bacterium are release with the death of the neutrophil and can cause tissue damage. The immune system evolved to control of infectious agents and to undertake the surveillance and destruction of neoplastic cells. We cannot survive without it and are at great risk when it is diminished either by naturally occurring (i.e., genetic) defects, environmental (e.g., radiation), infections (e.g., HIV infection) or by therapies (e.g., immunosuppressants used to modulate organ rejection in transplant recipients). The immunocompromised patients most commonly encountered in practice are either those with HIV infection, those with bone marrow suppression due to an underlying malignancy, such as acute leukemia, or those iatrogenically suppressed with therapeutic agents for a variety of reasons. The classic example is the recipient of an organ transplant. In order to prevent the body from rejecting the foreign transplant, the immune system must be suppressed to a certain degree. In so doing, the process of defense against invading microbes and tumor cells is compromised, sometimes with disastrous consequences.[88]

The complexity of the immune system is amazing. Its intricacies are staggering and they are still not completely understood. The interested reader is referred to several

excellent reviews of the subject.[42,77,104]

Clinical Signs and Symptoms of Infection. Signs and symptoms of infection may be generalized (i.e., systemic) or they may be focal or localized to a particular organ or organ system. Early Greek and Roman physicians recognized four cardinal signs of inflammation: These were Dolor (pain); Calor (heat); Rubor (redness); and Tumor (swelling).

The underlying mechanisms predisposing to these signs are complex and even today incompletely understood. The initiating pathophysiologic mechanism is the dilation of blood vessels caused by a complex cascade of vasoactive amines and other chemical mediators.[38] The local release of chemical mediators results in an increased blood flow to the site of injury or infection, with venous and capillary congestion; this, in part is responsible for the calor and rubor that is experienced. The increased permeability of vessels allows for the escape of fluid, blood, and proteins from the vascular system into the extracellular spaces; this, in part, resulted in the dolor and tumor that is experienced. Polymorphonuclear leukocytes (i.e., neutrophils) are attracted to the area of injury by chemotactic substances that are released when injury occurs; these leukocytes escape through the permeable vasculature into the extracellular spaces. Collection of these leukocytes and the extracellular fluids is referred to as pus formation.

General or Systemic Signs and Symptoms of Infection. In the acute phase of an infection, the patient may experience fever (often high-grade and spiking), chills, flushing (vasodilation), and an increase in pulse rate. Patients with subacute or chronic infections may present with minimal or vague symptoms—intermittent low-grade fever, weight loss or fatigue, and lassitude. Toxic reactions to bacterial products may produce eczematous or hemorrhagic skin reactions or a variety of neuromuscular, cardiorespiratory, or gastrointestinal signs and symptoms—initial indicators of an underlying infectious disease.

Local Signs of Infection. The cardinal signs of inflammation are unmistakable at the site of a local infection. Imagine an infection that will result from a thorn injury to the hand. There is localized redness and heat at the site of injury, the production of a swelling as the fluid and inflammatory cells fill the subcutaneous tissues. Finally, there will clearly be pain with and often even with palpation of and around the lesion. The presence of a draining sinus and the secretion of a purulent exudate are also indications of a local inflammatory or infectious process. Any of these signs and symptoms should direct the physician to collect material for direct microscopic examination and culture.

The specific signs and symptoms of infections that manifest in the several organ systems (respiratory, gastrointestinal, urinary tract, genital, or other) will be presented in detail in Chapter 2.

Indirect Effects of Infectious Agents on Humans. As humans have responded to

infectious agents over millennia, many changes in our genetic makeup have resulted. The development of the immune system is the most dramatic and important, but other examples can be found. Malaria is one of the most prevalent and devastating infections in the world, although not common in the United States. The appearance of hemoglobin S, which results in less severe infections by *Plasmodium falciparum*, probably was an adaptive change.[72] Unfortunately, when the infectious challenge no longer exists (i.e., areas wherein malaria has been eradicated), the untoward consequences of this hemoglobin, which manifests as sickle cell disease, are preeminent. Similarly, the Duffy blood group antigen is required for entry of *Plasmodium vivax* merozoites into red blood cells. Although it is a highly prevalent antigen, the recognition of this biologic phenomenon has provided clues that could be used for construction of vaccines to block entry of malarial parasites into their target cells.[79]

Phases of the Diagnostic Cycle

It is useful to categorize the laboratory assessment of clinical specimen into three phases: the preanalytic, the analytic, and the postanalytic (Fig. 1-1). Traditionally, microbiologists and other laboratorians have concentrated most of their efforts on the scientific measurement or assessment (i.e., the analytic phase). It is now very clear that what occurs prior to measurement (i.e., the preanalytic phase) and what occurs after the scientific determination is complete (i.e., the postanalytic phase) are just as important as the accuracy of the measurement. Monitoring of performance throughout the whole cycle is part of quality assurance for the laboratory, and is discussed later in this chapter.[32]

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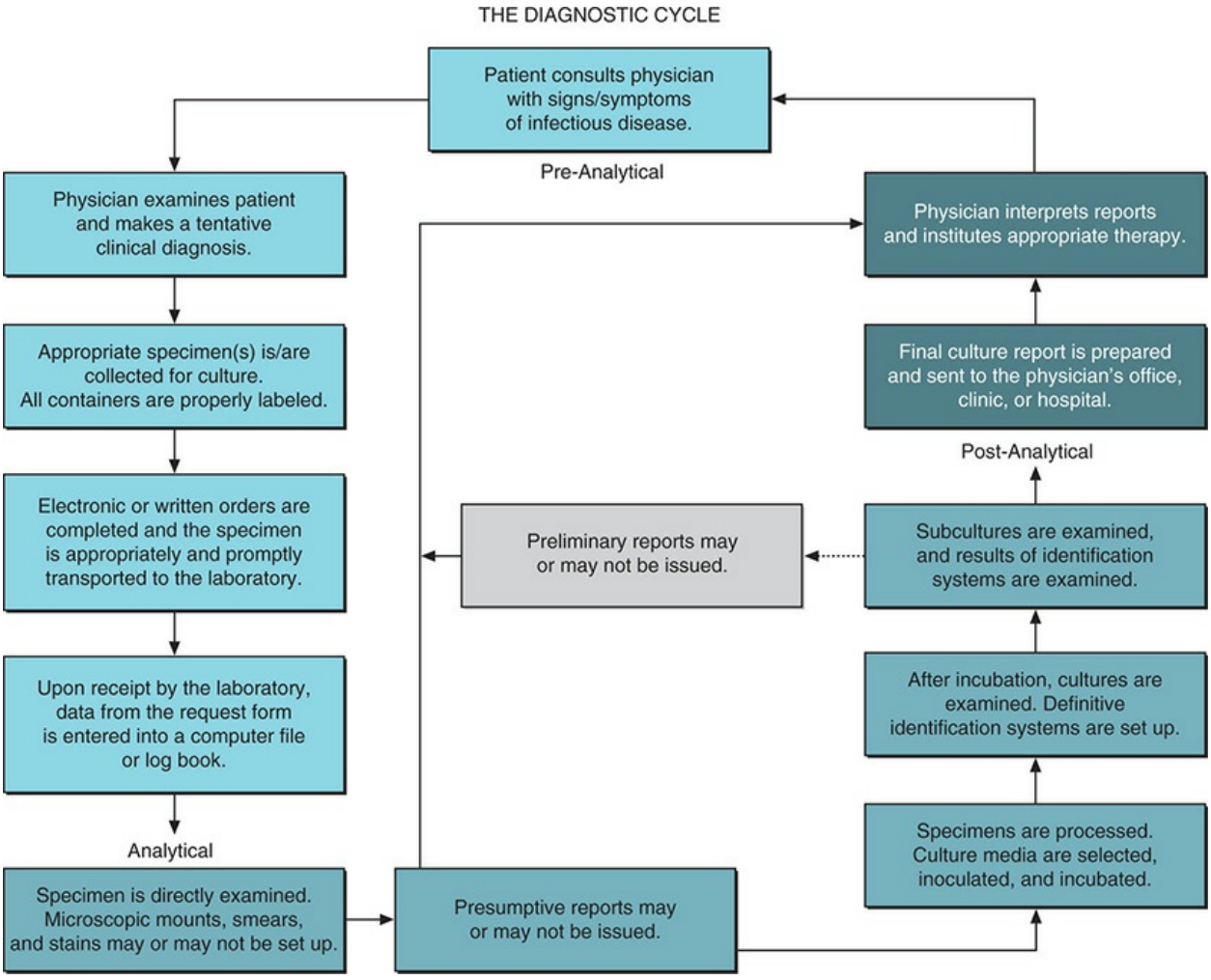

■ **FIGURE 1-1** The clinical and laboratory diagnosis of infectious diseases: a schematic overview of the diagnostic cycle.

Preanalytic Phase

Specimen Collection. Once an infectious disease is suspected, appropriate tests must be ordered. Culture, serologic studies, and/or molecular detection of nucleic acids are all possible diagnostic approaches. As discussed in Chapters 3 and 4, direct detection of antigen and nucleic acid in clinical specimens and use of these approaches for identification of isolated organisms are being utilized with increasing frequency. Pathologists, microbiologists, and medical technologists are available in most institutions and communities to assist physicians in selecting the proper specimens for culture and in ordering the appropriate tests to achieve the maximum recovery or detection of microorganisms.

The proper collection and transport to the laboratory of a specimen for examination is a critically important step in the ultimate confirmation of the etiologic agent that is

responsible for the infectious disease process.[82] A poorly collected specimen not only may result in failure to recover important agents, but may also lead to incorrect or even harmful therapy if treatment is directed toward a commensal or contaminating organism. For example, assume that *Klebsiella pneumoniae*, a recognized cause of human pneumonia, was recovered from the sputum of a patient with the clinical features of pneumonia. It would be considered the etiologic agent of disease. However, *K. pneumoniae* is also known to colonize the tonsils and nasopharynx. If the sputum in this theoretical case was improperly collected and consisted primarily of saliva, then the recovery of *Streptococcus pneumoniae* might not reflect the true cause of the pneumonia, but merely nasopharyngeal colonization. Furthermore, the treatment directed against *K. pneumoniae* would be improper, if the disease were actually caused by an agent unresponsive to such therapy, such as *Legionella pneumophila.* A scenario similar to that given above actually happened in Pennsylvania in 1976. Attendees at the annual convention of the Pennsylvania American Legion were infected at the convention hotel in Philadelphia, but became ill after they returned to their homes. They were treated with ineffective antibiotics directed against enteric bacilli that were colonizing the upper respiratory tract. The actual pathogen, *L. pneumophila*, was not known at the time and unfortunately the wrong therapeutic approach was employed based on the misleading laboratory results.[47]

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The following are fundamentals to be considered when collecting specimens:

1. The material must be from the actual site of infection, collected with a minimum of contamination from adjacent tissues, organs, or secretions. For example, throat swabs for streptococcal screening should be taken from the peritonsillar fossae and posterior pharyngeal wall, avoiding contact of the swab with other areas in the mouth. Contamination of sputum or lower respiratory specimens with oropharyngeal secretions must also be minimized. Other situations where an improperly collected specimen may produce misleading results include the following:

- **a.** Failure to culture the depths of a wound or draining sinus without touching the adjacent skin
- **b.** Inadequate cleansing of the periurethral tissue and perineum before collecting a clean-catch urine sample from a woman
- **c.** Contamination of an endometrial sample with vaginal secretions
- **d.** Failure to reach deep abscesses with aspirating needles or cannulas

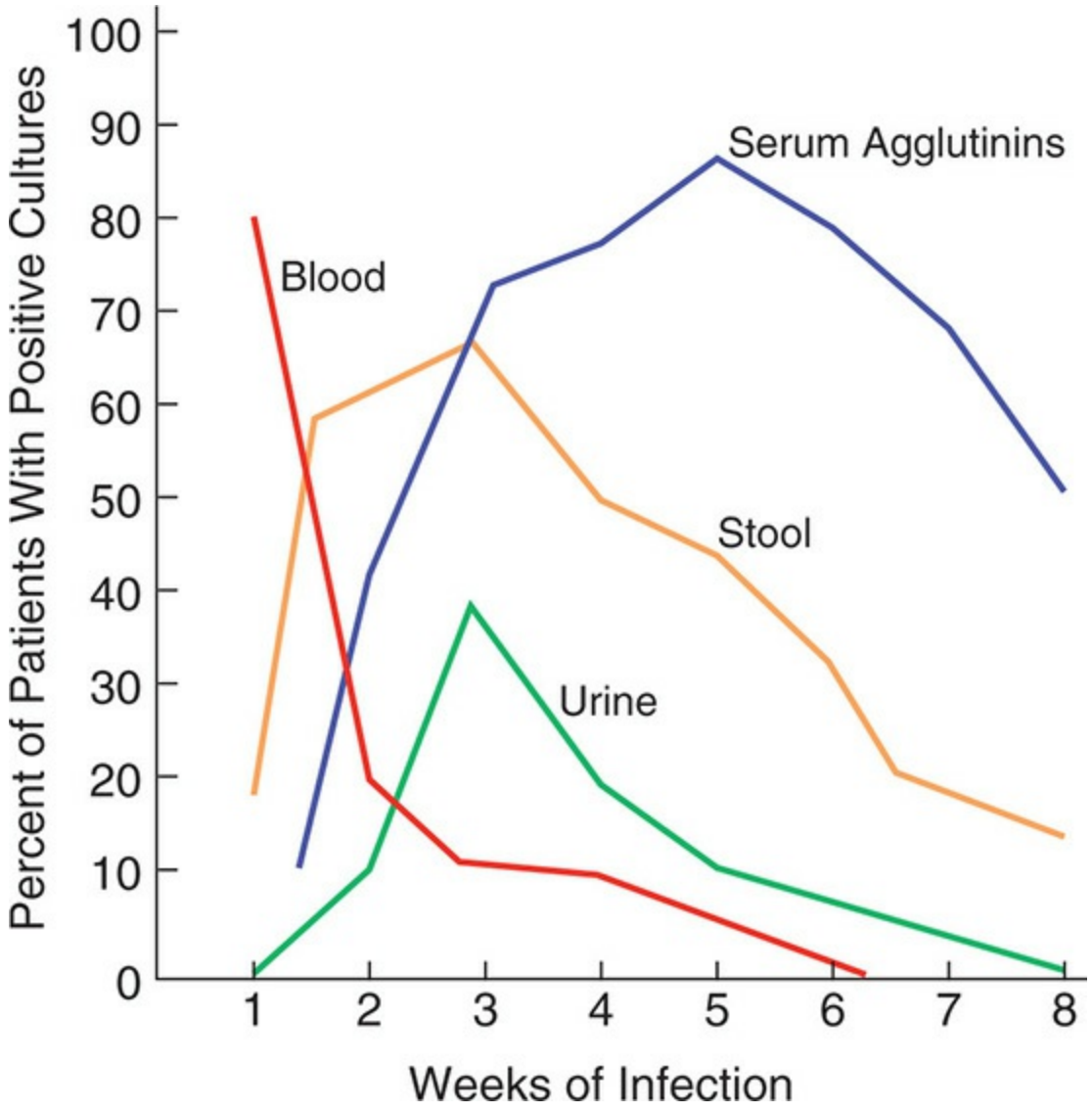
Swabs are, by and large, inferior in the collection of most specimens; the use of

aspiration needles and catheters should be encouraged whenever there are local accumulations of pus. Protected discard containers for collection of "sharps" should be readily accessible and appropriate devices used to minimizing injury when removing needles must be provided at the site of specimen acquisition. Syringes with needles attached should not be submitted to the laboratory, as they represent a hazard.

2. Optimal times for collection of specimens must be established to provide the best chance of recovering causative microorganisms. Knowledge of the natural history and pathophysiology of the infectious disease is important for some infections for determining the optimal time to collect the specimen. Although typhoid fever is now a rare disease in the United States, the progression of the infectious process in this disease is a prime example of the importance of proper timing of specimens (Fig. 1-2). The causative bacterium can be recovered optimally from the blood during the first week of illness. Culture of the feces or urine is usually positive during the second and third weeks of illness. Serum agglutinins begin to rise during the second week of illness, reaching a peak during the fifth week; they remain detectable for many weeks after clinical remission of the disease, but are infrequently used diagnostically in modern laboratories.

Twenty-four-hour collections of clinical materials for culture, particularly of sputum and urine, should not be done, because the risk of contamination or overgrowth of rapidly growing commensal bacteria is greater than if one well-collected specimen is promptly submitted to the laboratory.

3. A sufficient quantity of specimen must be obtained to perform the tests requested. Guidelines should be established to define a sufficient volume of material for testing. In most active bacterial infections, sufficient quantities of pus or purulent secretions are produced that volume should not be a problem. All too often, however, an unknowing clinician may submit a tiny sample, while discarding the remainder of a voluminous specimen.



■ **FIGURE 1-2** Diagnosis of typhoid fever by culture and serology.

In chronic or mild forms of infection, it may be difficult to procure sufficient material. Submission of a dry swab or scant secretions to the laboratory with the hope that something will grow is frequently an exercise in futility and, possibly, of considerable cost to the patient. Furthermore, the lesion may be incorrectly considered to be uninfected on the basis of a falsely negative culture, because of an inadequate specimen was obtained and submitted for study. For these reasons, it is a standard of practice to add disclaimer language to blood culture reports wherein a substandard amount of blood was collected for culture. All too frequently, 0.5 mL or less of material labeled "sputum" or "bronchial washings" is delivered to the laboratory with a request for routine, acid-fast bacilli (AFB), and fungal cultures. Such specimens may not represent pulmonary secretions from the site of infection, and the low volume may

be insufficient to enable performance of all the procedures requested. Tubes containing holding broth such as physiologic saline (nonnutrient) or phosphate yeast glucose (PYG) (nutrient) can be provided. The physician can directly inoculate whatever amount of material that can be collected. In this way, the specimen can be divided in the laboratory for inoculation to a variety of primary isolation media. In some institutions, several tubes are provided, each containing culture media optimal for the recovery of mycobacteria, fungi, and viruses. If the secretions obtained are minimal, the physician must choose which tube to inoculate based on clinical considerations.

When the sample size is too small to fulfill all of the requests adequately, it is worth a telephone call to the clinician so that priorities for culture may be established. If the specimen will be exhausted before all testing can be performed, then it is important to communicate which tests were not performed because of a lack of specimen. The report should indicate that the material submitted for examination was scanty.

4. Appropriate collection devices, specimen containers, and culture media must be used to ensure optimal recovery of microorganisms. Sterile containers should be used for

collection of most specimens. It is also important that containers be constructed for ease of collection, particularly if the patients are required to obtain their own specimens. Narrow-mouthed bottles are poorly designed for collection of sputum or urine samples. The containers should also be provided with tightly fitted caps or lids to prevent leakage or contamination during transport.

Swabs are commonly used for obtaining many types of cultures; however, they are generally inferior to other methods for collecting specimens, and their use should be discouraged as much as possible if aspirate or tissue is available as an alternative.

If swabs are used, certain precautions should be taken. Cotton swabs may contain residual fatty acids, and calcium alginate may emit toxic products that may inhibit certain fastidious bacteria. Swabs tipped with Dacron or Rayon polyester are often better choices. The newer flocked swabs represent a significant advance in swab technology and deliver significantly more specimen than former swab types. Specimens should not be allowed to remain in contact with the swab any longer than necessary. In addition to toxicity, the ability of swabs to absorb and then release specimens varies with the material used to construct the swabs.

Swabs should be placed in a transport medium or moist container to prevent drying and death of bacteria. Good recovery of most bacterial species from these tubes has been demonstrated for up to 48 hours or longer. The use of culture tubes containing semisolid Stuart's or Amies' transport medium, with or without charcoal, also serves

as an adequate means for holding swab cultures during transport. There are a few exceptions to this guideline. Skin scrapings and nail clippings for recovery of dermatophytic fungi should be submitted dry in a clean container to prevent the overgrowth of bacteria. Throat swabs are acceptable for the detection and/or recovery of *S. pyogenes* from patient with streptococcal pharyngitis. Similarly, swabs are acceptable specimens for the detection of most causes of vaginitis.

The ability to collect a specimen of virtually anything with a swab is an even larger problem than toxicity and retention of material. The likelihood is much higher with an aspirate or biopsy that the specimen that will yield meaningful results (i.e., detect the etiologic agent of infection). In contrast, a swab may have been used to sample either an inflammatory process (e.g., the tonsillar exudate) or colonizing microbiota (e.g., the side of the mouth). Whenever a potential pathogen (e.g., *Haemophilus influenzae*) is admixed with normal microbiota, it is often impossible to differentiate pathogen from flora. This is particularly true when the potential pathogen may be part of the normal microbiota in the anatomic location sampled. Figure 1-3 compares the results of culturing an aspirate and a swab from the same site.

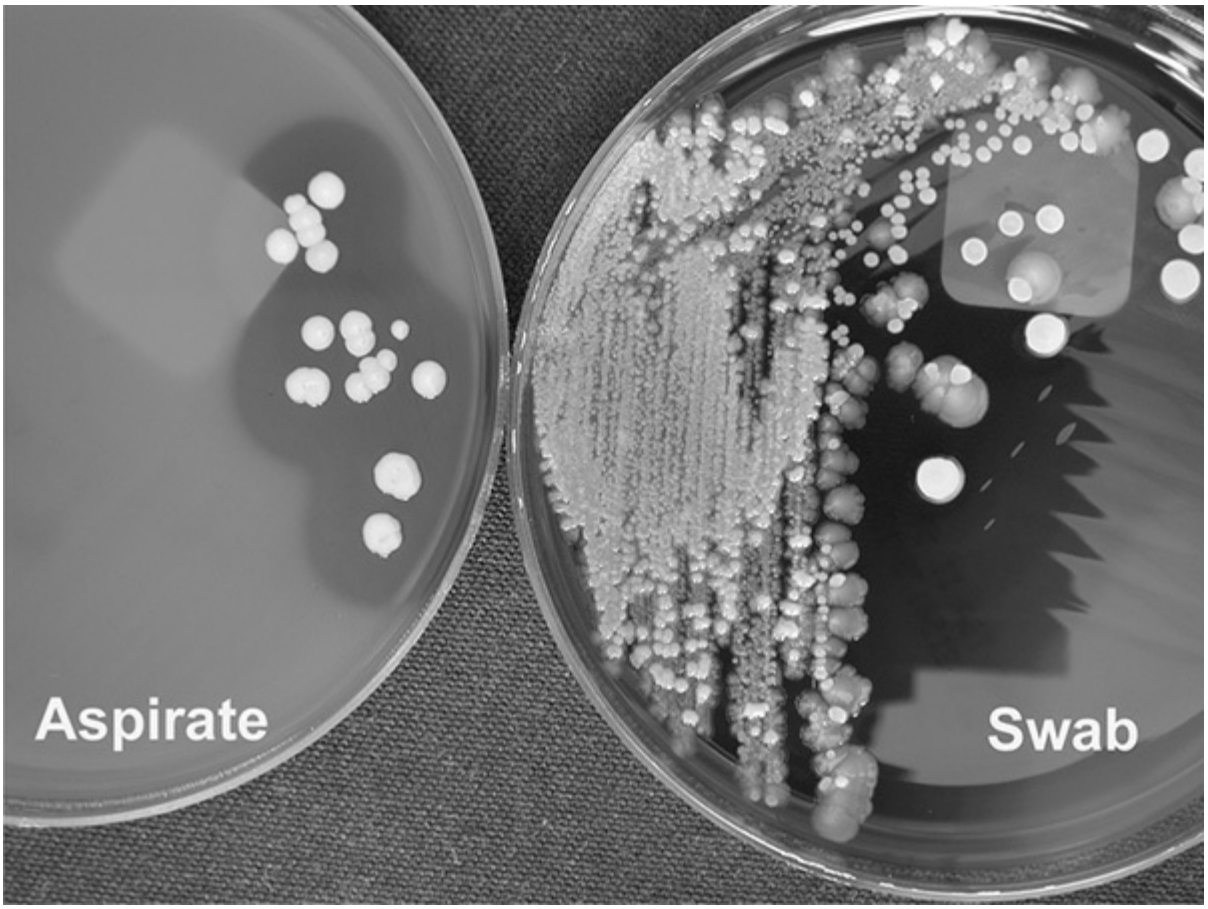
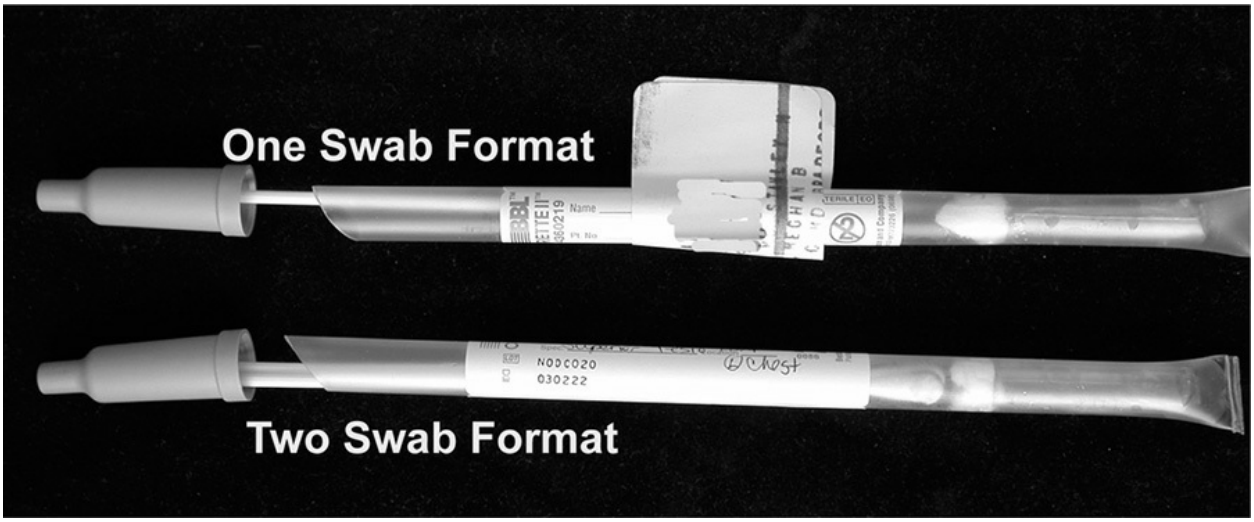


FIGURE 1-3 Comparison of aspirated pus and swab specimens. An infected joint site

was sampled with a swab and with aspirated material. The aspirate yielded a pure culture of *S. aureus*. The *Staphylococcus* was also recovered in the specimen collected by swabbing, but it was mixed in with other contaminating flora. Interpretation of the aspirate was straightforward; determining the significance of the swab culture is problematic, even with the presence of a likely pathogen.

Although it is possible to perform a smear and a culture from a single swab if the glass slide is flamed to sterilize it before the smear is prepared, it is not optimal because of the possibility of contaminating the culture. Many swab devices contain dual swabs that are both to be used in specimen collection and then submitted for smear and culture, so to assure adequate material for both tests. In that case, it is wise to provide two-swab transport systems to caregivers in order to minimize the instances where a single swab accompanies a request for smear and culture (Fig. 1-4).



■ **FIGURE 1-4** Swab transport systems. The simplest system contains a single swab in a transport tube that contains a nonnutrient medium to maintain moisture (**top**). A similar system is available with two swabs in a single transport tube (**bottom**); in this case, one swab can be used for culture and the second swab for a direct smear.

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TABLE 1-5 Transport Containers for Anaerobic Specimens

Container	Rationale or Description
	Fresh exudate or liquid specimens can be transported to the

Syringe and needle for aspiration	laboratory after bubbles are carefully expelled from the syringe and the needle is removed using an approved safety device; syringes with needles in place should not be submitted, as these represent a safety hazard. This procedure is valid only if the specimen can be transported to the laboratory without delay.
Tube or vial	Tube or vial contains semisolid holding medium, an atmosphere of 5% CO_2, a reducing agent, and resazurin indicator to give visual indication of anaerobiosis. The tube is used primarily for insertion of swab specimens; the vials are used for inoculation of liquid specimens. High-quality anaerobic transport vials are commercially available and are commonly used for the transport of specimens that will require anaerobic culture.
Swab/plastic jacket system	Plastic tube or jacket is fitted with a swab and contains either Cary–Blair, Amies transport, or prereduced (PRAS) medium. The culturette system also includes a vial or chamber separated by a membrane that contains chemicals resulting in generation of CO_2 catalysts, and desiccants to "scavenge" any residual O_2 that may get into the system. This approach is rarely used.
Bio-bag or plastic pouch	Transparent plastic bag containing a CO_2-generating system, palladium catalyst cups, and an anaerobic indicator. The bag is sufficiently large to enclose an inoculated Petri dish containing prereduced media, or a biochemical identification microtube tray such as for performing Minitek tests. Bag or pouch is sealed after inoculated plates have been inserted and the CO_2-generating system is activated. The advantage of these systems is that the plates can be directly observed through the thin, clear plastic of the bag for visualization of early growth of colonies.

The use of swabs for collection of specimens for recovery of anaerobic bacteria is discouraged; rather, aspiration with a needle and syringe is recommended. The collected specimens must be protected from exposure to ambient oxygen and kept from drying until they can be processed in the laboratory. Selected transport systems suitable for anaerobic specimens are listed in Table 1-5.

Constant education is required to discourage inappropriate use of swabs. The education can be as instructions for collection of specimens in a laboratory services directory, as newsletters, and as comments on reports for specimens that were submitted on swabs.

Although fluid or tissue is the preferred specimen type, in contrast to swabs, there have been advances in swab technology. Flocked swabs are swabs that have numerous microscopic folds which significantly increases the surface area of the swab, so that more clinical specimen can be obtained on the swab surface. These types of swabs are commercially available, and are often preferred to standard swabs. They have been validated for use in a variety of assay and are compatible with instruments of automation.

The operating room is one place where there is virtually no excuse for collection of a specimen with a swab. A campaign should be undertaken to remove swabs from the operating room and prevent their reintroduction. Communication with the operating room nurses is a useful means for accomplishing that goal. Appropriate sterile contains and collection tubes should be readily available in every operating room. Excellent commercially available transport tubes, such as the BBL Port-A-Cul transport vials and similar products are designed to protect both anaerobic and aerobic microorganisms prior to inoculation (Fig. 1-5).

Regardless of the transport system used, the major task is to reduce to a minimum the time delay between collection of specimens and inoculation of media. For example, if rectal swabs are used for the recovery of *Shigella* species from patients with bacillary dysentery, the material collected should optimally be inoculated directly onto the surface of MacConkey medium or into gram-negative enrichment broth. Even the use of a holding or transport medium may diminish the recovery of certain strains. Urethral or cervical secretions obtained for the recovery of *Neisseria gonorrhoeae* also should be inoculated directly onto the surface of chocolate agar and one of several selective culture media (see Chapter 11). Alternatively, a commercially available transport system that includes a tablet for generation of CO_2 can be employed (BD BBL Jembec system; BD, Franklin Lakes, NJ) for the recovery of *N. gonorrhoeae*. If a culture-based approach is taken for the detection of *Bordetella pertussis* from

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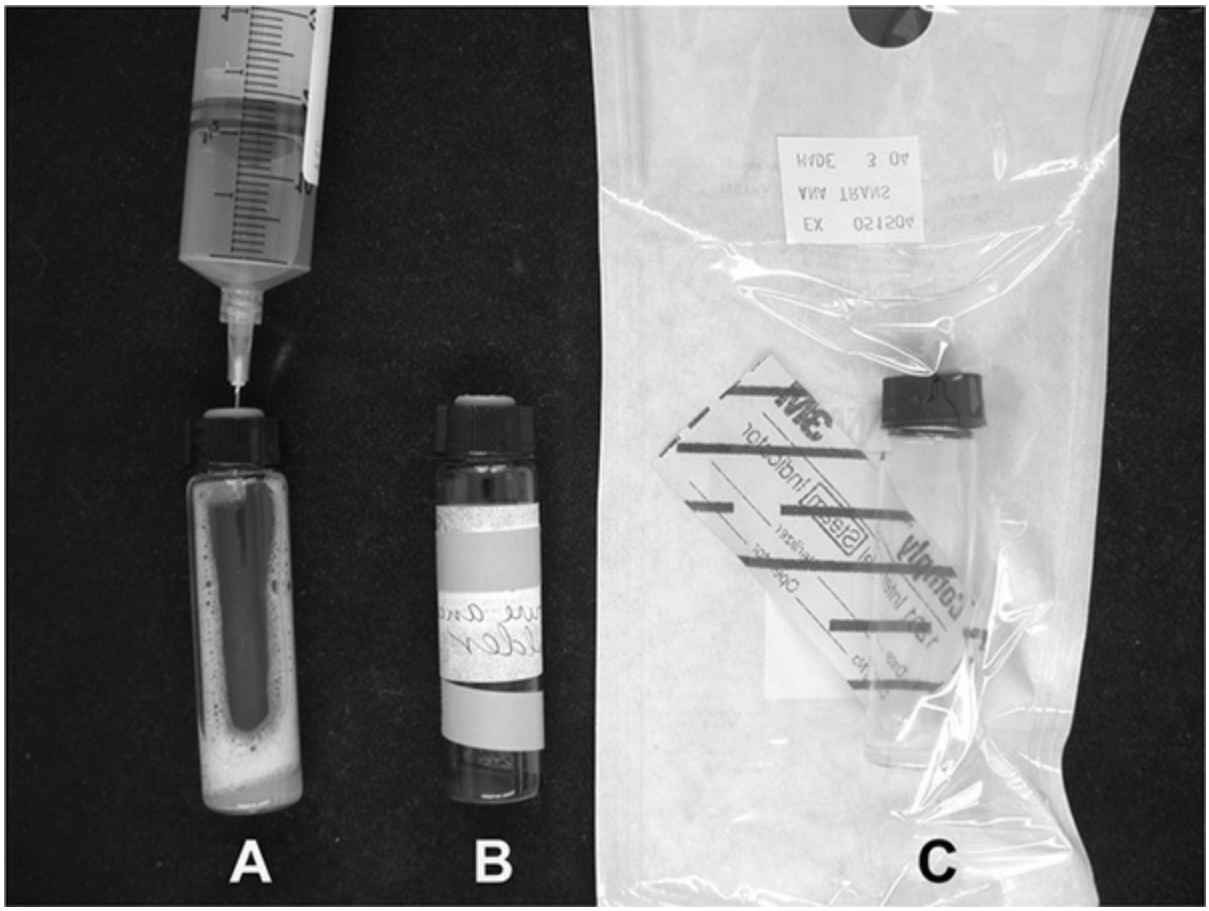
upper respiratory specimens, then fresh Bordet–Gengou, Regan–Lowe charcoal agar, or an equivalent should be used at the bedside or in the clinic, unless an appropriate transport system (such as Regan–Lowe medium) is used.[94]



■ **FIGURE 1-5** Transport system for aerobic and/or anaerobic culture. A vial is closed with a screw cap that contains a rubber diaphragm. A layer of agar in the bottom of the vial maintains moisture; a redox indicator in the agar visually displays the oxygenation of the atmosphere. Vial A contains aspirated fluid that has been inoculated through the rubber diaphragm. Vial B contains a resected graft segment that has been placed into the uncapped vial at surgery. Vial C has been sterilized in an envelope made for surgical instruments; it is ready for use in a sterile field.

5. Whenever possible, obtain cultures before the administration of antibiotics. Obtaining cultures before the use of antibiotics is particularly recommended for recovery of organisms that are usually highly susceptible to antibiotics, such as β-hemolytic streptococci from throat specimens, *N. gonorrhoeae* from genitourinary samples, or *H. influenzae* or *Neisseria meningitidis* from cerebrospinal fluid. It is also particularly important that a full complement of blood cultures are drawn on patient suspected of having endocarditis prior to the administration of antibiotics. The administration of antibiotics does not necessarily preclude recovery of microorganisms from clinical specimens, and even a compromised specimen is better than none at all, as long as clinicians understand that the results must be viewed with circumspection.

6. Smears should be performed in addition to cultures in most instances. Smears provide extremely useful information that supplements the culture. There are occasions when the smear is arguably more useful than the culture, as in examination of expectorated sputum. Smears allow assessment of the inflammatory nature of the specimen and provide an indication as to whether the results of culture are meaningful clinically. For example, sputum specimens contaminated with oral microbiota, as evidenced by excessive squamous epithelial cells, should not be cultured. If a culture were performed in the absence of a smear, which would have disclosed excessive squamous epithelium, then normal microbiota, such as *S. pneumoniae, Moraxella catarrhalis,* and *H. influenzae* could be reported and basis of the treatment would be completely erroneous. Similarly, a typical wound specimen that does not contain PMNs and yields mixed bacterial flora on culture should be recognized as a superficial sampling and not a valid representation of the organism(s) causing the infection.

In addition, a review of Gram's smear may serve as a quality indicator. A smear that contains many gram-negative bacilli that fail to grow in a conventional aerobic and anaerobic culture may indicate that the culture conditions were not optimal or the organisms were nonviable. Suboptimal culture conditions include an inappropriate atmosphere of incubation (anaerobes) or inappropriate media selection (fastidious organisms, such as *Legionella* or *Bordetella*). Investigation into increase transport times may disclose possible reasons for nonviability. Alternatively, the microbes could be nonviable because of appropriate antimicrobial therapy or because of an effective inflammatory response.

The federal fraud and abuse regulations require that a laboratory perform only the tests that have been requested. Therefore, it is necessary to convey the importance of the direct examination (i.e., a smear), as an accompaniment to the culture, to the ordering physicians. Mechanisms for enhancing appropriate use of the laboratory in the preanalytical phase are discussed below.

7. The specimen container must be properly labeled. Each specimen container must have a legible label, with the following minimum information:

Patient name
Patient identification number
Source of specimen
Clinician
Date/hour collected

The full name of the patient should be used; avoid initials. The identification number may be the hospital number, clinic or office number, home address, or social security number, depending on local policy. The name of the submitting physician or office contact is necessary should consultation or early reporting be required. The

specimen source should be noted so that the optimal culture media selection can be made. The date and time of collection should be recorded to assess the timeliness of specimen transport and processing. The tracking of specimens with timestamps at important points, such as collection, receipt, and processing completion, is invaluable to performing root-cause analysis should problems arise. Other potentially useful information includes the clinical diagnosis and the antibiotic treatment history of the patient, but the latter is rarely provided.

Specimen Transport. The primary objective in the transport of diagnostic specimens, whether within the hospital, from the clinic, or externally by courier to a distant reference laboratory, is to maintain the specimen as near its original state as possible. Quality control guidelines for manufacturers of devices used for collection and transport of specimens have been developed by the Clinical and Laboratory Standards Institute (CLSI).[36] Potential hazards to specimen handlers are minimized by using tightly fitting collection devices that are confined within proper protective containers. To maintain the integrity of the specimen, adverse environmental conditions, such as exposure to extremes of heat and cold, rapid changes in pressure (during air transport), or excessive drying should be avoided. The use of freezing specimens at −70°C is considered if there will be an extended transport time, but this is highly dependent on the microorganism suspected. Guidelines are available from quality reference laboratory providers, and should be followed to achieve optimal recovery of the organisms sought. Sputum samples that have been collected primarily for recovery of mycobacteria and fungi may be shipped without further treatment if collected in sterile propylene or polyethylene containers. To avoid breakage during transport, do not use glass containers.

Most fluid specimens should be transported to the laboratory as quickly as possible. In a hospital setting, a maximum 2-hour time limit between collection and delivery of specimens to the laboratory is recommended.[6,63] This time limit poses a problem for specimens collected in physicians' offices. Urine transport containers containing a small amount of boric acid may be used if rapid transport is not possible. Alternatively, urine specimens may be refrigerated for up to 24 hours before they are cultured. A holding or transport medium can be used for most other specimens, following the manufacturer's instructions. Stuart, Amies, and Carey–Blair transport media are most frequently used and are commercially available; instructions exist, however, for those interested in preparing media (Box 1-2).

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BOX 1-2

Stuart's Transport Medium

Sodium chloride	3 g
Potassium chloride	0.2 g
Disodium phosphate	1.25 g
Monopotassium phosphate	0.2 g
Sodium thioglycolate	1.0 g
Calcium chloride, 1% aqueous	10.0 g
Magnesium chloride, 1% aqueous	10.0 g
Agar	4.0 g
Distilled water to equal pH = 7.3	1.0 L

These media are essentially solutions of buffers with carbohydrates, peptones and other nutrients and growth factors excluded, designed to preserve the viability of bacteria during transport without allowing their multiplication. Sodium thioglycolate is added as a reducing agent to improve recovery of anaerobic bacteria, and the small amount of agar provides a semisolid consistency to prevent oxygenation and spillage during transport. Sodium borate solution can be recommended as a preservative for shipping specimens suspected of containing mycobacteria to distant laboratories.[98] Sucrose–phosphate–glutamate is a good transport buffer medium for recovery of certain viruses, such as herpesvirus. Universal transport media and M4 are common transport solutions for viral culture, and may also be used in conjunction with many of the molecular assays that are rapidly replacing viral culture. Culturette swabs have also been used successfully for viral specimens in some facilities.[107] New and updated joint guidelines from the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM) have been published; these extensive and well-done guidelines should be thoroughly reviewed and maintained in every microbiology laboratory.[4] These are available electronically online and free of charge.

Rather than recreate an excellent document, the reader is directed to *A Guide to Utilization of the Microbiology Laboratory for the Diagnosis of Infectious Diseases: 2013 Recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM).* This 100-page document provides guidelines on the recommended diagnostic procedures to secure an adequate specimen to achieve a diagnosis, as well as the optimum type of specimen and specimen transport.

Specimen Receipt and Preliminary Observations. There is an area is designated for the receipt of specimens in most clinical laboratories. Initial observations and handling should be performed in a biologic safety cabinet (BSC) (see below) because of the increasing possibility that laboratory personnel may incur a laboratory-acquired infection from specimens that contain pathogens. Personnel should wear protective clothing as appropriate—laboratory coats, rubber gloves and, in some instances, custom-fitted masks. In the distant past, these precautions were taken only for specimens carrying hazard labels. It is not possible, however, to determine if a patient may be harboring a transmissible agent or if a given sample contains a highly contagious pathogen. In addition, blood or body fluids that are submitted for bacterial culture may also contain HIV, HCV, or other blood-borne pathogens. Therefore, all clinical specimens should be handled in a safe and uniform way, which has been termed *universal precautions*. Following these guidelines helps protect the technologist performing the processing from the unsuspected pathogen, as well as the suspected ones.

The processing of specimens includes the following: (1) the entry of essential data into a log, which today is usually a computer database that is part of the laboratory information system (LIS); (2) visual examination and determination of whether all criteria for acceptance are met (see section on criteria for specimen rejection immediately below); and (3) for certain specimens, the microscopic examination of direct mounts or stained smears to establish a presumptive diagnosis.

Criteria for Rejection of Specimens. Criteria for rejection of unsuitable specimens for culture must be established in all laboratories.[112] Although general guidelines exist and accrediting agencies have established standards, each laboratory director must decide which parameters to utilize, depending on local conditions. Request slips and specimen labels must be checked upon specimen receipt to see that all essential information is included. Should there be a problem, collection of a fresh sample is the best course of action. If the specimen cannot be re-collected, a responsible person should be contacted to make corrections. A comment should be entered on the final report that the specimen was received with a (specified) problem, and the name of the person who corrected the problem should be appended. If the type of specimen can be ascertained, it may be acceptable in certain cases to issue a report: "specimen appears to be ----------." If not, the specimen should be rejected. Whenever discrepancies occur, a written record of how the situation was handled and names of the individual contacted should be entered on the back of the requisition, in a log, or in the computer database.

A list of specimen types or culture requests that should appear on the rejection list and should not be processed further appears in Box 1-3.

When a specimen is to be rejected, the person who submitted the specimen should be contacted and made aware of the problem. Every effort should be made not to

reject specimens that are difficult to re-collect, such as cerebrospinal fluid or bronchial washings. If a problem cannot be resolved expeditiously, cultures should be set up to avoid losing the integrity of the specimen. The decision can be made afterward whether to report the results. If appropriate, the condition of the specimen should be indicated on the report. The clinician will then have the responsibility for interpreting the report in light of the qualifying information.

Rejection criteria should be clearly communicated in the laboratory service directory. Instruction of hospital personnel on the importance of submitting relevant specimens for culture should be conducted when possible. Recurring problems and their solutions should be published in laboratory newsletters or other publications that may reach hospital personnel and staff physicians. Targeted re-education of particular groups that consistently breach protocol or submit substandard specimens is sometimes necessary. When this is needed, collegially approaching the group to establish "best practices" in patient care is a method to begin the dialogue in a nonconfrontational manner.

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BOX 1-3

Specimen Types or Culture Requests That Should Be Rejected

1. Any specimen received in formalin. The only exception might be large specimens in which the time of exposure to formalin is short (less than 1 hour). In these instances, the tissue should be bisected with a sterile knife or scissors and an innermost portion sampled for culture.
2. Twenty-four-hour sputum collections. It is difficult to prevent contamination, and individual collections containing a high concentration of microorganisms will be diluted out by subsequent, less-concentrated samples.
3. Smears of secretions from uterine cervix, vaginal canal, or anus for Gram's stain detection of *Neisseria gonorrhoeae*.
4. A single swab submitted for multiple requests; for example, "aerobes, anaerobes, fungus, and tuberculosis."
5. Submission in an improper, nonsterile, or obviously contaminated container in which portions of the specimen have leaked out. Any leaking container that contains a clinical specimen should be handled with extreme care.
6. Culture plates that are overgrown or dried out.
7. Specimens that are obviously contaminated, as evidenced by the presence of foreign materials, such as barium, colored dyes, or oily chemicals.
8. The following specimens are not acceptable for anaerobic culture: gastric washings, midstream urine, prostatic secretions collected transurethrally, feces

(except for the recovery of *Clostridium difficile* in the rare instance that toxigenic culture is needed), ileostomy or colostomy swabs, throat, nose, or other oropharyngeal specimens (except specimens obtained from deep tissue during oral surgery), superficial skin, and environmental cultures.

Cost-Effective Approaches in the Preanalytic Phase. Discussing cost-effectiveness in clinical microbiology has been unpopular for many years and associated with "cutting corners" and providing suboptimal testing. It was considered antiacademic, impure, or even dangerous by traditional microbiologists. Another way to view the issue, however, is the application of clinical relevance to diagnostic microbiology. The point is not to identify every organism that might be recovered or to perform susceptibility tests on every organism that will grow in the laboratory. The point is to provide clinicians with information that will allow them to provide the best care for their patients. In the process, the work can usually be done more economically than if everything possible is done. Thus, clinical relevance usually equals cost-effectiveness. Cost-effectiveness does not mean cheap; it means the best value for money.

These concepts are not new. Dr. Bartlett, a distinguished pathologist and the director of the microbiology laboratory at Hartford Hospital in Connecticut for many years, wrote meaningfully on the subject in the mid-1970s. His seminal book on the subject is still worthwhile reading for clinical microbiologists.[6,7] Many others have followed his lead, and importantly, led with best practices for best patient care, rather than seeking yet another means to cut costs.

Clinical relevance and cost-effectiveness should inform laboratory practice at every stage in the process. Some suggestions for the preanalytical phase are detailed in Table 1-6. Newer concepts, some of them imported from industry and streamline processes, decrease variability and contribute to improved outcomes. The investigation and time investment to explore these strategies may result in substantial savings in materials and time.[84] The clever laboratory director will then reuse the saved time and money to justify new clinical services or insourcing tests that will provide even better service to their patients and providers. Conditions vary in every institution, so each laboratory director must decide which possibilities are appropriate and investigate these with the local leadership. Most importantly, the director should ensure that there is professional leadership driving the change, in contrast to purely cost-saving initiatives from administration.

Analytic Phase

Microscopic Examination. The reasons for performing microscopic examination of clinical materials have been emphasized.[6,8]

1. The number and percentage of segmented neutrophils that are present indicate the magnitude and type of inflammatory response. The quality of the specimens can be validated.
2. The observation of bacteria, hyphae and/or yeast forms, parasitic structures, or viral inclusions may provide sufficient information to render an immediate presumptive diagnosis.
3. Direct microscopic examination may also give immediate presumptive evidence that species of anaerobic

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TABLE 1-6 Cost-Effective Approaches in the Preanalytical Phase of Testing

Action	Rationale	Comments
Selective culturing of CSF for fungi[70]	Low yield in immunocompetent patients who have normal CSF chemistry and cell count values	*Cryptococcus neoformans* may produce chronic infection without pleocytosis in the CSF.
Selective culturing of CSF for mycobacteria[70]	Low yield in immunocompetent patients who have normal CSF chemistry and cell count values	In populations with a low risk of tuberculosis, the yield will be even less.
Selective culturing of feces for bacterial pathogens or examination for O&P[70,105]	Low yield in patients who have been hospitalized more than 3 days	Exceptions exist, which include immunocompromised patients and the elderly.
Selective culturing of endotracheal suction aspirates or expectorated sputum[70]	Recovery of contaminating oropharyngeal flora increased if more than 10 squamous epithelial cells per low power field observed.	Several different criteria have been applied by investigators.
Selective use of broth back-up for bacterial cultures	Questionable usefulness except for cultures wherein there may be biofilm formation (e.g., CSF shunt cultures) or delayed growth (e.g., Actinomyces, P. acnes, and possibly valves from patients with endocarditis)	Broth cultures may be used in other situations if they are examined only when an organism seen in smears is not recovered on plates. They should never be performed on specimens from mucosal surfaces.

Nonuse of bacterial antigen tests on CSF[70,111]	Poor sensitivity and specificity limit usefulness	
Selective culturing of peritoneal fluid	Pattern of organisms recovered and susceptibility profiles predictable in patients with secondary peritonitis (community-acquired)	Cultures indicated in primary peritonitis (spontaneous bacterial peritonitis) and in peritonitis acquired in the hospital
Selective culturing of throat specimens	Streptococcus pyogenes is the major cause of pharyngitis: "routine culture" with multiple media will likely provide misleading information.	Culture for Corynebacterium diphtheriae, Neisseria, or herpes simplex virus should be requested specifically if appropriate Arcanobacterium hemolyticum causes pharyngitis in older children and adolescents, but is isolated on media for recovery of Streptococcus pyogenes.
Selective culturing for anaerobic bacteria	Anaerobic cultures should be performed routinely only on tissues or aspirated fluid/pus.	Never perform anaerobic cultures on specimens from sites that may be contaminated with mucosal flora or feces
Limits on frequency of culture	Throat, urine, wound limited to one per 24 hours; blood limited to two to three cultures for exclusion of bacteremia (10 mL/bottle, 2 bottles/set and two sets used per blood culture order with the sets drawn approximately 15 minutes apart)	Results of the first cultures should be evaluated prior to ordering additional cultures, unless the patient is critically ill and/or the clinical condition has changed in a way that suggests a new infection.
Limits on frequency of O&P exams	Yield from exams for Giardia minimal after three specimens, which should be collected every other day	Less than three exams probably adequate for other intestinal parasites.
	Repeat testing is not warranted within at least 7 days following	There is no value in repeat testing

Limits on frequency of testing for C. difficile[96]	a negative result from a PCR-based assay, because of the high negative predictive value. Repeat testing should only be performed if there is a clinical change suggestive of new-onset C. difficile enterocolitis.	following a positive PCR result; there is no "test of cure" for this disease and C. difficile may remain as part of the microbiota of the patient without causing disease.
Limits on testing of CSF for herpes simplex DNA[106,110]	Yield minimal if CSF cell counts normal and no evidence of focal lesions by MRI exam	Exceptions have been described, especially in children.
Limits on duplicate specimens submitted from the same site on the same day	Either pool the specimens or select the best specimen (based on Gram's stain and/or transport time)	If there is any uncertainty as to the equivalence of the specimens, consult the clinician before processing; when in doubt, process separately immediately and consult at leisure; preserve specimens for at least 24 hours
Limits on repeat specimens from a nonsterile site within a defined time period	Refer subsequent specimens to the previous evaluation; if the subsequent specimen has been inoculated to media and substantial differences from the original specimen are present, consult clinician about further action	Preserve specimen for at least 24 hours; preserve inoculated plates that are incompletely evaluated for a defined period (e.g., 7 days)
Elimination of culture of indwelling urinary catheter tips[114]	Reject the specimen for culture	Inform the clinician that urine should be submitted

CSF, cerebrospinal fluid; O&P, ova and parasites; MRI, magnetic resonance imaging. For more information, the reader should consult references 4, 62, and 81.

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bacteria are present. With these clues in hand, the clinician can make more rational decisions about initial antimicrobial therapy.

In the past, the examination of wet mounts of unstained materials by phase contrast or darkfield microscopy was used for demonstrating motility, spirochetes and endospores, but this in uncommon performed today. Giemsa's, Wright's, or acridine orange (AO) stains may be helpful in observing bacterial forms that stain poorly or that have little contrast with background material.

Direct Gram's stains of clinical material may also be used to determine whether a specimen is representative of the site of infection. This technique has been applied to the evaluation of sputum samples. Bartlett has devised a grading system for evaluating sputum samples for the relative number of squamous epithelial cells and segmented neutrophils in direct Gram-stained sputum samples, (Box 1-4).[6] Using this system, negative numbers are assigned to a smear when squamous epithelial cells are observed, indicating contamination with oropharyngeal secretions (saliva). Positive numbers are assigned for the presence of segmented neutrophils, indicating the presence of active inflammation. The magnitude of these negative and positive determinations depends on the relative numbers of epithelial cells and segmented neutrophils as shown in the outline of Bartlett's grading system. A final score of 0 or less indicates either lack of inflammatory response or presence of significant salivary contamination, thus invalidating the specimen. Representative photomicrographs of Gram-stained sputum preparations illustrating this grading system are shown in Image Plate 1-1.

BOX 1-4

Bartlett's Grading System for Assessing the Quality of Sputum Samples

No. of Neutrophils per 10× Low-Power Field	Grade
<10	0
10–25	+1
>25	+2
Presence of mucus	+1
No. of Epithelial Cells per 10× Low-Power Field	
10–25	−1

>25	−2
Total[a]	

[a]Average for the number of epithelial cells and neutrophils in about 20 or 30 separate 10×microscopic fields is determined; then a total is calculated. A final score of 0 or less indicates lack of active inflammation or contamination with saliva. Unsatisfactory specimens should be rejected, the provider should be notified and a repeat sputum specimen should be requested, if clinically indicated. The denotation of "if clinically indicated" is important, because if a message such as "Repeat sputum culture" is given, then this may be performed, even if the clinically findings of the patient do not warrant repeat testing.

BOX 1-5

Murray and Washington's Grading System for Assessing the Quality of Sputum Samples

	Epithelial Cells Per Low-Power Field	Leukocytes Per Low-Power Field
Group 1	25	10
Group 2	25	10–25
Group 3	25	25
Group 4	10–25	25
Group 5	<10	25

A similar grading system has been proposed by Murray and Washington[86] (Box 1-5). The large number of epithelial cells in groups 1 to 4 of this system indicates contamination with oropharyngeal secretions and invalidates the samples (i.e., the specimen should be rejected). Only group 5 specimens are considered clinically relevant. In a clinical study, Van Scoy[115] recommended that sputum samples containing more than 25 neutrophils be accepted for culture even if more than 10 epithelial cells are present (group 4). These suggested criteria for evaluation of sputum have been evaluated by applying them to matched pairs of respiratory secretion obtained by expectoration and by transtracheal aspiration, a technique that bypasses

the oropharyngeal flora.[53]

The sputum grading system cannot be employed if pulmonary infections caused by mycobacteria, fungi, *Legionella* spp., and viruses are suspected. Additionally, the significance of PMNs is altered in a few situations: (1) when the patient is neutropenic from disease or therapy, (2) when the patient does not mount an effective inflammatory response, and (3) where a foreign body causes irritation to a mucosal surface. Neutropenia can result from an inherited deficiency or because the inflammatory cells or their precursors are destroyed, either by a disease process or by chemotherapy for a disease. In some conditions, the ability of neutrophils to migrate to the site of an infection is impaired. From the information ordinarily supplied by clinicians, it is rarely possible for the clinical microbiologist to determine whether a patient is neutropenic. One of the exceptions is the incompletely understood deficiency in mobilization of neutrophils that is found in infancy.[39] The exact age at which a full neutrophilic response can be mounted is not clearly defined, but in the very youngest children (less than 2 months of age) decisions to reject specimens or evaluate isolates incompletely should not be based on the absence of neutrophils in the specimen.

The two situations in which foreign bodies modify the interpretations associated with the presence of neutrophils. These are when an endotracheal catheter is in place the lower respiratory tract and when there is an indwelling catheter in the lower urinary tract, such as a Foley catheter. In each of these situations, the presence of neutrophils in a specimen may reflect irritation from the catheter rather than from an

infectious agent, although both factors may be present. In the respiratory tract, the inflammation may come from local infection (tracheitis) rather than from pneumonia, even if there is an infectious element. If the respiratory tract neutrophils cannot be used as an indication of pneumonia, then the diagnosis of pneumonia must be made clinically and radiographically, as discussed in Chapter 2. In the lower urinary tract, the presence of leukocytes cannot be used as an indication of a clinically important infection if a catheter is in place[108] or even if repeated intermittent catheterization is performed.[54] The critical determinant for therapy of urinary tract infections in the chronically catheterized patient is whether the patient is symptomatic (see Chapter 2).

Microscopic Techniques. A number of techniques may be used in the direct microscopic examination of clinical specimens, either to demonstrate the presence of microorganisms or to observe certain biochemical, physiologic, or serologic characteristics. The techniques commonly used in clinical microbiology laboratories are outlined in Table 1-7. Because the refractive index of bacteria and other

TABLE 1-7 Techniques for Direct Examination of Unstained Specimens

Methods and Materials	Purpose	Techniques
Saline mount Sodium chloride, 0.85% (aqueous) Glass microscope slides, 3 × 1-inch Coverslips Paraffin–petrolatum mixture (Vaspar)	To determine biologic activity of microorganisms, including motility or reactions to certain chemicals, or serologic reactivity in specific antisera. The latter includes the quellung (capsular swelling) reaction used to identify different capsular types of Streptococcus pneumoniae and Haemophilus influenzae.	Disperse a small quantity of the specimen to be examined into a drop of saline on a microscope slide. Overlay a coverslip and examine directly with a 40× or 100× (oil immersion) objective of the microscope, closing the iris diaphragm to reduce the amount of transmitted light. To prevent drying, ring the coverslip with a small amount of paraffin–petrolatum before overlaying the specimen drop on the slide.
Hanging-drop procedure Hanging-drop glass side (This is a thick glass slide with a central concave well.) Coverslip Physiologic saline or water Paraffin–petrolatum mixture	The hanging-drop mount serves the same purpose as the saline mount, except that there is less distortion from the weight of the coverslip and a deeper field of focus into the drop can be achieved. This technique is generally used for studying the motility of bacteria.	A small amount of paraffin–petrolatum mixture is placed around the lip of the well on the undersurface of the hanging-drop slide. Cells from a bacterial colony to be examined are placed in the center of the coverslip, into a small drop of saline or water. The slide is inverted and pressed over the coverslip, guiding the drop of bacterial suspension into the center of the well. The slide is carefully brought to an upright position for direct examination under the microscope.
Iodine mount Lugol's iodine solution: Iodine crystals, 5 g Potassium iodide, 10 g Distilled water, 100 mL	Iodine mounts are usually used in parallel with saline mounts when examining feces or other materials for intestinal protozoa	A small amount of fecal matter or other material is mixed in a drop of the iodine solution on a microscope slide. This is mixed to form an even suspension, and a coverslip is placed over

Dissolve KI in water and add iodine crystals slowly until dissolved. Filter and store in tightly stoppered bottle. Dilute 1:5 with water before use. Microscope slides, 3 × 1-inch Coverslips	stains the nuclei and intracytoplasmic organelles so that they are more easily seen. Iodine mounts cannot be used to the exclusion of saline mounts because iodine paralyzes the motility of bacteria and protozoan trophozoites.	and a coverslip is placed over the drop. The mount is then examined directly under a microscope. If this is to be delayed or if a semipermanent preparation for future study is desired, the edges of the coverslip can be sealed with the paraffin–petrolatum mixture.
Potassium hydroxide (KOH) mount Potassium hydroxide, 10% (aqueous) Microscope slides, 3 × 1-inch Coverslips	The KOH mount is used to aid in detecting fungal elements in thick mucoid material or in specimens containing keratinous material, such as skin scales, nails, or hair. The KOH dissolves the background keratin, unmasking the fungal elements to make them more apparent.	Suspend fragments of skin scales, nails, or hair in a drop of 10% KOH. Add coverslip over the drop and let sit at room temperature for about half an hour. The mount may be gently heated to accelerate the clearing process. Do not boil. Examine under a microscope for fungal hyphae or spores.
India Ink preparation India Ink (Pelikan brand) or Nigrosin (granular) Microscope slides, 3 × 1-inch Coverslips	India ink or nigrosin preparations are used for the direct microscopic examination of the capsules of many microorganisms. The fine granules of the India ink or nigrosin give a semiopaque background against which the clear capsules can easily be seen. This technique is particularly useful in visualizing the large capsules of Cryptococcus neoformansin cerebrospinal fluid, sputum, and other secretions.	Centrifuge the cerebrospinal fluid or other fluid specimens lightly to concentrate any microorganisms in the sediment. Emulsify a small quantity of the sediment into a drop of India ink or nigrosin on a microscope slide and overlay with a coverslip. Do not make the contrast emulsion too thick, or the transmitted light may be completely blocked. Examine the mount directly under a microscope, using the 10× objective for screening and the 40× objective for confirmation of suspicious encapsulated microorganisms.
		The secretion to be examined is obtained from the patient. In

Darkfield exam Compound microscope equipped with a darkfield condenser Microscope slides, 3 × 1-inch Coverslips Physiologic saline Applicator sticks or curet Paraffin–petrolatum mixture	Darkfield examinations are used to visualize certain delicate microorganisms that are invisible by brightfield optics and stain only with great difficulty. This method is particularly useful in demonstrating spirochetes from suspicious syphilitic chancres for Treponema pallidum.	the case of a chancre, the top crust is scraped away with a scalpel blade and a small quantity of serous material is placed on a microscope slide. Ring a coverslip with paraffin–petrolatum mixture and place over the drop of material. Examine the mount directly under a microscope fitted with a darkfield condenser with a 40× or 100× objective. Spirochetes will appear as motile, bright "corkscrews" against a black background.

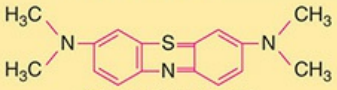
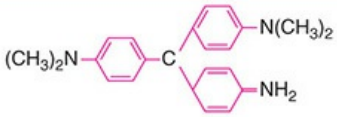
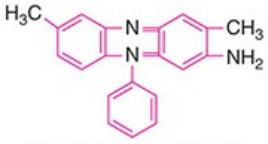
microorganisms is similar to that of the mounting medium, they are not visible when examined by brightfield illumination. Therefore, microorganisms are most commonly stained prior to attempts at visualization. However, sometime, an unstained assessment is made and can be enhanced with certain manipulations of the light source. Often, it is helpful to reduce the amount of light entering the field by closing the iris diaphragm, thereby increasing the contrast between the object being observed and the background. The common practice of lowering the condenser to achieve this effect should be discouraged.

Direct Stains. Biologic stains are generally required to visualize bacteria adequately and sometimes demonstrate the fine detail of internal structures. The introduction of stains in the mid-19th century was, in large part, responsible for the major advances that have occurred in clinical microbiology and in other fields of diagnostic microscopy during the past 100 years. Today, we are so dependent on biologic stains that it is difficult to imagine how the study of bacteria would have progressed without their introduction. The chemical formulas, components, and purposes of the stains commonly used in the microbiology laboratory are shown in Table 1-8.

Stains consist of aqueous or organic preparations of dyes or groups of dyes that impart a variety of colors to microorganisms, plant and animal tissues, or other substances of biologic importance. Dyes may be used as direct stains of biologic materials, as indicators of pH shifts in culture media, as oxidation–reduction indicators to demonstrate the presence or lack of anaerobic conditions, or to demonstrate physiologic functions of microorganisms using so-called supravital techniques.

Almost all biologically useful dyes are derivatives of coal tar. The fundamental chemical structure of most dyes is the benzene ring. Dyes are generally composed of two or more benzene rings connected by well-defined chemical bonds that are associated with color production (chromophores).

TABLE 1-8 Common Biologic Stains Used in Bacteriology

Stain	Chemical Formula	Ingredients		Purpose
Loeffler's methylene blue	 Tetramethyl thionin	Methylene blue Ethyl alcohol, 95% Distilled water	0.3 g 30 mL 100 mL	This is a simple direct stain used to a variety of microorganisms, specifically used to detect bacteria in cerebrospinal fluid smears in suspected cases of bacterial meningitis.
Gram's stain	 Crystal Violet (Hexamethylpararosanilin)  Dimethyl Phenosafranin	Crystal violet Crystal violet Ethyl alcohol, 95% NH_4 oxalate Distilled water Gram's iodine Potassium iodide Iodine crystals Distilled water Decolorizer Acetone Ethyl alcohol, 95% Counterstain Safranin O Ethyl alcohol, 95% Add 100 mL to distilled water	 2 g 20 mL 0.8 g 100 mL 2 g 1 g 100 mL 50 mL 50 mL 2.5 g 100 mL 100 mL	This is a stain used to demonstrate the staining properties of bacteria of all types. Gram-positive bacteria retain the crystal violet dye after decolorization and appear deep blue. Gram-negative bacteria are not capable of retaining the crystal violet dye after decolorization and are counterstained red by the safranin dye. Gram-staining characteristics may be atypical in very young, old, dead, or degenerating cultures.

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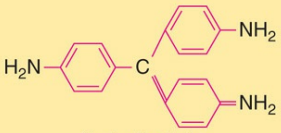
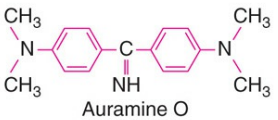
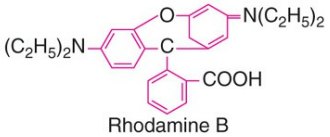
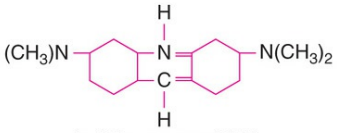
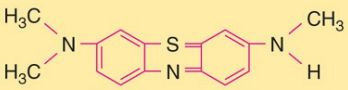
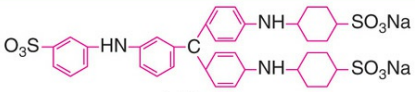
Ziehl–Neelsen acid-fast	 Carbolfuchsin (Triaminotriphenylmethane)	Carbolfuchsin Phenol crystals Alcohol, 95% Basic fuchsin Distilled water Acid alcohol, 3% HCl, concentrated Alcohol, 70% Methylene blue Methylene blue Glacial acetic Distilled water	 2.5 mL 5 mL 0.5 g 100 mL 3 mL 100 mL 0.5 g 0.5 mL 100 mL	Acid-fast bacilli are so called because they are surrounded by a waxy envelope that is resistant to staining. Either heat or a detergent (Tergitol) is required to allow the stain to penetrate the capsule. Once stained, acid-fast bacteria resist decolorization, whereas other bacteria are destained with the acid alcohol.
Fluorochrome	 Auramine O  Rhodamine B  Acridine orange (AO)	Auramine O Rhodamine B Glycerol Phenol Distilled water AO powder Sodium acetate buffer (pH 3.5) (Add about 90 mL 1 M HCl to 100 mL 1 M Na acetate)	1.5 g 0.75 g 75 mL 10 mL 50 mL 20 mg 190 mL	This fluorochrome dye stains mycobacteria selectively by binding to the mycolic acid in the cell wall. This stain demonstrates mycobacteria better than conventional acid-fast stains and permits screening of smears at lower magnification because organisms are more easily seen. Acridine orange is a stain particularly well adapted for the demonstration of bacteria in blood culture broth, cerebro-spinal fluid, urethral smears, or other exudates where they may be present in relatively small numbers, as low as 10^4 CFU mL, or when they are obscured by a heavy background of polymorphonuclear leukocytes or other debris. At pH below 4.0, bacteria and yeast cells stain brilliant orange against a black, light green, or yellow background.
Wright—Giemsa's	Polychrome methylene blue Methylene blue Methylene azure Eosin  Methylene azure B	Powdered Wright's stain Powdered Giemsa's stain Glycerin Absolute methyl alcohol Mix in brown bottle and let stand for 1 month before using.	9 g 1 g 90 mL 2910 mL	Wright—Giemsa's is commonly used for staining the cellular elements of the peripheral blood smear. It is useful in microbiology for the demonstration of intracellular organisms such as *Histoplasma capsulatum* and *Leishmania* species (Image Plate 1G). The stain is also useful in demonstrating intracellular inclusions in direct smears of skin or mucous membranes, such as corneal scrapings for trachoma.
Lactophenol	 Aniline blue	Phenol crystals Lactic acid Glycerol Distilled water Dissolve ingredients, then add: Aniline blue	20 g 20 g 40 mL 20 mL 0.05 g	Because of the sulfonic groups, aniline blue the dye is strongly acidic and has been used as a counter stain for unfixed tissues, bacteria, and protozoa, in combination with other dyes. Currently used for the direct staining of fungal mycelium and fruiting structures which take on a delicate light blue color.

Although the underlying mechanism of the color development is not totally understood, it is theorized that certain chemical radicals have the property of absorbing light of different wavelengths, acting as chemical prisms. Some of the more common chromophore groupings found in dyes are: C=C, C=O, C=S, C=N, N=N, N=O, and NO_2. (Note the presence of these groups in the chemical formulas of the stains shown in Table 1-8.) The depth of color of a dye is proportional to the number of chromophore radicals in the compound.

Dyes differ from one another in the number and arrangement of these rings and in

the substitution of hydrogen atoms with other molecules. For example, there are three key single substitutions for one hydrogen atom of benzene that constitute the basic structure of most dyes: (1) substitution of a methyl group to form toluene (methylbenzene),

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(2) substitution of a hydroxyl group to form phenol (carbolic acid), and (3) the substitution of an amine group to form aniline (phenylamine). Most stains used in microbiology are derived from aniline and are called aniline dyes.

All biologic dyes have a high affinity for hydrogen. When all the molecular sites that can bind hydrogen are filled, the dye is in its reduced state and is generally colorless. In the colorless state, the dye is called a leuko compound. Looking at this concept from the opposite view, a dye retains its color only as long as its affinities for hydrogen are not completely satisfied. Because oxygen generally has a higher affinity for hydrogen than many dyes, color is retained in the presence of air. This allows certain dyes, such as methylene blue, to be used as an oxidation–reduction indicator in an anaerobic environment, because the indicator becomes colorless in the absence of oxygen.

In broad terms, dyes are referred to as acidic or basic. These designations do not necessarily indicate their pH reactions in solution, but rather, whether a significant part of the molecule is anionic or cationic. From a practical standpoint, basic dyes stain structures that are acidic, such as the nuclear chromatin in cells; acidic dyes react with basic substances, such as cytoplasmic structures. If both nuclear and cytoplasmic structures are to be stained in a given preparation, combinations of acidic and basic dyes may be used. A common example is the hematoxylin (basic) and eosin (acidic), the characteristic H&E, stain used in the examination of tissue sections.

The Use of Stains in Microbiology. Microbiologists commonly perform direct microscopic examination on specimens submitted for culture. Not only may it be possible to provide the physician with a rapid presumptive diagnosis, but also the detection of specific microorganisms may serve as a guide for selecting appropriate culture media and provide a valuable quality control comparison with isolates recovered. The positive findings for various staining procedures and diseases in selected specimens that are submitted to microbiology laboratories are listed in Table 1-9. The following is a brief description of the stains most commonly used.

Gram's Stain. Gram's stain, discovered over 100 years ago by Hans Christian Gram, is most commonly used for direct microscopic examination of specimens and subcultures (Table 1-8). The staining procedure is explained in Box 1-6.

Crystal violet (gentian violet) serves as the primary stain, binding to the bacterial cell wall after treatment with a weak solution of iodine, which serves as the mordant to bind the dye. Some bacterial species, because of the chemical nature of their cell

walls, have the ability to retain the crystal violet even after treatment with an organic decolorizer, such as a mixture of equal parts of acetone and 95% ethyl alcohol. Dye-retaining bacteria appear blue-black when observed under the microscope and are called gram positive. Certain bacteria lose the crystal violet primary stain when treated with the decolorizer, presumably because of the high lipid content of their cell wall and less abundant peptidoglycan.

TABLE 1-9 Diagnosis of Infectious Disease by Direct Examination of Culture Specimens

Specimen	Suspected Disease	Laboratory Procedure	Positive Findings
Throat culture	Diphtheria	Gram's stain	Delicate pleomorphic gram-positive bacilli in Chinese letter arrangement
		Methylene blue stain	Light-blue-staining bacilli; with prominent metachromatic granules
	Acute streptococcal pharyngitis	Direct fluorescent antibody technique (after 4–6 hours incubation in Todd–Hewitt broth)	Fluorescent cocci in chains; use positive and negative controls with each stain
Oropharyngeal ulcers	Vincent's disease	Gram's stain	Presence of gram-negative bacilli and thin, spiral-shaped bacilli
Sputum Transtracheal aspirates Bronchial washings	Bacterial pneumonia	Gram's stain	Variety of bacterial types; *Streptococcus pneumoniae* (i.e., gram-positive lancet-shaped diplococci) are particularly diagnostic
	Tuberculosis Pulmonary mycosis	Acid-fast stain Gram's stain, Wright–Giemsa's stain, or Calcofluor white Gram–Weigert's stain	Acid-fast bacilli Budding yeasts, pseudohyphae, true hyphae, or fruiting bodies
Cutaneous wounds or purulent drainage from subcutaneous sinuses	Bacterial cellulitis	Gram's stain	Variety of bacterial types; suspect anaerobic species
	Gas gangrene (myonecrosis)	Gram's stain	Gram-positive bacilli suggesting *Clostridium perfringens* (i.e., gram-positive, boxcar-shaped bacilli); spores usually not seen
	Actinomycotic mycetoma	Direct saline mount Gram's stain or modified acid-fast stain	"Sulfur granules" Delicate, branching gram-positive filaments; *Nocardia* species may be weakly acid-fast
	Eumycotic mycetoma	Direct saline mount Gram's stain or lactophenol cotton blue mount	White, grayish, or black grains True hyphae with focal swellings or chlamydospores

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Cerebrospinal fluid	Bacterial meningitis	Gram's stain	Small gram-negative coccobacilli (*Haemophilus* species) Gram-negative diplococci (*Neisseria meningitidis*) Gram-positive diplococcic (*Streptococcus pneumoniae*)
		Methylene blue stain	Bacterial forms that stain blue-black
		Acridine orange stain	Bacterial forms that glow brilliant orange under ultraviolet illumination
	Cryptococcal meningitis	India ink or nigrosin mount	Encapsulated yeast cells with narrow-necked budding
	Listeriosis	Gram's stain Hanging-drop mount	Delicate gram-positive bacilli Bacteria with tumbling motility
Urine	Yeast infection	Gram's stain or Wright–Giemsa's stain	Pseudohyphae or budding yeasts
	Bacterial infection	Gram's stain	Variety of bacterial types
	Leptospirosis	Darkfield examination	Tightly coiled motile spirochetes
Purulent urethral discharge	Gonorrhea	Gram's stain	Intracellular gram-negative diplococcic
	Chlamydial infection	Direct fluorescent antibody stain of smear	Elementary bodies
Purulent vaginal discharge	Yeast infection	Direct mount or Gram's stain	Pseudohyphae or budding yeasts
	Trichomonas infection	Direct mount	Flagellates with darting motility
	Gardnerella vaginalis	Pap stain or Gram's stain Measure pH of vaginal secretions	"Clue cells" or pH of vaginal secretions >5.5
Penile or vulvar ulcer (chancre)	Primary syphilis	Darkfield mount of chancre secretion	Tightly coiled motile spirochetes
	Chancroid	Gram's stain of ulcer secretion or aspirate of inguinal bubo	Intracellular and extracellular small gram-negative bacilli
Eye	Purulent conjunctivitis	Gram's stain	Variety of bacterial species
	Trachoma	Giemsa stain of corneal scrapings	Intracellular perinuclear inclusion clusters
Feces	Purulent enterocolitis	Gram's stain	Neutrophils and aggregates of staphylococci
	Cholera	Direct mount of alkaline peptone water enrichment	Bacilli with characteristic darting motility; no neutrophils
	Parasitic disease	Direct saline or iodine mounts. Examine purged specimens.	Adult parasites or parasite fragments; protozoa or ova
Skin scrapings, nail fragments, or plucked hairs	Dermatophytosis	10% KOH mount	Delicate hyphae or clusters of arthroconidia
	Tinea versicolor	10% KOH mount or lactophenol cotton blue mount	Hyphae and spores resembling spaghetti and meatballs
Blood	Relapsing fever (*Borrelia*)	Wright's or Giemsa stain Darkfield examination	Loosely coiled spirochetes
	Blood parasites: malaria, trypanosomiasis, filariasis	Wright's or Giemsa stain	Intracellular parasites (malaria, babesia) Extracellular forms: trypanosomes or microfilaria
		Direct examination of anticoagulated blood for the presence of microfilaria	

These decolorized bacteria are then stained with the safranin counterstain and appear pink or red when observed under the microscope; they are called gram negative (Image Plate 1-2A). The visualization of certain fastidious gram-negative bacilli, such as *Legionella* species, can be improved by adding 0.05% carbolfuchsin to the safranin counterstain. These Gram reactions, when observed in conjunction with the morphologic form (cocci and bacilli) and arrangement of bacterial cells, can be used to make presumptive identifications.

Common applications of Gram's stain have been reviewed by Friedly.[48] Gram-positive cocci in clusters suggest staphylococci, whereas cocci in chains suggest streptococci.

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BOX 1-6

Gram's Stain Technique

1. Make a thin smear of the material for study and allow to air-dry.
2. Fix the material to the slide by passing the slide three or four times through the flame of a Bunsen burner or dry on a slide warmer. This is done so that the material does not wash off during the staining procedure. Some workers now recommend the use of alcohol for the fixation of material to be Gram-stained, wherein the slide is flooded with methanol or ethanol for a few minutes)
3. Place the smear on a staining rack and overlay the surface with crystal violet solution.
4. After 1 minute of exposure to the crystal violet stain, wash thoroughly with distilled water or buffer.
5. Overlay the smear with Gram's iodine solution for 1 minute. Wash again with water.
6. Hold the smear between the thumb and forefinger and flood the surface with a few drops of the acetone–alcohol decolorizer, until no violet color washes off. This usually takes 10 seconds or less.
7. Wash with running water and again place the smear on the staining rack. Overlay the surface with safranin counterstain for 1 minute. Wash with running water.
8. Place the smear in an upright position in a staining rack, allowing the excess water to drain off and the smear to dry. Alternatively, one may blot dry.
9. Examine the stained smear under the 100× (oil) immersion objective of the microscope. Gram-positive bacteria stain dark blue; gram-negative bacteria appear pink-red.

Gram-positive, lancet-shaped diplococci, when seen in smears made from respiratory and CSF specimens are highly suggestive of *S. pneumoniae*; these characteristics have a lower positive predictive value in other specimens, because *Acinetobacter,* which sometimes retains the crystal violet in Gram's stain and occasionally enterococci are similar in appearance. Gram-negative, kidney-shaped diplococci are characteristic of *Neisseria* species or *M. catarrhalis*. Large, "boxy" gram-positive bacilli suggest

Bacillus or *Clostridium* species, whereas small gram-positive bacilli suggest *Listeria* species. The coryneforms (diphtheroids) are suspected, if small gram-positive bacilli in "Chinese-letter" or "picket fence" arrangements are observed. Curved, gram-negative bacilli in diarrheal stool specimens suggest *Vibrio* species, whereas very small, helical or corkscrew forms are supportive of a *Campylobacter* species. Gram-negative bacilli are some of the bacteria most commonly encountered in clinical laboratories and include the *Enterobacteriaceae,* which are plump, the nonfermentative bacilli, which are thinner in diameter than *Enterobacteriaceae*, *Haemophilus* species, which are coccobacillary, and a variety of fastidious species. Selected Gram-stained images are included in Image Plate 1-3, which are discussed in more detail in a later section of this chapter.

Gram's stain is a deceptively simple procedure, but the appropriate performance, which includes interpretation, is highly complex. Considerable experience, careful training, and correlation with culture results are essential to achieve a high level of proficiency. The intermittent performer of Gram's stain will not usually do as well as someone who views them regularly, particularly with respect to the subtleties that really only come with experience and correlation with culture. For example, an interesting study examined the performance of medical house staff and experienced microbiologists in the diagnosis of community-acquired pneumonia.[45] The house staff were better than the nursing personnel at obtaining a purulent sputum specimen, but their preparation and interpretation of the Gram-stained smears was inferior to that of the microbiologists.

There are some microbiologic and technical reasons for the importance of experience. Most of the time bacterial morphology matches the classical descriptions. Occasionally, this is not the case. With good training, practice, and a willingness to learn from mistakes, the microbiologist will learn the pitfalls. Some of the classical challenges that may be encountered in Gram's stain interpretations are summarized in Table 1-10. A cardinal rule is that the final interpretation must be made on the basis of staining color, bacterial morphology, and known variants. To complicate matters still further, a variety of artifacts can resemble infectious agents and be reported mistakenly as a microbe. Some of these potential sources of error are illustrated in Image Plate 1-4. If the possibility of an artifact is considered, a useful maneuver is to stain another smear with AO (see below); with this stain it can be established whether the structure contains DNA and is, therefore, biologic. Although the staining procedure is simple, the decolorization step can cause problems if not performed properly. If acetone, rather than acetone–alcohol is used as the decolorizer, particular care must be taken because it acts very quickly. Under-decolorization can be monitored by observing the nuclei of inflammatory cells; if they are not completely gram negative, then the smear has not been adequately decolorized. The only recipe for detecting over-decolorization is cross-checking the Gram reaction and the morphology of the

bacteria with known staining characteristics. If the quality assurance program (see below) includes a review of smears that do not correlate with cultures, improper decolorization and nonbacterial artifacts that have been reported erroneously can be detected and the reader of the smear can learn from the mistake.

Gram's stain can also be used to identify nonbacterial forms such as fungi, trichomonads, *Strongyloides* larvae, *Pneumocystis jiroveci* cysts, and *Toxoplasma gondii* trophozoites, although it is not as sensitive as other special stains used for visualizing these organisms. These various applications demonstrate the versatility of Gram's stain.

Acid-Fast Stains. The cell walls of mycobacteria essentially coats them with a thick, waxy material that resists

TABLE 1-10 Pitfalls in the Interpretation of Gram's Stains

Organism	Classic Presentation	Variant Presentation	Comments
Streptococcus pneumoniae	Gram-positive, lancet-shaped, diplococci	Elongated cocci, resembling short bacilli	May be misinterpreted as mixed organisms; over-decolorized cells may be mistaken for gram-negative coccobacilli.
Acinetobacter spp.	Gram-negative coccobacilli	Gram-negative cocci; gram-variable staining is common	May be mistaken for *Neisseria* spp. and reported as gram-negative cocci; search the smear to find some organisms that demonstrate elongated forms, which are not seen in *Neisseria*
Clostridium perfringens	Boxcar-shaped gram-positive bacilli	Gram-positive cocci	May be mistaken for *Streptococcus pneumoniae* and reported as gram-positive cocci; in addition to a coccal form, cells retain crystal violet tenaciously during decolorization.
		Gram-variable or gram-negative bacilli	May be mistaken for gram-negative bacilli; the boxcar shape is a clue that the organism is gram-positive; other *Clostridia* and *Bacillus* spp. may also appear similar.
Yeast, especially *Cryptococcus neoformans*	Gram-positive round or oval cells with budding	Gram-variable cells	May be mistaken for artifacts; size and shape distinguish them from bacteria.

staining; once stained, however, the bacterial cells resist decolorization by strong organic solvents such as acid–alcohol. Consequently, these bacteria are known as acid-fast, a phenomenon first discovered in 1881 by Ziehl and Neelsen.

Special treatment is required for the primary stain, carbolfuchsin, to penetrate the waxy material of the acid-fast bacilli. Heat is used in the conventional Ziehl–Neelsen technique. After the carbolfuchsin is overlaid on the surface of the smear to be stained, traditionally the flame of a Bunsen burner is passed back and forth beneath the slide. The smear is heated to steaming, stopping short of boiling. The Kinyoun modification of the acid-fast stain is called the "cold method" because a surface-active detergent, such as Tergitol, is used rather than heat treatment.

With either of these stains, the acid-fast bacilli appear red against either a green or

blue background depending on the counterstain used (Image Plate 1-2B). Although this method is satisfactory for most mycobacteria, certain weakly acid-fast strains of rapidly growing species (e.g., *Mycobacterium fortuitum* or *M. chelonae*) may stain better with the Ziehl–Neelsen method (see Chapter 19). In addition, certain bacteria, such as *Nocardia* spp., characteristically exhibit partial or weak acid-fastness.

Fluorescent Stains. Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TMRI) are two commonly used fluorochromes that, on excitation with ultraviolet or short-wavelength visible light, emit light waves in the visible range, with absorption maxima of 490 nm and 555 nm, respectively. These fluorochromes bind chemically with a variety of proteins, including antigens and antibodies, providing a fluorescent signal that can be visualized in direct smears of biological materials. Fluorochrome/protein ratios vary with different reagents for optimal staining of the desired objects with a minimum of nonspecific background distraction. The current development of monoclonal antibodies, which are specific for their respective antigens, has led to the preparation of fluorescent reagents for direct and indirect detection of several pathogens including *Legionella* species, *T. pallidum*, *T. gondii*, and several viruses, including varicella zoster, herpes simplex, influenza, cytomegalovirus, and respiratory syncytial viruses, among others.

Fluorescence microscopy is an exacting technique that requires a microscope of high quality, the proper combination of microscope objectives, bright and darkfield condensers, a mercury arc or halogen ultraviolet light source, and appropriate combinations of exciter and barrier or suppression filters.[22] Achromatic objectives are satisfactory for most applications, except in research applications where expensive apochromatic lenses may be needed to achieve maximum illumination and resolution. The selection of microscope slides and coverslips of proper thickness and the use of low-fluorescing immersion oils and mounting fluids are critical for optimal performance.

The choice of filters in fluorescence microscopy is also critical to successful work. Four filters are required in sequence: (1) one to absorb heat (to prevent damage to the exciter filter), (2) an exciter filter with a wave bandwidth appropriate for the wavelength of light produced by the excited fluorochrome, (3) a red-absorbing filter to block any red light emitted by the blue excitation filters, and (4) a barrier filter to absorb any of the residual short-wavelength incident excitation light (which would damage the eyes of the microscopist), allowing only the longer-wavelength visible light to pass. Suboptimal performance of a fluorescence microscope system is often due to poor selection of filter combinations. Manufacturers of fluorescence equipment provide information and consultation so that users can achieve optimal performance. Fluorescence systems that employ (1) epi-illumination, (2) blue light halogen lamps that do not require expensive transformers, and (3) interference filters with maximum absorption peaks at the longer visible wavelengths are now available within a price

range acceptable to most clinical laboratory directors.

Fluorochrome Stains for Mycobacteria. The fluorochrome dyes auramine and rhodamine can be used to demonstrate acid-fast bacilli. Viewed by fluorescence microscopy,

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the bacterial cells appear yellow, red, or orange (depending on the filter combination and dyes used); the background is dark when potassium permanganate is used as the counterstain (Image Plate 1-2C). Use of the fluorescence procedure facilitates the screening of smears, particularly when a 25× objective is utilized. This objective provides magnification low enough to scan wide microscopic fields, yet sufficiently high to see the yellow light points of light emanating from the fluorescing bacterial cells (Image Plate 1-2C). Higher magnification can be used to confirm suspicious objects observed with the 25× lens. It is also a common practice in some laboratories to confirm mycobacteria detected by this method with a traditional acid-fast stain, as described above.

The acid-fast stains can also be used to identify nonbacterial microorganisms. The oocysts of *Cryptosporidium* species and *Cystoisospora* (formerly *Isospora*) *belli*, two coccidian organisms that are known etiologic agents of gastroenteritis, are acid-fast and can be readily detected in appropriately stained preparations of stool (Image Plate 22-4BJ).

Acridine Orange. The AO stain is used with increasing frequency in microbiology laboratories to detect bacteria in smears prepared from fluids and exudates in which bacteria are expected to be in low concentration (10^3 to 10^4 colony-forming units [CFU]/mL) or are trapped within a heavy aggregate of background debris, making them difficult to visualize by conventional staining procedures. The AO stain was originally used by microbiologists to demonstrate bacteria in soil samples. As in the application of fluorochrome dyes for studying acid-fast bacilli, smears stained with AO and examined under ultraviolet light can be more rapidly and efficiently screened at low-power magnifications (100×), reserving magnifications of 450× or higher when suspicious forms are visualized. The stain detects both living and dead bacteria, but does not indicate whether they are gram negative or gram positive. Once bacteria have been detected using the AO stain, a Gram's stain must be used to determine their differential-staining characteristics (Image Plate 1-2D).

Lauer and associates have found the AO stain to be more sensitive than Gram's stain in detecting bacteria in CSF sediments, particularly when gram-negative bacteria are present.[70] The AO stain has also been useful to screen urine specimens for significant bacteriuria.[59] In Box 1-7 preparation of an AO stain is outlined.

Toluidine Blue and Methylene Blue. Toluidine blue, a dye closely related to azure

A and methylene blue, is used for staining lung biopsy imprints and respiratory secretions to detect *P. jiroveci*. Methylene blue stains may be performed on spinal fluid sediments along with Gram's stains. This may be useful to detect the gram-negative bacterial cells of *H. influenzae* and *N. meningitidis* that often do not stand out against the red-staining background in Gram's stains. With methylene blue, polymorphonuclear leukocytes stain blue; the bacterial cells are also deep blue and easier to detect against the light gray-staining background (Image Plate 1-2E). Methylene blue stains should be considered as an adjunct to Gram's stains in laboratories where the inaccessibility to a fluorescence microscope precludes the use of the AO procedure.

Calcofluor White. Calcofluor white, a colorless dye used in industry to whiten textiles and paper, has two properties that make it useful in microbiology: (1) binding to beta 1-3, beta 1-4-polysaccharides (specifically cellulose and chitin); and (2) fluorescence when exposed to long-wavelength ultraviolet and short-wavelength visible light. Calcofluor white is a valuable fluorochrome stain for the rapid detection of fungi in wet mounts, smears, and tissues, because the cell walls of fungi and plants are rich in chitin. The stain has been most useful in detecting yeast cells, hyphae, and pseudohyphae in skin and mucous membrane scrapings. When mixed with 10% potassium hydroxide, mounts of skin scrapings can be screened for dermatophytes rapidly. Viewed microscopically under ultraviolet light, fungal structures display a brilliant apple-green or a blue-white color (Image Plate 1-2F), depending on the wavelength of the exciter light and the filter combinations used. The fungi are readily differentiated from background debris, cells, and tissue fragments based on their morphologic features, which are well highlighted. Calcofluor white has the added advantage that tissue sections can be stained subsequently with periodic acid–Schiff stain (PAS), Gomori methenamine silver (GMS), or other special stains without interference, should confirmation of findings or the availability of permanent slides be desired. The calcofluor white-staining technique is rapid and provides good definition of fungal fine structures and better contrast from the background than the widely used lactophenol aniline blue stain.[56]

BOX 1-7

Preparation of an AO Stain

Ingredients: AO powder 20 mg, sodium acetate buffer 290 mL, HCl 1M

Reagent preparation: Add 20 mg AO powder (JT Baker Chemical Co., Phillipsburg, NJ) to 290 mL of sodium acetate buffer (stock solution of 100 mL of 2 molar [M]$CH_2COONa.3H_2O$ and 90 mL of 1M HCl); 1M HCl should be added as necessary to maintain the differential staining of the bacteria against the

background debris.[84]The staining solution should be stored in a brown bottle at room temperature.

Procedure: The stain is performed by flooding air-dried and methanol-fixed smears of the material to be examined with the AO stain for 2 minutes, followed by a washing with tap water. The stained slides are dried and examined with a microscope equipped with an ultraviolet light source.

Silver Impregnation Stains. Certain bacteria, for example, spirochetes (including the etiologic agent of Lyme disease, *Borrelia burgdorferi*) and the small bacillary organisms associated with cat scratch disease (*Bartonella henselae*), are not readily stained by conventional methods. These organisms are either too slender to be visualized by brightfield microscopy, they are not present in sufficient concentrations to be detected, or their chemical composition does not interact with the stains. Darkfield microscopy has been used to identify *T. pallidum*, the etiologic agent of syphilis, and

other nontreponemal spirochetes, such as *Leptospira interrogans*, the cause of leptospirosis. One limitation of the darkfield procedure is the necessity to examine wet, moist specimens containing living organisms very quickly, because visualization of the moving bacteria is essential to detection. The silver stain has been used to observe these organisms in tissue sections and immunofluorescent or immunohistochemical reagents are available for some pathogens, such as *T. pallidum*.

The Warthin–Starry, Dieterle, and Steiner silver impregnation stains have been used for years to demonstrate spirochetes in formalin-fixed tissue sections. They perform equivalently. However, we (author GWP) have demonstrated that the detection of *T. pallidum* by silver precipitation stains is inferior to detection using an immunohistochemical method.

Wright–Giemsa's Stain. The Wright–Giemsa's stain is commonly used for staining the cellular elements of the peripheral blood smear. This stain has little use for staining bacteria, but is used primarily to detect the intracellular yeast forms of *Histoplasma capsulatum* or the intracellular amastigotes of *Leishmania* species or *Trypanosoma cruzi* (Image Plate 1-2G). The stain is also helpful for demonstrating certain intracellular viral inclusions (Table 1-8).

Periodic Acid–Schiff. The PAS stain is based on the oxidation of hexoses and hexosamines by periodic acid, which cleaves the pyranose rings in these molecules, producing dialdehydes that react with Schiff reagent. Schiff reagent is a triphenylmethane dye prepared from basic fuchsin or *p*-rosaniline by reduction with

sulfuric acid. Most substances that contain hexoses or hexosamines are PAS-positive, staining red against a green or blue background, depending on the counterstain used. The stain is most frequently used for staining tissue sections to demonstrate fungi (Image Plate 1-2H). The PAS stain highlights glycogen, which is a limitation of the stain in glycogen-rich tissues, such as the liver. Pretreatment of glycogen-rich tissues with diastase, which substantially reduces the amount of glycogen in such tissues, is needed prior to PAS staining.

Processing Specimens. After a specimen for culture has been received in the microbiology laboratory, the following decisions must be made:

1. Selection of primary culture media appropriate for the particular specimen.
2. Determine the temperature, atmosphere, and length of incubation to recover organisms of potential significance.
3. Determine which of the isolates recovered on primary media require further characterization.
4. Determine whether antimicrobial susceptibility tests are required.

No single approach can be expected to serve the needs of all laboratories and clinical settings. The protocol in a 50-bed rural community hospital will differ from that used in a large, tertiary care medical center. Everyone recognizes, however, the difficulties in maintaining quality services in an environment of more and more stringent demands for containment of costs. Laboratory directors and supervisors must identify and eliminate the clinically irrelevant work to optimize the limited resources. In addition, the establishment of evidence-based, cost-effective strategies will hopefully help to eliminate the "pan-culture" of every clinical specimen (i.e., the indiscriminate ordering of cultures from all accessible body sites in the hope of recovering a pathogen). For the past several decades, many microbiologists have been attempting to practice what Bartlett calls "processing control," "restricting the processing and reporting of culture specimens to the production of predictably useful information."

Selection of Primary Culture Media. Only a few media are required for daily use in the diagnostic laboratory. Agar plates are commonly used. Inoculation of broth media for primary recovery of organisms should be limited to those few specimens for which supplemental broth media have proven useful (Table 1-6). The past practice of inoculating thioglycolate broth routinely to recover pathogens has been abandoned in most laboratories. In almost all instances, the recovery of an organism in broth culture after 4 or 5 days of incubation will have little clinical relevance. Incubation of broths for prolonged periods also leads to the frequent recovery of contaminants.[85] Bacterial isolates in very low concentrations are rarely significant, and the prolonged time for recovery will usually render the information irrelevant for effective management. In

some laboratories, broth media are inoculated for certain types of specimens, but they are examined only if growth is not detected on agar plates or if bacterial morphotypes observed in a direct smear of patient material are not recovered on agar.

There are situations in which broth cultures are useful or even essential. The most obvious one is in the culture of blood, where a single pathogen is expected in most situations and commensal flora are not expected. A few other clinical situations meet these guidelines and broth cultures may be useful: spontaneous bacterial (primary) peritonitis (as opposed to peritonitis after disruption of an abdominal viscus),[10] peritoneal infections in patients who are receiving peritoneal dialysis,[2] cultures from prosthetic material that may harbor organisms in a biofilm state, cardiac valves from patients with endocarditis who have been extensively treated with antibiotics and septic arthritis.[60]

The media used may be selective or nonselective. Nonselective media are free of inhibitors and support the growth of most microorganisms encountered in clinical laboratories. Five percent sheep blood agar is the most commonly used nonselective medium and is included in the battery of primary isolation media for virtually every clinical specimen. Horse blood agar, sheep blood agar supplemented with additives such as IsoVitaleX (or a similar supplement that includes nicotinamide adenine dinucleotide [NAD] and a heme product), or chocolate agar (agar to which partially hemolyzed blood or hemoglobin powder has been added) is necessary for the recovery of *H. influenzae* because it has no inhibitory effects on bacterial growth and is a rich source of Factor X. Chocolate agar is also important for recovery of *N. gonorrhoeae* and other fastidious bacteria.

Blood agar can be made selective by adding antibiotics or inhibitory chemicals. It is a general rule that inhibitory agars should not be used alone, because they usually inhibit the organisms of interest—just less so than other microbiota. Often the inhibition is only partial, so growth on a selective agar should not be taken as proof that the targeted organism has been isolated. For instance, enterococci and yeast often break through and produce small colonies on MacConkey agar, especially if the formulation does not include crystal violet.

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Media can also be made differential by addition of certain dyes, sugars, or other chemicals, thus providing some clues to the identity of the isolated microbes. Some of the commonly used selective and differential agars are summarized in Table 1-11.

Techniques for Transfer and Culturing of Clinical Specimens. Once a specimen is found acceptable for culture (i.e., did not demonstrate any of the various criteria for rejection), appropriate portions must be transferred to the culture media described previously. Media inoculation is

TABLE 1-11 Commonly Used Differential and Inhibitory Agars

Inhibitory (I) or Differential (D) Agar	Added Compounds (I or D)	Organisms Inhibited	Organisms Enriched	Comments/Chapter Reference
Bacteroides bile esculin (BBE) (I, D)	Bile salts (I); esculin (D)	Most bacteria	*Bacteroides fragilis* group	Bile stimulatory for *B. fragilis*
Campy-BAP (I)	Bacitracin, novobiocin, colistin, cephalothin, polymyxin B (I)	Most bacteria	*Campylobacter jejuni*	Incubation at 42°C also selects for *C. jejuni*
CCFA (I, D)	Cycloserine, Cefoxitin (I); Fructose (D)	Most bacteria	*Clostridium difficile*	Yellow colonies; very inhibitory
CHROMagar (D)	Various (D)	NA	Various	Identification of colonies suggested by color
CIN (I)	Cefsulodin, Irgasan, novobiocin (I)	Most bacteria	*Yersinia* spp., *Aeromonas* spp.	Several formulations available
CNA (I)	Colistin, nalidixic acid (I)	Gram-negative bacteria	Gram-positive bacteria	Most strains of *Staphylococcus saprophyticus*[48] and some strains of *S. aureus* are inhibited.
EMB (I, D)	Eosin (I); eosin, methylene blue, lactose, sucrose (D)	Gram-positive bacteria	Gram-negative bacteria	Slightly selective (inhibitory);[49] lactose or sucrose fermenting organisms produce blue-black colonies (sucrose fermenters, such as *Yersinia enterocolitica*, appear identical to lactose fermenters); strong lactose fermenters (such as *Escherichia coli* or *Candida kefyr*) produce a characteristic green sheen.
Hektoen enteric (HE) (I, D)	Bile salts (I); lactose, sucrose, salicin, bromthymol blue, acid fuchsin (D); sodium thiosulfate, ferric ammonium citrate for hydrogen sulfide production (D)	Gram-positive bacteria	Enteric pathogens[a]	Moderately inhibitory; lactose (sucrose, salicin) fermenters produce green colonies; hydrogen sulfide producers form black colonies.
LKV (I)	Kanamycin, vancomycin (I)	Aerobic bacteria; anaerobic gram-positive bacteria	Anaerobic gram-negative bacilli, particularly *Bacteroides*	Laked blood added as a source of nutrients; vancomycin-resistant enterococci may be selected on this medium.
MacConkey (I, D)	Bile salts, crystal violet (I); lactose, neutral red (D)	Gram-positive bacteria	Enteric pathogens[a]	Moderately inhibitory (selective); lactose fermenters produce red colonies; formulations without crystal violet available
Mannitol-salt (I, D)	NaCl (I); Mannitol, phenol red (D)	Gram-negative bacteria; gram-positive bacteria other than *Staphylococcus*	*Staphylococcus aureus*	Coagulase-negative staphylococci grow on salt agar, but do not ferment mannitol.
Mycobiotic/Mycosel (I)	Chloramphenicol, cycloheximide (I)	Bacteria; saprophytic fungi (and a few fungal pathogens)	Dermatophytes, dimorphic fungi	
PC (*Pseudomonas* [*Burkholderia*] *cepacia*) (I, D)	Crystal violet, bile salts for gram-positive bacteria (I); polymyxin B, ticar cillin for gram-negative bacteria (I); pyruvate (D)	Gram-positive bacteria; most gram-negative bacteria	*Burkholderia cepacia*	Highly selective; pink colonies not completely specific for *B. cepacia*

PEA (I)	Phenylethyl alcohol (I)	Gram-negative bacteria	Gram-positive bacteria	
Salmonella–Shigella (SS) (I, D)	Bile salts, brilliant green (I); lactose, neutral red (D); sodium thiosulfate, ferric ammonium citrate for hydrogen sulfide (D)	Gram-positive bacteria	Enteric pathogens[a]	More inhibitory (selective) than MacConkey and EMB agars; *Shigella* spp. may be inhibited.
Sorbitol–MacConkey (I, D)	Bile salts, crystal violet (I); sorbitol, neutral red (D)	Gram-positive bacteria	*Escherichia coli* O157 (sorbitol negative)	Screening agar for shigatoxin-producing *E. coli*
TCBS (I, D)	Sodium thiosulfate, sodium citrate, NaCl for gram-negative bacteria (I); bile salts, NaCl for gram-positive bacteria (I); sucrose, thymol blue–bromthymol blue (D)	Gram-positive bacteria; most gram-negative bacteria	*Vibrio* spp.	Sucrose fermenting species appear yellow; species that utilize citrate appear blue.
GC selective media (Modified Thayer–Martin, Martin–Lewis, etc.) (I)	Vancomycin for gram-positive bacteria (I); colistin for gram-negative bacteria (I); trimethoprim for swarming *Proteus* (I); nystatin, amphotericin B, or anisomycin for yeast (I); supplements for growth	Gram-positive bacteria; gram- negative bacilli	*Neisseria gonorrhoeae, N. meningitidis*	Multiple formulations available. Some strains of *N. gonorrhoeae* are inhibited by vancomycin. Optimally, chocolate agar should also be inoculated.
XLD (I, D)	Bile salts, NaCl (I); lactose, sucrose, xylose, phenol red (D); sodium thiosulfate, ferric ammonium citrate for hydrogen sulfide production (D)	Gram-positive bacteria	Enteric pathogens[a]	Performs similar to Hektoen enteric agar

[a]Enteric pathogens: *Salmonella* spp., *Shigella* spp., and *Yersinia* spp.

usually carried out in a designated part of the laboratory and should be carried out in a BSC (see below). The best policy is to handle all specimens as if they were highly infectious (i.e., universal precautions). Personnel should be required to wear rubber gloves and other personal protective equipment when handling clinical specimens. The wearing of a surgical mask is optional, but is unnecessary for most operations in the diagnostic microbiology laboratory, with the exception of some of the worked necessary in mycobacteriology.

The inoculation area should be equipped with all of the necessary implements and stocked with appropriate culture media. Most media must be refrigerated for prolonged storage, but must be allowed to warm to room temperature before inoculation.

Although full-time personnel working in the setup area may have memorized the culture media required for each specimen type, it is essential to have appropriate charts and instructions readily available, and included in a bench manual for use by those who perform the tasks infrequently. Every attempt should be made to use well-trained personnel, under close supervision, for the processing of specimens. Errors or

misjudgments made during this phase of the diagnostic cycle can negate all the expertise one may apply in the assessment and interpretation of cultures. Expert microbiologists and technologists have occasionally been deterred in making a definitive diagnosis because inadequate or incorrect media were selected for a specimen.

Techniques for Culturing Specimens. The equipment required for the primary inoculation of specimens is relatively simple. A Nichrome or platinum inoculating wire or loop is recommended (Fig. 1-6), with one end inserted into a cylindrical handle for easy use. Disposable, sterile plastic loops are an acceptable alternative. The surface of agar media in Petri plates may be inoculated with the specimen by several methods, one of which is shown in Figure 1-7. The primary inoculation can be made with a loop, swab, or other

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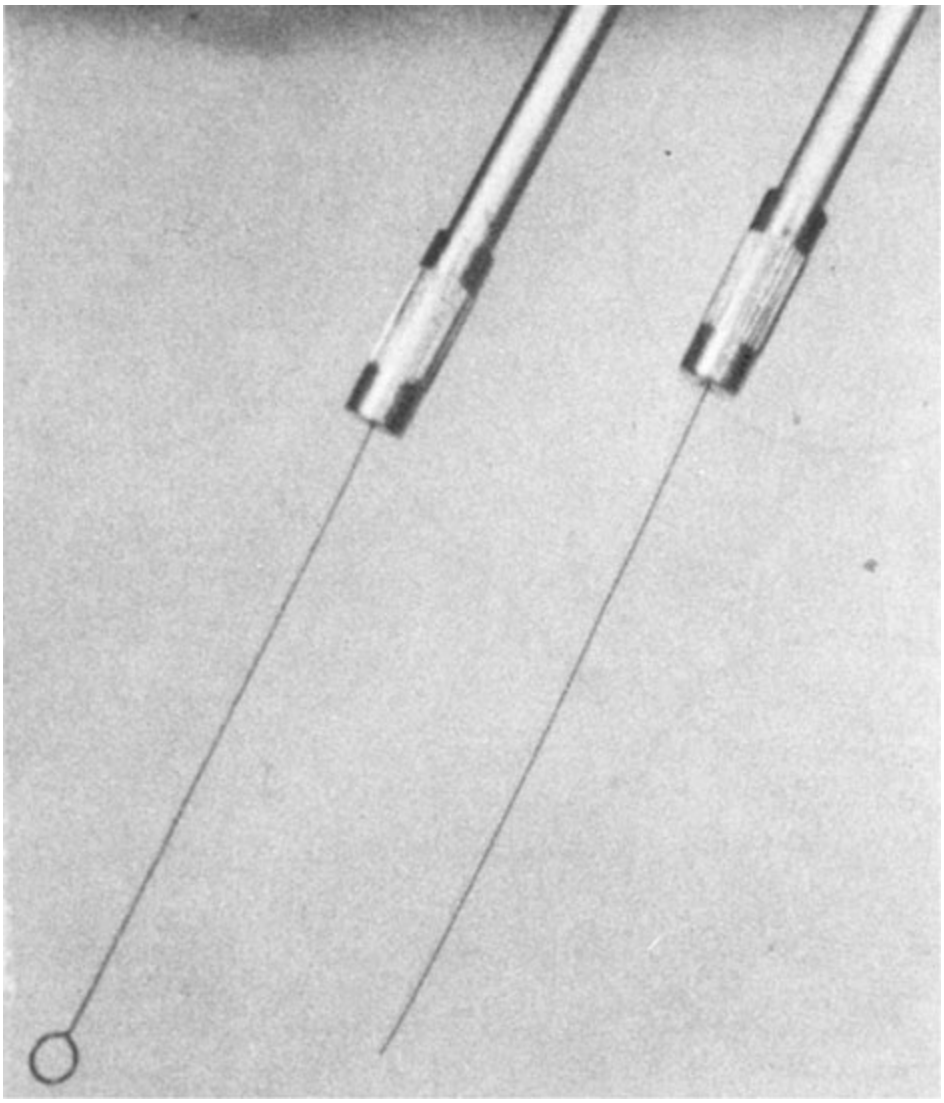
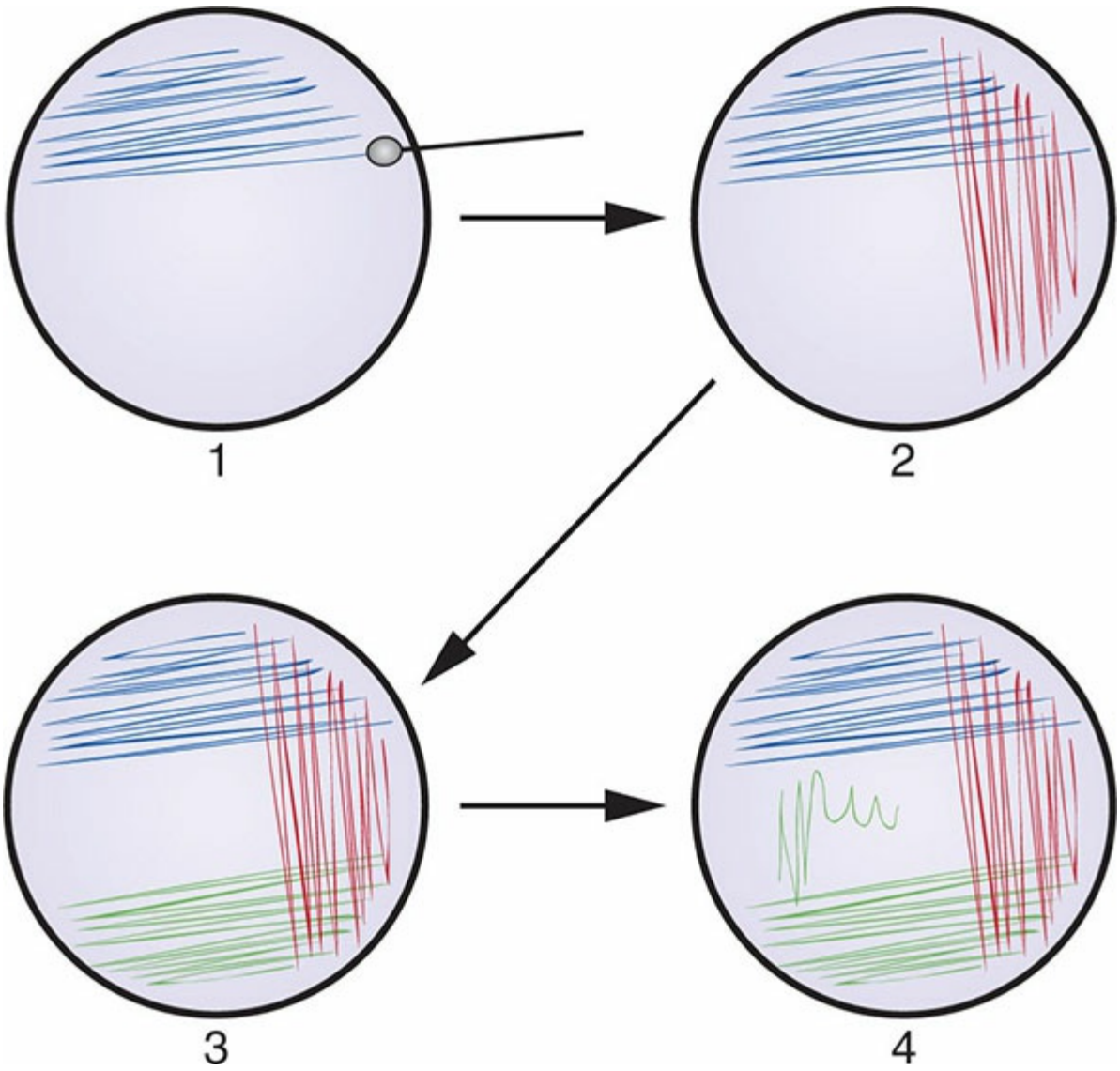


■ **FIGURE 1-6** Loop and straight wires commonly used for the transfer of isolates and subculturing. Although these may also be used for the inoculation of specimens, disposable loops are also commercially available.

■ **FIGURE 1-7** The surface of an agar plate being inoculated with a specimen contained within an inoculating loop. Inoculation is accomplished by first touching the surface of the

agar in one small area, then streaking the surface with a back-and-forth motion in a pattern shown in F igure 1-8.



■ **FIGURE 1-8** Streaking pattern for the inoculation of specimens onto culture plates to obtain isolated bacterial colonies.

suitable device. Once the primary inoculum is made, a loop or straight wire can be used to spread the material into the four quadrants of the plate, as illustrated in Figure 1-8. The inoculum is successively streaked with a back-and-forth motion into each quadrant by turning the plate at 90-degree angles. The loop or wire should be sterilized between each successive quadrant streak. The purpose of this process is to dilute the inoculum sufficiently on the surface of the agar medium so that well-isolated colonies

of bacteria are obtained. The isolated colonies can then be subcultured individually to other media to obtain pure populations for further study. When streaking blood agar plates with throat swabs submitted for streptococcal screens, multiple stabs should be made in the areas of inoculation to disclose the oxygen-labile hemolysins, enhancing the detection of β-hemolytic streptococci. Also, bits of tissue that have been submitted for the recovery of fungi should be submerged beneath the surface of the agar (i.e., pushed into the agar). The initial growth of many species of fungi is enhanced in the microaerophilic atmosphere just beneath the agar surface. In addition, the filamentous fungi are frequently not disassociated from the tissue in which they are invasive and may not be recovered, if portions of tissue are not added to the agar plates.

The streaking technique used to inoculate agar media for semiquantitative colony counts is illustrated in Figure 1-9. This is most commonly performed for urine cultures. Nonferrous (Nichrome or platinum) or disposable plastic inoculating loops, calibrated to contain either 0.01 or 0.001 mL of fluid, are immersed into an uncentrifuged urine sample.[1] The loop is then carefully removed and the entire volume within the loop is delivered to the surface of an agar plate by making a single streak across the center. The inoculum is spread evenly at right angles to the primary streak; then the plate is turned 90 degrees and the inoculum is spread to

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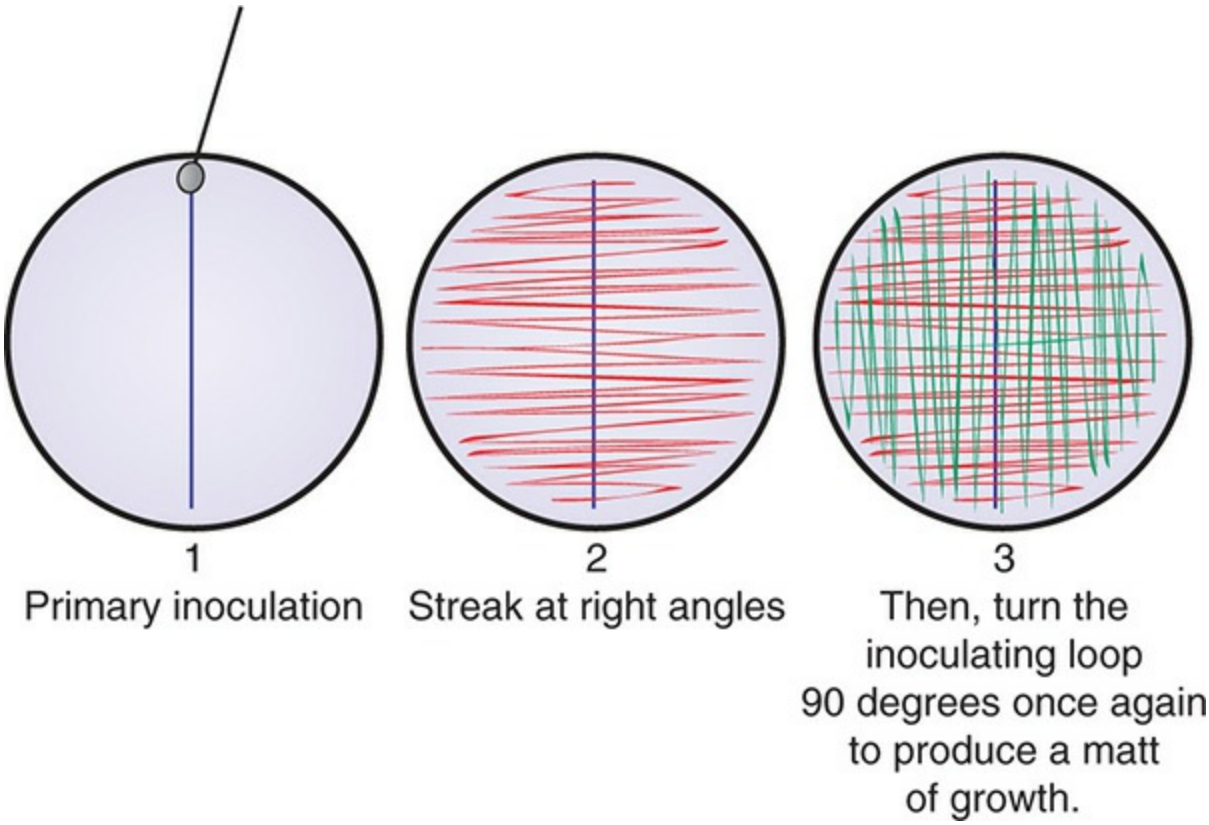


■ **FIGURE 1-9** Culture plates demonstrating streaking patterns of specimens for which a semiquantitative bacterial count is to be performed.

cover the entire surface. In some laboratories, two plates are inoculated, one with the 0.01-mL loop and the other with the 0.001-mL loop, serving as a quality control check. Although the inoculating loops are calibrated to deliver the volume of urine prescribed, accuracy has an error rate of as much as ±50%, particularly when using the 0.001-mL loop. Vertical sampling from a small container may deliver only 50% of the prescribed volume; horizontal sampling at a 45-degree angle from a large container may deliver 150% of volume. Microbiologists should be aware of these potential errors and derive a standard angle for sampling in their laboratory, based on the volume of containers being used.

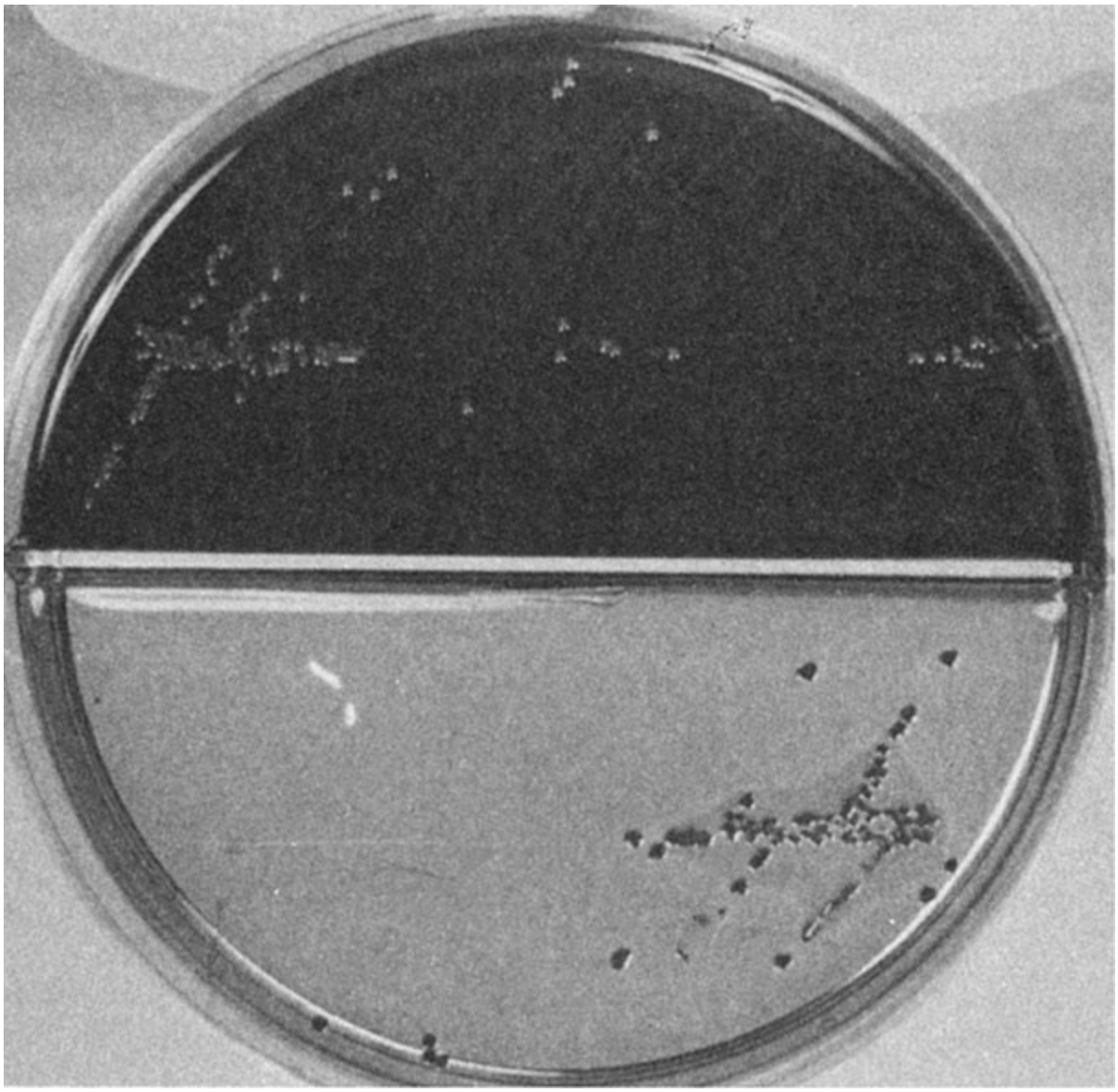
Accuracy and precision studies on the volume of inoculum can be done (1) photometrically, by adding a loop of gentian violet sampled from a 60-mL reservoir of dye to 2 mL of water in a cuvette and reading it in a spectrophotometer set at 590 nm, or (2) manometrically, by noting the change in weight when a loop of water is delivered to a filter paper disk placed in the pan of a highly sensitive analytical balance. Devices are available to deliver a standard inoculum from fluid samples.[73]

After 18 to 24 hours of incubation, the number of bacteria in urine samples is estimated by counting the number of colonies on the surface of the agar. As illustrated

in Figure 1-10, approximately 50 colonies are present. If a 0.001-mL loop had been used to inoculate the medium, the number of colonies would be multiplied by 1,000. Therefore, the count in this illustration is 50,000 CFU/mL.

Although semiquantitative techniques are used most commonly with urine, they have been employed in other situations also. Quantitating the bacteria recovered from the lower respiratory tract by bronchoscopic techniques may help in interpretation of these cultures.[21] When bacteria were present at a concentration greater than 10^3 to 10^4 CFU, the probability that they were causing pneumonia was greater than when lesser quantities were present. Considerable work can be avoided by not evaluating small quantities of contaminating nasopharyngeal, throat, and oral microbiota.

Semiquantitative cultures are commonly employed to evaluate the likelihood that intravascular catheters are the source of a bacteremia. Although a variety of techniques have been employed, the most prevalent approach is to roll the resected segment of catheter on the surface of agar.[74] There is a statistical association of isolated bacteria with catheter-related bacteremia if more than 15 colonies of the bacterium are present. In this approach, the catheter is removed in order to perform the test. Quantitation of bacteria in blood drawn through an intravascular catheter, which is left in place, has also been used to assess the role of the catheter in an infectious process.[46]



■ **FIGURE 1-10** Blood agar–MacConkey agar biplate previously streaked for a semiquantitative colony count demonstrates approximately 50 colonies on each side of the plate. If a 0.001-mL calibrated semiquantitative urine inoculating loop had been used for streaking each medium, the colony count would be 50,000 colony-forming units (CFU) per mL.

Quantitation may also be useful in virology for determining the significance of a virus, such as cytomegalovirus, that can produce persistent or latent infection.[11] In addition, the effectiveness of antiviral chemotherapy can be monitored by following the quantity of virus present.[61] Many of the quantitative tests for viral disease (viral load testing) employ molecular detection and measurement of viral nucleic acid rather

than culture.

Media in tubes may be liquid, semisolid (0.3% to 0.5% agar), or solid (1% to 2% agar). Semisolid agar is most commonly used for motility testing. Broth medium in a tube can be inoculated by the method shown in Figure 1-11. The tube should be tipped at an angle of approximately 30 degrees and an inoculating loop touched to the inner surface of the glass, just above the point where the surface of the broth makes an acute angle. When the culture tube is returned to its upright position, the area of inoculation is submerged beneath the surface. Laboratory directors and microbiology supervisors, in an evidence-based manner, must determine which specimens should be transferred to broth cultures routinely (Table 1-6).

Slants of agar medium are inoculated by first stabbing the depth of the agar, followed by streaking the slant from bottom to top with an S-motion as the inoculating wire is removed (Figs. 1-12 and 1-13). When inoculating semisolid

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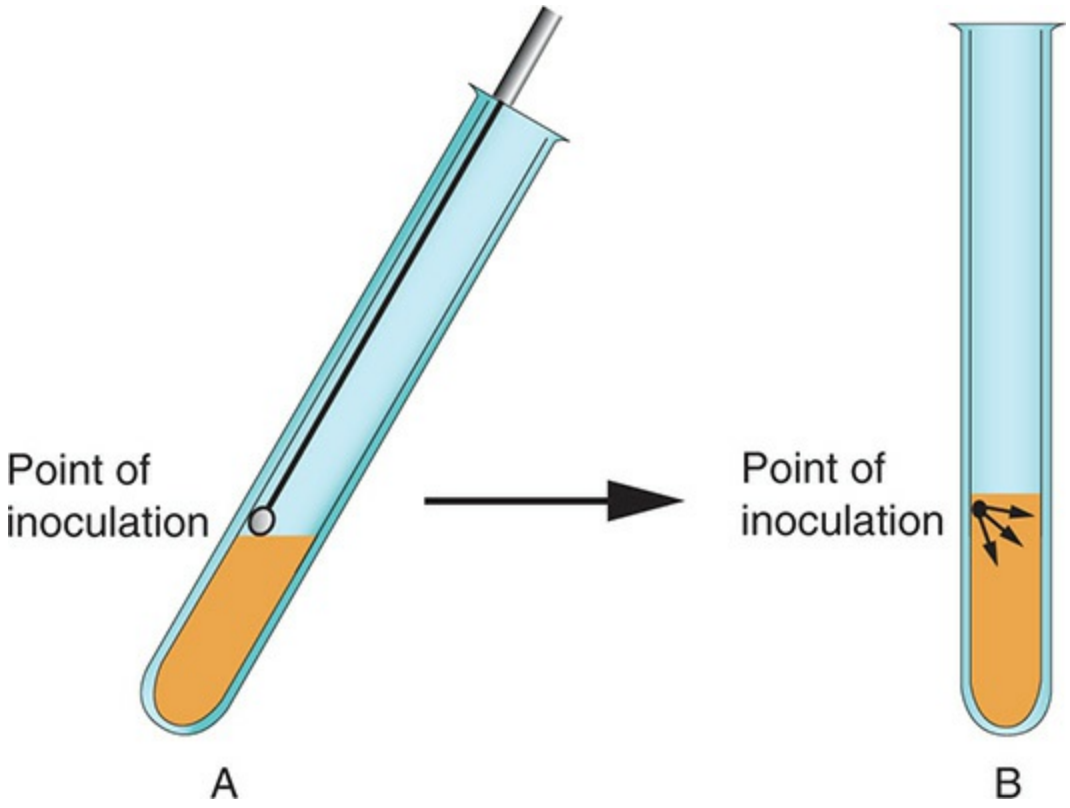
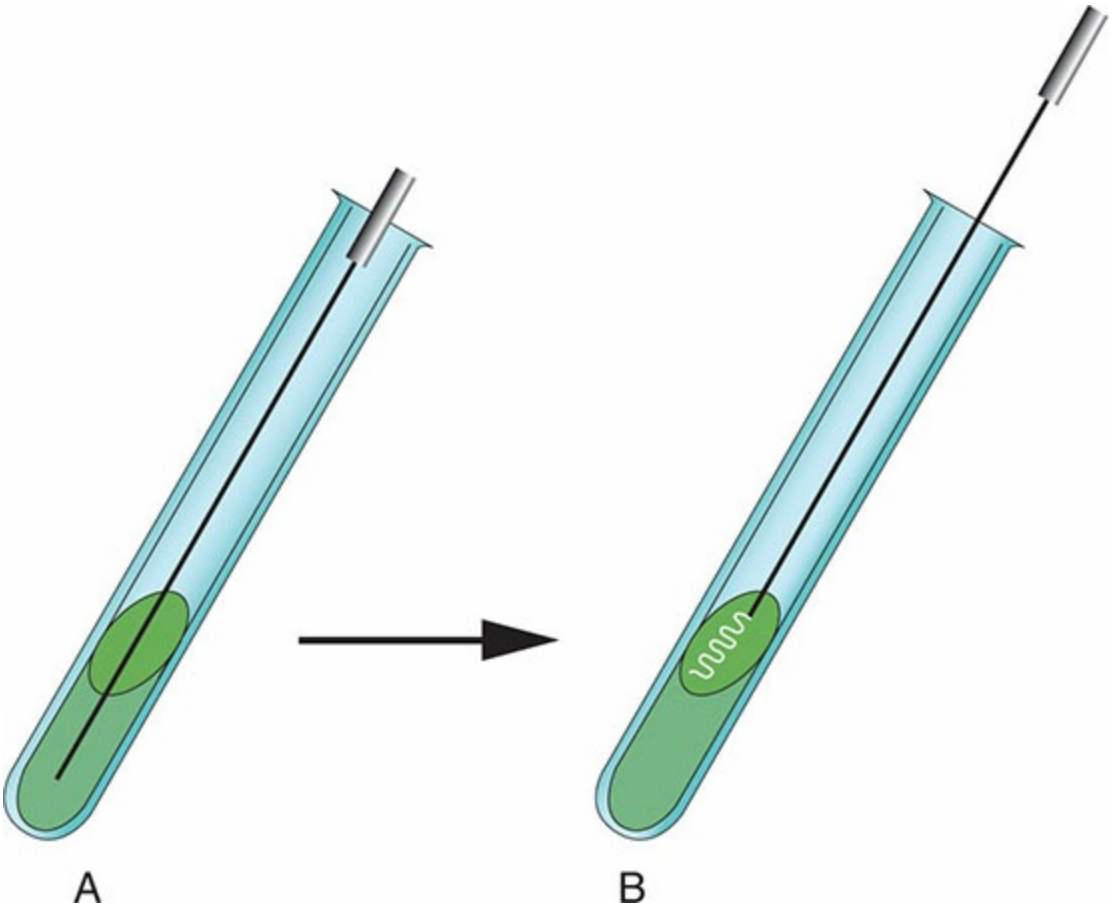


■ **FIGURE 1-11** Technique for inoculating a tube of broth medium. **A.** Slant the tube and inoculate the tube by touching the moist inner surface of the glass tube at the acute angle of the meniscus. **B.** Return the tube to an upright position, which has the effect of submerging the point of inoculation under the surface.



■ **FIGURE 1-12** The technique for inoculating an agar slant with a straight inoculating wire: **A.** Stab the deep of the agar slant to within 2 to 3 mm of the bottom of the glass. If the bottom of the glass is touched, atmospheric air may enter, negating the anaerobic conditions. **B.** Slowly remove the wire and streak the agar surface with a back-and-forth "S" motion.

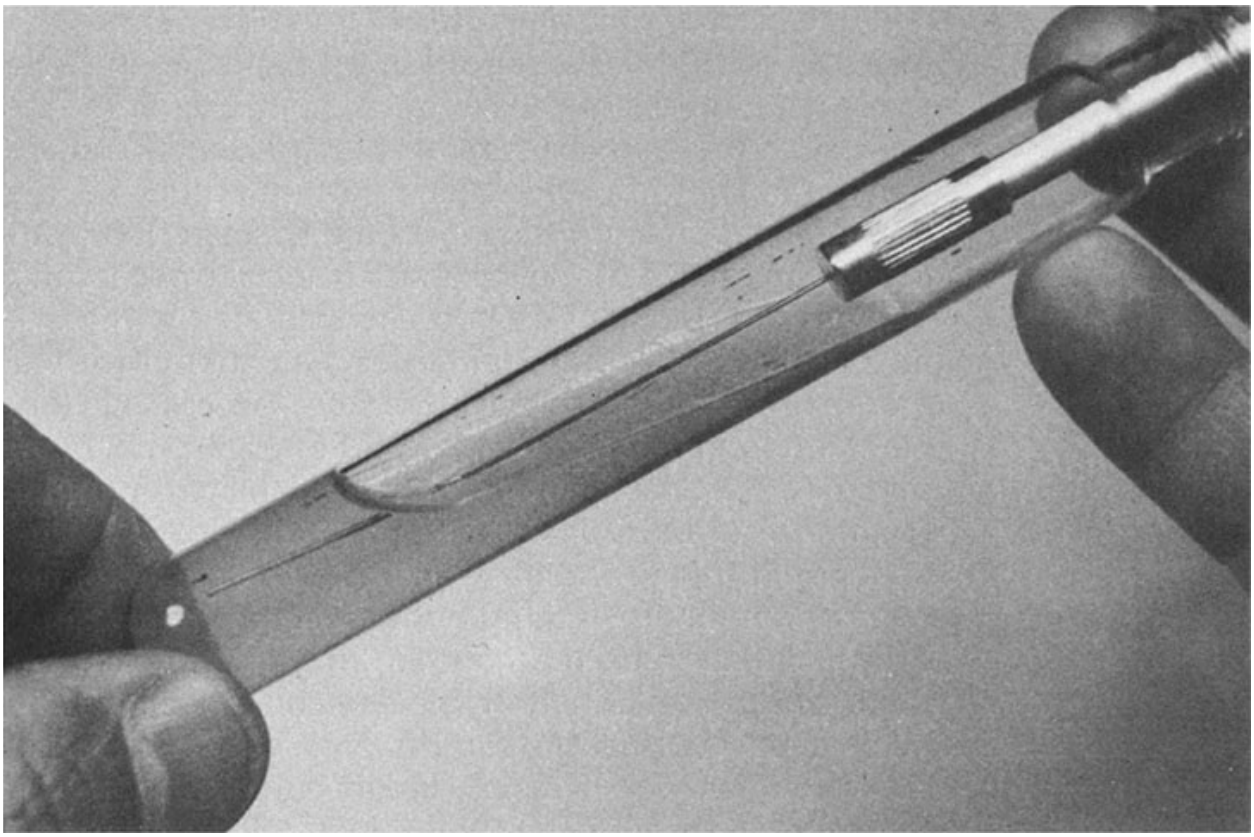
tubed agar for motility testing, it is important that the inoculating wire be removed along the exact track used to stab the medium. A fanning motion can result in a growth pattern along the stab line that may be falsely interpreted as bacterial motility.

Certain specimens may require centrifugation or filtering to concentrate any microbes present. Tenacious, mucoid sputum samples can be liquefied with *N*-acetylcysteine (Mucomyst, WellSpring Pharmaceutical Corp., Neptune, NJ) to facilitate even streaking of the agar surface. Sputum samples to be processed for the recovery of *Mycobacterium* spp. must also be treated with sodium hydroxide to minimize the overgrowth of bacterial contaminants. Other specimens, such as urine and stool suspensions submitted for the recovery of mycobacteria, can also be briefly treated with NaOH to eliminate colonizing bacteria. Similarly, antibiotic-containing media can be used to control bacterial overgrowth.

Body fluids, such as those obtained by thoracentesis and paracentesis, should first be allowed to settle, after which aliquots of the sediment are centrifuged to concentrate further any bacteria that are present. Requests may be made to provide blood culture bottles for the direct inoculation of certain specimens, as previously discussed. This practice is appropriate in those few situations where small numbers of a single pathogen are expected. It should be discouraged in other situations for several reasons:

1. If mixed bacterial species are present, bacteria that grow more rapidly will overgrow the slower growing bacteria in the broth.
2. The semiquantitative relationships of isolated bacterial types that may be seen on agar plates are lost in a broth culture.
3. The direct smear that is so important to assessment of the inflammatory nature of the specimen and the types of bacteria present are lost if the entire specimen is inoculated into a broth.

Cerebrospinal fluid specimens should be centrifuged and portions of the sediment transferred to appropriate culture media; or preferably, the fluid should be passed through a 0.45-mm microbiologic filter to trap and concentrate any bacteria or yeast that may be present. The challenge with filtration is



■ **FIGURE 1-13** The technique for inoculating an agar deep with a straight wire, as illustrated in F igure 1-12A. The deep of the medium is stabbed with the wire to within 2 to 3 mm of the bottom of the tube; then, after slowly removing the needle, the agar surface is streaked with a back-and-forth "S" motion, as illustrated in F igure 1-12B.

that contamination may occur when the filter is manipulated either upon inoculation of the medium initially or when the position of the filter is moved to examine for growth.

Microorganisms differ in their optimal temperatures of incubation. In small laboratories, where resources may be limited, it may not be possible to provide all of the incubation temperatures optimal for growth of all clinical isolates. Most microorganisms grow at 35°C; thus, if only one incubator is provided, it should be set at 35°C. Even organisms such as *Campylobacter jejuni*, which grows optimally at 42°C, will grow at 35°C if an additional 24 or 48 hours of incubation are allowed. In this case, however, the differential effect of incubation at higher temperatures where other bacteria do not grow is lost. The growth of most organisms is enhanced by an atmosphere of 5% to 10% CO_2. If only an ambient air, non-CO_2 incubator is available, the culture tubes and plates can be placed in a candle extinction jar and the entire assembly placed in the incubator. Conversely, an ambient air environment can be maintained in a CO_2 incubator by placing the cultures into a holding jar with a tightly fitted lid (an anaerobic jar or chamber is suitable). It should be realized, however, that organisms such as *C. jejuni*, which requires a reduced oxygen tension of 5% or below (i.e., are microaerophilic), will have difficulty growing in a candle jar, in which the oxygen concentration is in the range of 10%.

Many microorganisms grow optimally within a narrow temperature range; others have a relatively wide spectrum within which they can be recovered. The optimum temperature for growth of *C. jejuni* is 42°C, as just mentioned. Most fungi grow best at 30°C; however, most can be recovered at room temperature or 35°C on appropriate media. *Yersinia enterocolitica* grows optimally at a temperature slightly above room temperature. Most strains will also grow, however, at 35°C, even though colonies may appear small or require an additional 24-hour incubation period. Thus, only infrequently will access to a single incubator compromise the ability of a microbiologist to recover most clinically important bacteria. In large laboratories where several incubation temperatures can be provided, recovery of organisms will often be more rapid, and the appearance of colonies more characteristic. Occasionally, incubation at room temperature for an extended time may be necessary to demonstrate certain biochemical or physical characteristics, such as pigment production and motility. These various adjustments will be learned through experience and by trial and error.

Probably more important than the specific temperature of incubation is prevention

of wide fluctuations in temperature. Incubators should be carefully controlled for temperature, with no more than ±1 to 2°C fluctuations from day to day. Incubators should be located so that the control dials are protected and cannot be easily disturbed by cleaning personnel during off hours.

Humidity control within the incubator is also important. Most organisms grow maximally when the humidity is 70% or higher, and culture media tend to deteriorate more rapidly when undue drying occurs. The recovery of *H. pylori*, in particular, and to a lesser extent, *N. gonorrhoeae* requires an atmosphere with high humidity. Most incubators purchased during recent years have built-in water reservoirs by which humidity in the chamber can be regulated. If not, open pans of water can be placed on shelves to provide moisture through evaporation. Incubators must also be checked periodically for inadvertent spills that can cause contamination or a buildup of chemicals from reagents that may be inhibitory to bacterial growth.

Interpretation of Cultures. Interpretation of primary cultures after 24 to 48 hours of incubation requires considerable skill. From initial observations, the microbiologist must assess the nature of isolated colonies and decide whether additional procedures are required. The relevant parameters include the characteristics and relative number of each type of colony recovered on agar media; the purity, the Gram-stain features of each type of colony; and changes in the media, which reflect the metabolic activities of the bacteria within the colony (e.g., pink colonies on MacConkey due to lactose fermentation).

In the process of collecting specimens from patients or in handling those specimens in the laboratory extraneous organisms may be introduced from the environment or from the indigenous flora of the individuals who handle the culture. In blood cultures, for instance, resident skin flora is frequently encountered and must be interpreted carefully. In addition, "contaminants" may be introduced in the process of manufacturing or handling microbiologic products, such as agar plates. It may be apparent that a colony is extraneous if it is present on the second or third streak area of a plate—with no growth on the initial inoculation zone. These colonies can usually be ignored, although special attention must be given to important pathogens that are unusual "contaminants." A more difficult challenge arises when a single colony of a typical skin organism, such as coagulase-negative *Staphylococcus*, is found in the primary inoculation zone. There is, of course, a one-third or one-fourth probability of a plate contaminant ending up in the first zone. If the specimen is a critical one, such as cerebrospinal fluid and has been retained in the laboratory, it can be reinoculated on to the surface of a new agar. Individual laboratory directors need to assess how apparent contaminants should be handled in the local setting. If the decision is made to report an isolate wherein there is a high likelihood that it is a contaminant, some will append a comment such as "… ., probable contaminant." The clinician can then integrate this laboratory information with the clinical findings to make a final

interpretation of the significance.

Unusual situations present themselves on occasion. For instance, mixed organisms that resemble indigenous upper respiratory tract flora may be recovered from a normally sterile site. It is hard to avoid the suspicion that someone "sneezed" on the plate, but further evaluation must depend on evaluation of all factors, perhaps in consultation with the clinician. A most unusual laboratory phenomenon is illustrated in Figure 1-14.

Characteristics of Macroscopic Colonies. The gross assessment of colony characteristics is usually performed by inspecting growth on the surface of agar plates. This examination is carried out by holding the plate in one hand and observing the surface of the agar for the presence of bacterial growth (Fig. 1-15). Standard culture plates are 100 mm in diameter and are convenient to hold in one hand. Each plate must be studied carefully, because the bacteria initially recovered from specimens are often in mixed culture and a variety of colonial types may be present. Pinpoint colonies

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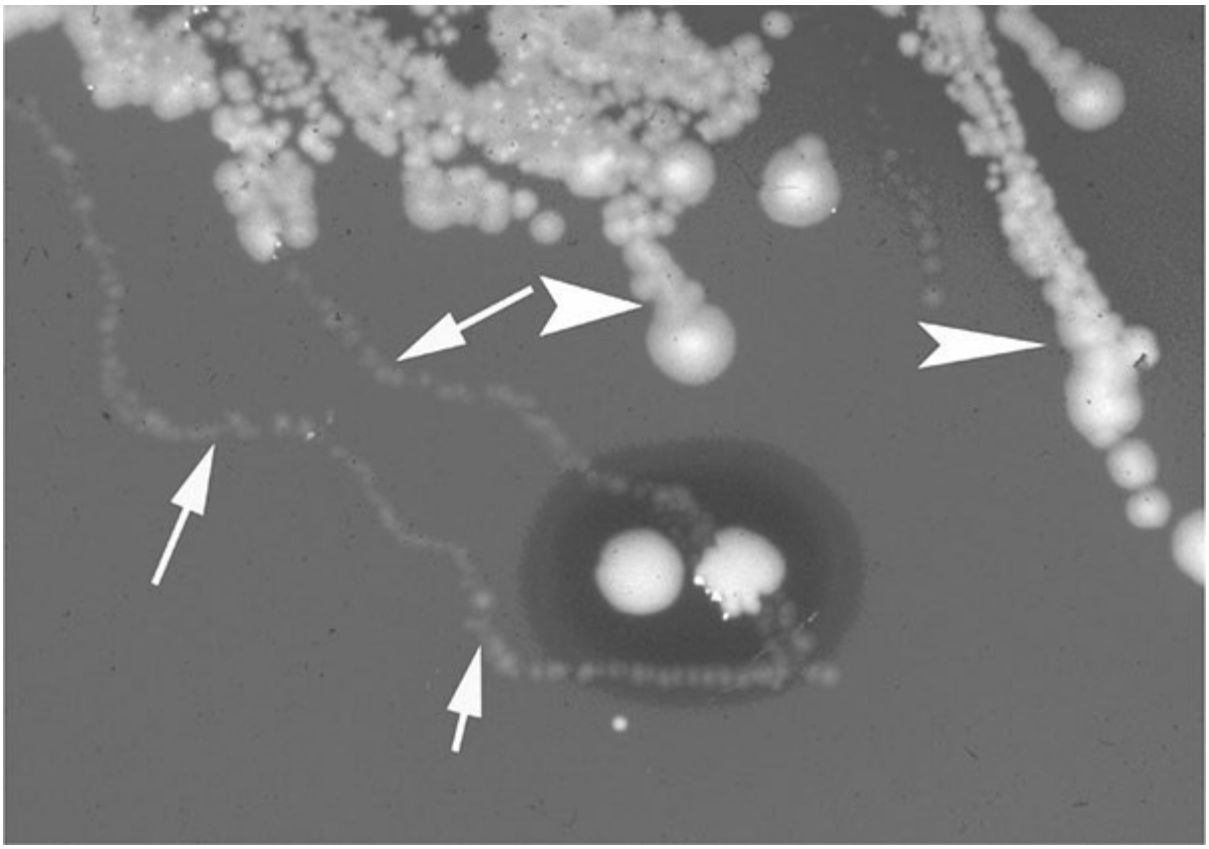
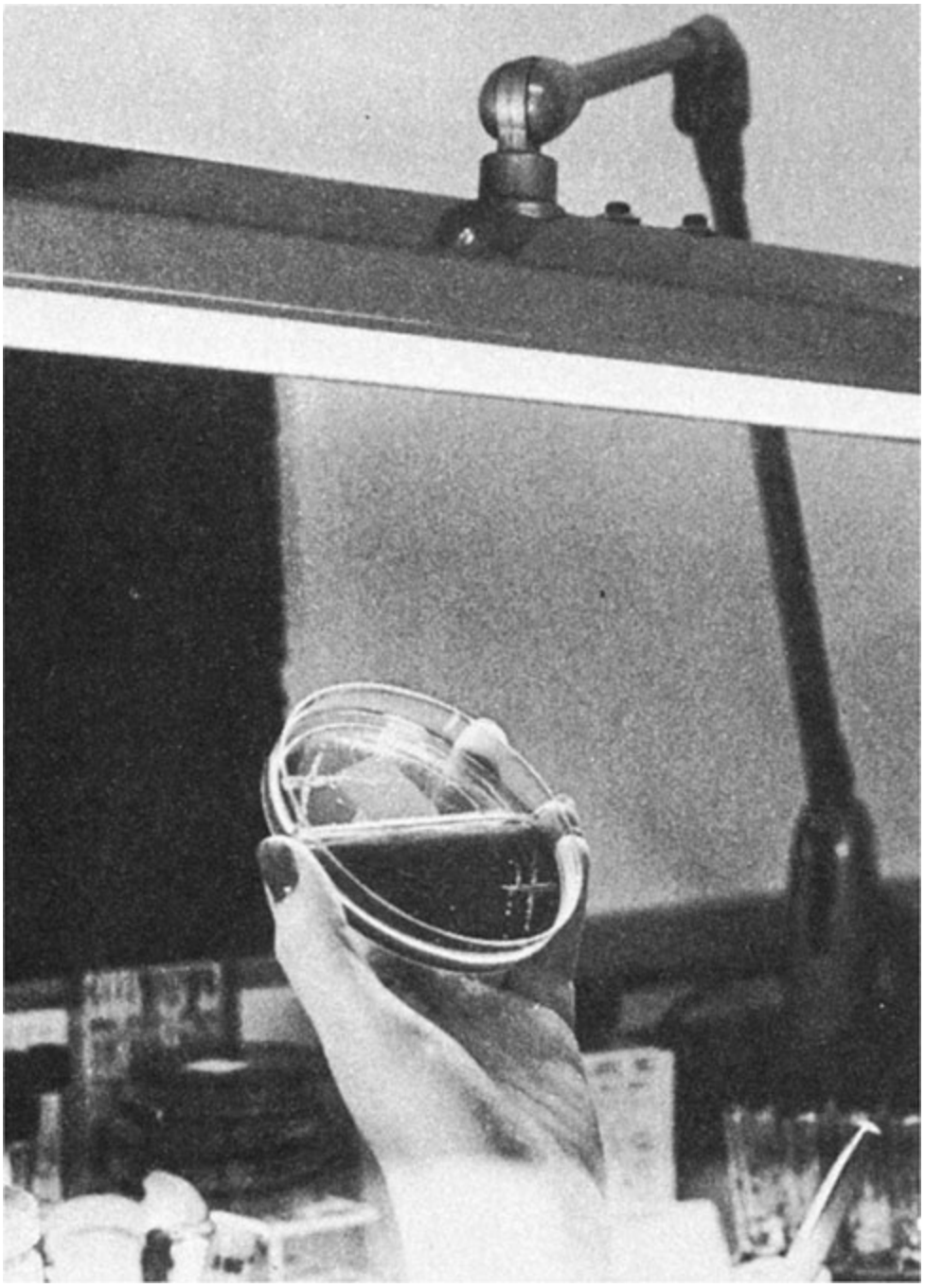


■ **FIGURE 1-14** An agar plate, which had been inoculated with a skin specimen, contains

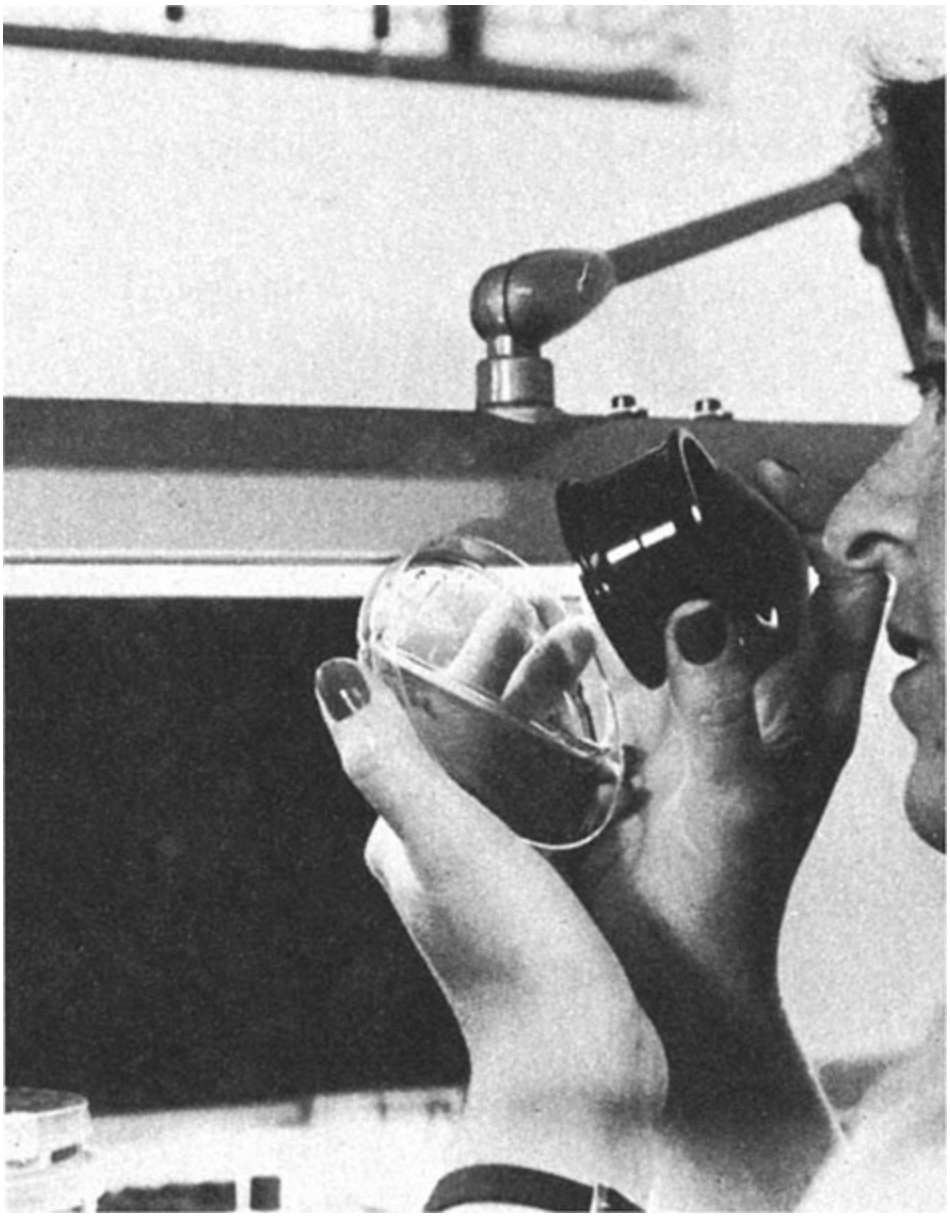
mixed bacterial microbiota from the skin. An irregular, meandering track of bacteria can be seen among the isolated colonies (*arrow*). In addition, unusual linear patterns of mature colonies may represent the same process (*arrowheads*). The most likely explanation is that a mite was present in the specimen that carried bacterial cells around the plate. This has also been seen in fungal cultures. Free-living amoebae have also been described to produce this phenomenon.



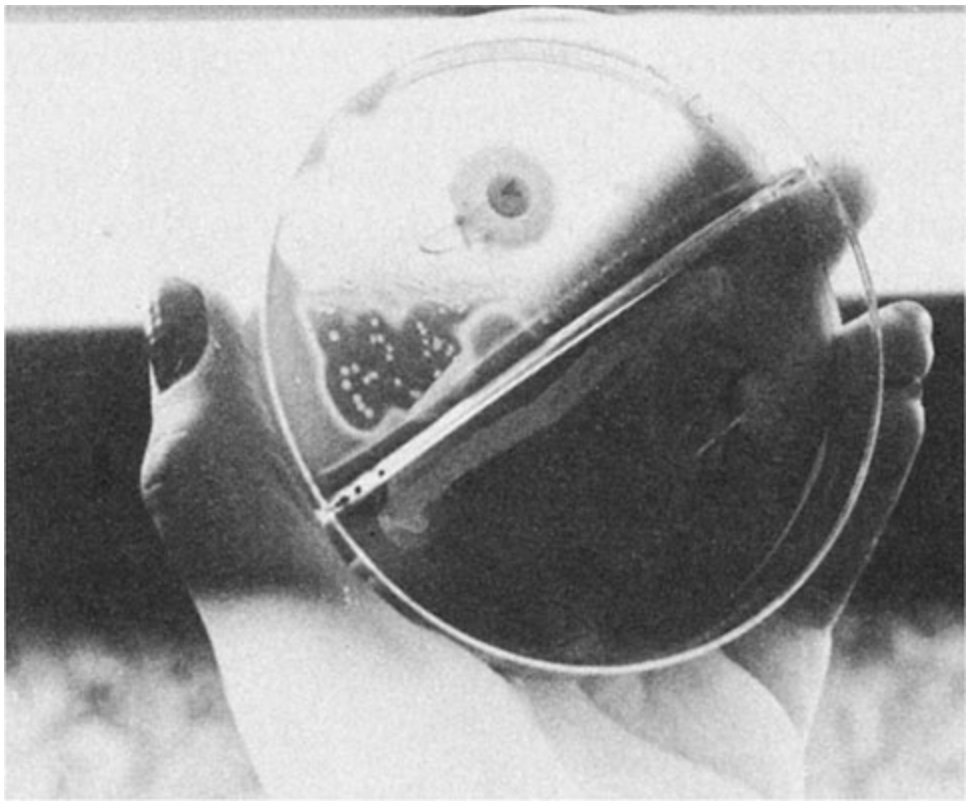
■ **FIGURE 1-15** The technique for examining the surface of an agar plate by direct, oblique-reflected light.

of slow-growing bacteria may be overlooked among larger colonies, particularly if there is any tendency for growth to spread over the surface of the plate.

During examination, plates should be tilted in various directions, under bright, direct illumination, so that light is reflected from various angles. This aids in the detection of small colonies. We highly recommend use of a hand lens, mounted magnifying lens or a dissecting microscope to assist in the detection of tiny or immature colonies and to observe their characteristics better (Fig. 1-16). Blood agar plates should also be examined with transilluminating bright light from behind the plate to detect hemolytic reactions in the agar (Fig. 1-17).



■ **FIGURE 1-16** The technique, using a hand lens, for examning colonies growing on the surface of an agar plate.



■ **FIGURE 1-17** The technique for examining colonies growing on the surface of an agar plate using transmitted light is helpful in assessing the hemolytic properties of colonies growing on blood agar.

Figure 1-18 provides terms and illustrations helpful when describing bacterial colonies. Additional guidelines are outlined in Boxes 1-8 through 1-10.

Although difficult to describe specifically, odors produced by the action of certain bacteria in plating media and in liquid media can be very helpful in the tentative identification of the microorganisms involved). Sniffing of plates is not recommended, but it does occur. The odors associated with certain bacteria are presented (Table 1-12). If performed against recommendations, it should always be performed

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Form	punctiform		irregular	
	circular		rhizoid	
	filamentous		spindle	
Elevation	flat		pulvinate	
	raised		umbonate	
	convex		umbilicate	
Margin	entire		erose	
	undulate		filamentous	
	lobate		curled	

■ **FIGURE 1-18** Illustrations of a variety of a variety of colony morphologies.

BOX 1-8

Characteristics of Colonies Used in the Identification of Bacteria

Size: diameter in millimeters
Form: punctiform, circular, filamentous, irregular, rhizoid, spindle-like
Elevation: flat, raised, convex, pulvinate, umbonate, umbilicate
Margin (edge of colony): entire, undulant, lobate, erose, filamentous, curled
Color: white, yellow, black, buff, orange, other
Surface: glistening, dull, other
Density: opaque, translucent, transparent, other
Consistency: butyrous, viscid, membranous, brittle, other

See also Figure 1-18 and Image Plate 1-5.

BOX 1-9

Reactions in Agar Media Used in the Identification of Bacteria

Hemolysis on blood agar

Alpha: partial clearing of blood around colonies with green discoloration of the medium; outline of red blood cells intact
Beta: zone of complete clearing of blood around colonies owing to lysis of the red blood cells
Gamma: no change in the medium around the colony; no lysis or discoloration of the red blood cells

Pigment production in agar medium

Water-soluble pigments discoloring the medium
Pyocyanin
Fluorescent pigments
Nondiffusable pigments confined to the colonies

Reaction in egg yolk agar

Lecithinase: zone of precipitate in medium surrounding colonies
Lipase: "pearly layer," an iridescent film in and immediately surrounding colonies, visible by reflected light
Proteolysis: clear zone surrounding colonies

BOX 1-10

Changes in Differential Media

Various dyes, pH indicators, and other ingredients are included in differential plating media to serve as indicators of enzymatic activities and aid in identifying bacterial isolates.

Examples:

MacConkey agar: Lactose nonfermenting colonies appear clear, whereas lactose-fermenters appear pink because of the production of acid, subsequent change in pH and in some instances precipitation of dyes.
Hektoen-Enteric agar: Lactose and sucrose nonfermenting colonies appear green, whereas lactose or sucrose-fermenters appear yellow because of the production of acid, subsequent change in pH.

Bacteria that have the ability to produce hydrogen sulfide (H_2S) are black or have black precipitant in the center of the colony, whereas those without this ability do not.

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TABLE 1-12 Characteristic Odors of Selected Microbes[a]

Microbe	Odor
Alkaligenes faecalis	Freshly cut apples
CDC EF-4 (N. animaloris/N. zoodegmatis)	Popcorn-like
Candida spp.	Yeast
Citrobacter spp.	Dirty sneakers

Clostridium difficile	Putrid, fecal
Corynebacterium spp., DF-3	Fruity
Eikenella corrodens	Bleach
Haemophilus spp.	Wet fur
Nocardia spp.	Musty basement
Pasteurella multocida	Pungent (indole)
Peptostreptococcus anaerobius	Fecal
Pigmented Bacteroides group	Acrid
Proteus spp.	Burnt chocolate
Pseudomonas aeruginosa	Grape juice
Staphylococcus spp.	Dirty sneakers
Certain viridans group Streptococcus spp.	Butter/butterscotch
Streptomyces spp.	Musty basement

[a]Foremost, the smelling of plates is discouraged. It should be noted that certain organisms (not listed here) pose a hazard if smelled. Therefore, if the microbiologist intends to smell the plate, he or she should be sure that the colonial features and other characteristics, such as Gram's stain morphology, exclude the possibility of these hazardous bacteria. See text for discussion.

with caution, by raising the lid of the Petri dish slightly to detect the odor. Some odors, such as those of *Nocardia* and *Streptomyces*, are so strong that they can be smelled even without removing the lid of the Petri dish. Laboratory workers are cautioned that a few species have demonstrated the ability to infect laboratory workers and isolates with even the remotest possibility of resembling these bacteria should never be sniffed. If there is any suspicion that *Bacillus anthracis*, *Francisella tularensis*, *Burkholderia pseudomallei*, *Brucella* spp., or *N. meningitidis* might be present, the culture should be removed to a BSC, where all subsequent work can be done without danger of transmission to the worker. Of course, plates and tubes should never be opened in the

mycobacteriology laboratory, until appropriate precautions are taken. Similarly, plates and tubed media that grow molds should be handled in a BSC with appropriate personal protective apparel.

By assessing the described colonial characteristics and action on media, the microbiologist can make a preliminary identification of the bacteria isolated by primary culture. These characteristics are helpful in selecting other appropriate differential media and tests to complete the identification of the isolates. Table 1-13 lists some of the commonly encountered colonial types, along with the groups of bacteria associated with each, additional tests required for definitive identification and reference to the relevant frame in Image Plate 1-5.

The initial inspection of colonies for preliminary identification of bacteria is one of the cornerstones of diagnostic microbiology and is discussed in detail in later chapters devoted to specific groups of pathogenic bacteria and other microorganisms.

Separation of Bacterial Morphotypes in Mixed Cultures. When agar plates are struck for isolation, it will be a simple matter to select isolated colonies for subculture. On

TABLE 1-13 Preliminary Bacterial Identification by Colonial Types

Colonial Type	Bacterial Group	Additional Tests	Frame of Plates Illustrating Type
Convex, entire edge, 2–3 mm, creamy, yellowish, zone of β-hemolysis	*Staphylococcus*	Catalase Coagulase DNase Mannitol utilization Tellurite reduction Novobiocin resistance Furazolidone resistance	1-5A
Convex or pulvinate, translucent, pinpoint in size, butyrous, wide zone of β-hemolysis	*Streptococcus*	Catalase Bacitracin "A" disk 6.5% NaCl tolerance Bile–esculin CAMP test Hippurate hydrolysis L-Pyrrolidonyl-β-naphthylamide (PYR)	1-5B, C
Umbilicate or flat, translucent, butyrous or mucoid, broad zone of α-hemolysis	*Pneumococcus*	"P" disk Bile solubility	1-5G

Pulvinate, semiopaque, gray, moist to somewhat dry, β-hemolysis may or may not be present	*Escherichia coli* and other *Enterobacteriaceae*	Multiple tests Indole Methyl red Voges–Proskauer reaction Citrate Decarboxylases Urease Phenylalanine Carbohydrate fermentations	1-5D
Flat, gray; spreading as thin film over agar surface; burned chocolate odor	*Proteus*	Phenylalanine deaminase Urease Lysine deaminase	
Flat, opaque, gray to greenish, margins erose or spreading, green-blue pigment, grapelike odor	*Pseudomonas*	Cytochrome oxidase Fluorescence of carbohydrate assimilations Denitrification DNase Hydrolysis of acetamide Growth at 42°C	

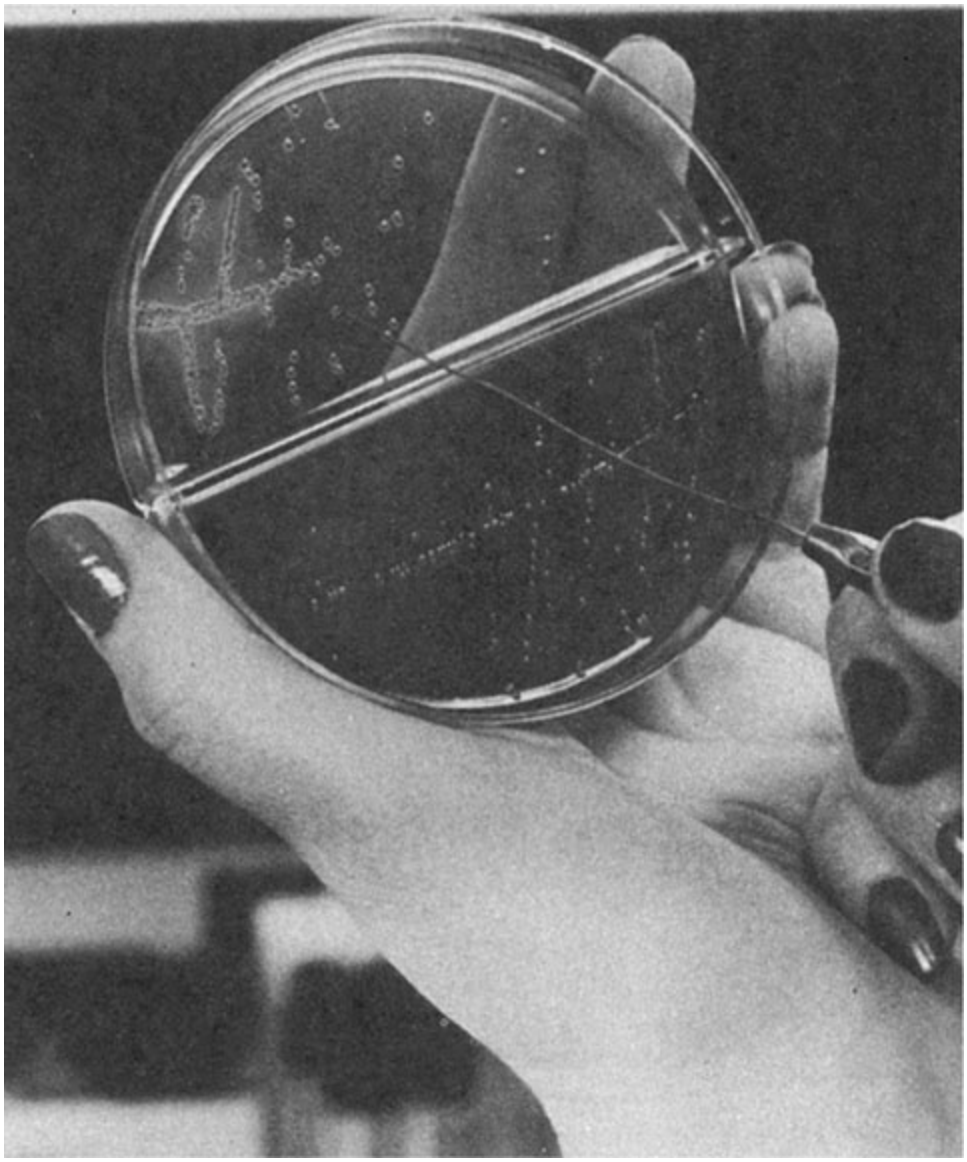
occasion, however, growth may be so crowded that it is difficult to pick individual isolated colonies. Several manipulations are available to the microbiologist in this eventuality (Table 1-14).

Gram's Stain Examination of Cultures. Preliminary impressions, based on observation of colony characteristics, can be further confirmed by studying Gram-stained smears, a technique that is relatively simple to perform. The top and center of the colony to be studied is first touched with the end of a straight inoculating wire, taking care not to touch the adjacent agar or nearby colonies (Fig. 1-19). The portion of the colony to be sampled is emulsified in a small drop of water or physiologic saline on a microscope slide to disperse the individual bacterial cells (Fig. 1-20). After the slide has air dried, the film is fixed to the glass surface either by using heat, quickly passing the slide four or five times through the flame of a Bunsen burner, or by flooding with methanol or ethanol for a few minutes. The fixed smear is then placed on a staining rack and Gram's stain is performed as described in Box 1-6.

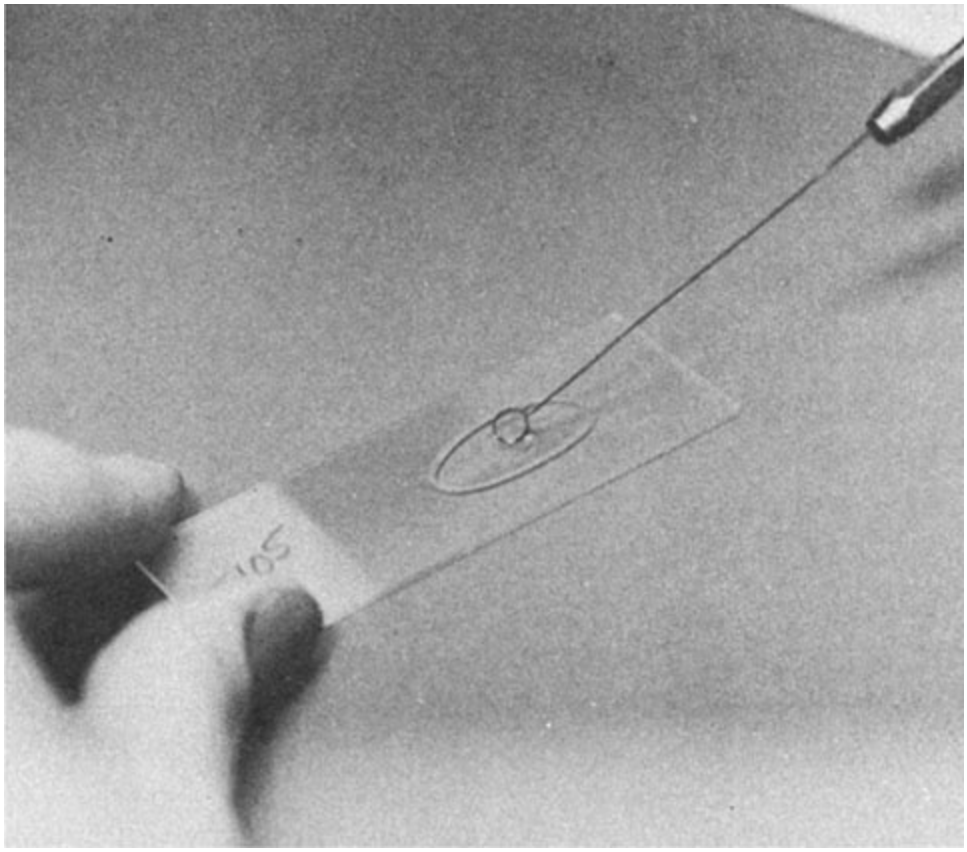
TABLE 1-14 Techniques for Preparation of Pure Isolates From Mixed Cultures

Technique	Description
Straight subculture	Carefully touch the surface of the desired colony with the tip of an inoculating needle (not a loop); streak a new plate for isolation
Use of selective media	Subculture the colony of interest onto an agar that will inhibit growth of the unwanted colonies; e.g., subculture of a gram-negative bacillus onto MacConkey agar to separate it from gram-positive cocci

Use of inhibitory chemicals incorporated into agar (analogous to selective media)	Subculture the colony of interest onto an agar that contains antibiotics or chemicals that will inhibit growth of the unwanted colonies
Use of antibiotic disks (analogous to an agar plate containing antibiotics but more flexible)	Subculture the colony of interest onto the surface of an agar plate and position an antibiotic-impregnated disk that is designed to inhibit the unwanted bacteria



■ **FIGURE 1-19** The technique for picking an isolated bacterial colony with a straight wire for subculture to another medium.



■ **FIGURE 1-20** The technique for preparing a smear for Gram's stain. The top portion of an isolated bacterial colony, as illustrated in F igure 1-19, is touched with an inoculating wire or loop, and then the tip of the loop is submerged in a drop of water or physiologic saline on a glass slide. The inoculum is then emulsified and the preparation is left to air-dry before heat fixation and staining.

The stained smear should be examined microscopically using an oil immersion objective. In addition to the Gram's stain reaction of the bacterial cells (gram-positive bacteria appear blue; gram-negative bacteria appear red or pink), three other characteristics are helpful in making a preliminary identification of isolates: (1) size and shape of the bacterial cells, (2) arrangement of the bacterial cells, and (3) presence or lack of specific structures (e.g., spores, metachromatic granules, capsule, or other features).

In making a preliminary identification of bacterial isolates, the microbiologist should evaluate each of these characteristics. A series of photomicrographs of several stains illustrating a number of the morphologic cells types and spatial arrangements of bacteria commonly encountered in clinical laboratories is shown in Image Plate 1-3.

With the information derived from the examination of bacterial colonies and the Gram's stain, the microbiologist now can be guided to the particular tests most likely to provide the correct identification. For example, a raised, creamy, hemolytic colony on blood agar that consists of gram-positive cocci in clusters is most likely *Staphylococcus* (Image Plate 1-3C). A pinpoint translucent β-hemolytic colony on blood agar that consists of gram-positive cocci in chains is most probably a *Streptococcus* species (Image Plate 1-3D).

The microbiologist soon learns, however, not to rely solely on the examination of Gram-stained smears because the staining reactions may be variable, particularly with very young or older colonies. Gram's stain morphology of bacteria is most characteristic when smears are prepared from a young broth subculture (4 to 6 hours), a time when the bacterial cells are in log phase of growth. Often the Gram's stain morphology is less characteristic when smears are prepared from colonies growing on agar surfaces.

Microbiologists should provide as much preliminary information to the physician as possible. In selected cases, such as when observing bacteria in a blood culture broth or directly in infected CSF, this type of preliminary information can be quite useful in directing specific antibiotic therapy before the final identification or antimicrobial susceptibility test results are available.

Procedures for Preliminary Identification of Bacterial Isolates. The initial observation and interpretation of microbial growth on culture media should be used to determine whether the microorganism(s) recovered merit further identification and whether antibiotic susceptibility tests should be performed. Subsequent tests are then used for the purpose of identifying an isolate, by assessing the biochemical or metabolic activity of the organism. This has traditionally been done by inoculating the primary isolate onto a series of differential test media or into solutions. Today, many of these will not be performed and the isolate may be submitted directly for identification by mass spectrometry. Although mass spectrometry is time and cost-effective, it is not perfect. Therefore, the retention of the knowledge and ability to identify of bacteria by traditional methods is warranted, particularly for isolates wherein the morphologic or other features are inconsistent with the identification by mass spectrometry. Furthermore, the use of colony morphology with key reactions (i.e., spot tests) is a recognized method for the identification of bacteria in certain culture settings, as described in the CLSI M32A and below, and in these settings even obviate the need for mass spectrometry.

Direct Biochemical Procedures for Making Preliminary Bacterial Identifications. Certain preliminary observations or a direct rapid test can be performed on select colonies. Frequently, an isolate can be identified to a level that is clinically useful based on these assessments alone. For example, the lactose-utilizing properties of gram-negative bacilli can be directly evaluated from MacConkey agar by

observing the red pigmentation of the colonies; H_2S production may be detected on Hektoen and xylose lysine deoxycholate (XLD) agars by observing colonies with black centers. The decarboxylation of lysine can also be suspected when observing colonies growing on XLD agar. A red halo around the colony, indicating an alkaline pH shift, indicates the decarboxylation of lysine.

The following are direct tests that can be performed on isolated colonies recovered on primary culture plates:

The Catalase Test. A few drops of 3% hydrogen peroxide are placed directly on a colony. Rapid effervescence indicates production of molecular oxygen and a positive test (Chart 1-1). Accurate results may be difficult to obtain if the test is performed on colonies growing on blood agar because of the presence of peroxidase in erythrocytes. The peroxidase reaction produced by erythrocytes is delayed and weak, however, and can usually be readily differentiated from the immediate and highly active reactions produced by catalase-positive bacteria. The catalase test is most often used to differentiate staphylococci (positive) from streptococci (negative) or *Bacillus* spp. (positive) from aerotolerant *Clostridium* species (negative).

The Bile Solubility Test. Two methods for determining bile solubility are commonly used. As an initial screen, a few drops of a 10% solution of sodium deoxycholate solution are placed on colonies of possible *S. pneumoniae*. Pneumococcal colonies lyse completely and disappear after about 30 minutes (Chart 1-2). This test is sometimes difficult to interpret

and the tube bile solubility test may be performed. An inoculum from the unknown bacterial colony can be suspended in a 10% solution of deoxycholate (bile salts) until turbidity is achieved. The clearing of the turbidity within 30 to 60 minutes after incubation at 35°C indicates bile solubility (Chart 1-2). A control viridans group *Streptococcus*, which does not dissolve in bile, should be tested concurrently.

The Slide Coagulase Test. A colony suspected of being *Staphylococcus* species is emulsified in a drop of rabbit plasma on a glass slide. Bacterial clumping within 2 minutes indicates the presence of bound coagulase and constitutes a positive test result (Chart 1-3). A negative slide coagulase test should be followed with a conventional tube coagulase test, if the colonial morphology is suggestive of *Staphylococcus aureus* in any way. Agglutination tests for detection of staphylococcal protein A can also be used as a marker for *S. aureus*. Bacteria with the appropriate morphology of *S. aureus* that are coagulase-positive may be reported as *S. aureus*. If this is done, technologists need to be aware of slide coagulase-positive isolates that may mimic *S. aureus,* so that additional testing may be performed. Coagulase-negative staphylococci are often reported as such, unless there is a clinical reason to identify these to the species level.

The Direct Spot Indole Test. A small portion of the colony to be tested is transferred from a nonselective medium, such as blood or chocolate agar, to a strip of filter paper that has been or will be saturated with Kovac's reagent or *p*-dimethylamino cinnamaldehyde (PACA) solution. The immediate development of a red color with Kovac's reagent indicates the presence of indole and a positive test (Chart 1-4). PACA is more sensitive than Kovac's reagent, and a positive test reaction is indicated by the rapid development of a blue color. In many laboratories, dry-appearing, lactose-positive, spot indole-positive colonies appearing after 24 hours of incubation on MacConkey agar, particularly on isolates from the urinary tract, are identified as *E. coli*, and further tests are usually not performed. In these cases the spot indole must be performed on colonies growing on parallel blood agar plates, because the pigmentation of the lactose-positive colonies on MacConkey agar will make interpretation of the color reaction difficult and indole is a derivative of tryptophan, which is present in blood containing media.

Cytochrome Oxidase Test. A portion of the colony to be tested is smeared on the reagent-impregnated area of an oxidase test strip. The immediate development of a blue color indicates cytochrome oxidase activity and a positive test result (Chart 1-5). Cytochrome oxidase tests are useful for the initial categorization of many bacterial species that have a distinctive colonial morphology. Bacterial species that produce cytochrome oxidase include *Aeromonas* spp., *Plesiomonas* spp., and *Pseudomonas* spp. Dimethyl oxidase is the reagent typically used in this test. Tetramethyl oxidase is a reagent that is useful for the identification of *Pasteurella* spp., which are dimethyl oxidase negative, tetramethyl oxidase positive. All members of the *Enterobacteriaceae* are oxidase negative.

MUG Test. The MUG (4-methylumbelliferyl-β-D-glucuronidase) test is based on the detection of the production of β-glucuronidase. This test can be used as a screen for *E. coli* as an alternative to the spot indole test. A heavy suspension of the unknown organism is inoculated into the MUG reagent, which has been suspended in tubes or impregnated in dehydrated disks. The reagent will fluoresce from release of 4-methylumbelliferone, if glucuronidase is present. Indole can also be detected by adding Kovac's indole reagent to the MUG tube, making this combination test a valuable method for screening lactose-fermenting enteric bacilli.

PYR Test. L-pyrrolidonyl-b-naphthylamide (PYR) substrate provides a simple method for the rapid identification of enterococci. The production of a red color after adding N, N-methylamino cinnamaldehyde reagent is indicative of PYR positivity. This is used predominantly for the identification of the genus *Enterococcus,* but other bacteria, such as *S. pyogenes,* are also positive (Chart 1-6).

Identification of Bacteria to the Level of Species and Selection of Differential Characteristics. The final characterization of an unknown bacterial isolate is usually accomplished by testing for enzyme systems that are characteristic of each species. These enzyme systems are detected by inoculating a small portion of a well-isolated

bacterial colony into a series of culture media that contain specific substrates and chemical indicators. By this means, the microbiologist can detect changes in pH produced by utilization of chemical substrates or color changes produced by specific by-products. The clinical microbiologist must select appropriate sets of differential characteristics that will permit the identification of each group of bacteria. One of the major advances in diagnostic microbiology has been the miniaturization of biochemical systems, so that multiple characteristics can be examined expeditiously and relatively inexpensively. Before these advances a small number of tests were performed in macroscopic tubes or plates, followed by subsequent testing based on the initial results. This process was slow, expensive, and more prone to error than modern approaches.[87]

Identification of Microorganisms Using Mass Spectrometry. The commercialization and Food and Drug Administration (FDA)-approval of mass spectrometry instruments with associated microbial libraries has changed the ways microbiologists identify microorganisms. It is not an exaggeration to state that the identification of some bacteria (e.g., *Listeria monocytogenes*) that took days and cost dollars can now be completed in minutes and for pennies. There are a variety of types and modifications of mass spectrometry. The matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) method and instrumentation is used for the identification of microorganisms in the two commercially available, FDA-approved systems.

In brief, the microorganisms are placed in a matrix material and ionized by a laser. Biomolecules, largely proteins, are accelerated and separated and the "time of flight" for each molecule is recorded. The composite of the time of flight signals for the molecules of the organism creates a spectrum, which remarkably correlates with the identification of the microbe. It is also reliable, reproducible and the manufacturers have made these instruments simple to use. There are well recognized limitations, which must be considered in laboratory procedures and utilize these instrument, but this is no different than any other technology. Numerous manuscripts and presentations have documented the ability of this technology correctly identify a wide array of microorganisms and to be meaningfully integrated into the clinical laboratory.

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Identification of Organisms Other Than Bacteria

Fungi. Yeast share many characteristics with bacteria and similar approaches are used for identification. A few screening tests are commonly used on yeast isolates to identify or exclude commonly recovered and/or particularly important organisms. The germ-tube test has been the primary screening tests for *Candida albicans* for many years, although it is recognized that *Candida dubliniensis* is also germ-tube positive.

Urease testing, including a rapid version, is the other commonly used screening test. It is commonly used to examine respiratory isolates, because the most frequent pathogen (*Cryptococcus neoformans*) produces urease, whereas the most frequent commensal yeast isolate (*Candida* spp.) does not contain this enzyme with rare exceptions. Traditionally, the morphologic characteristics of the yeast isolated are examined (i.e., the cornmeal agar morphology) in conjunction with the assimilation and/or fermentation profile to achieve a final identification. The use of mass spectrometry in the clinical laboratory is changing the approach to routine identification, largely obviating the need for many biochemical reactions and cornmeal agar assessment. The evidence-based approach to the identification of yeast using this technology will be developed over the next few years.

The mainstay of identification of mold isolates, however, is the morphologic study of largely asexual reproductive characteristics. Biochemical testing and temperature studies play subsidiary roles, but occasionally are needed to achieve species-level identification. The feasibility of mass spectrometry for the identification of filamentous fungi is being explored, but is likely to be limited, since the morphologic assessment is also quick and inexpensive for those with the appropriate skill set.

Mycobacteria. As specialized bacteria, the traditional approaches to identification have been similar for these organisms. The biochemical testing, however, is considerably more difficult with mycobacteria than with conventional bacteria predominantly because of the extended incubation time needed for adequate growth for testing. Most laboratory directors choose to either refer these specimens to specialized laboratories for identification and/or susceptibility testing, or to use molecular methods. DNA probes are commercially available for several of the more commonly encountered mycobacteria. These are particularly useful for the rapid identification or exclusion of *M. tuberculosis* following the detection of mycobacterial growth. Broad-range polymerase chain reaction (PCR) followed by DNA sequencing has been the standard for identification of mycobacteria in many larger or reference laboratories. Mass spectrometry has also been evaluated and may be used for the identification of mycobacteria in some instances. The definitive role of this technology in the routine mycobacteriology laboratory remains to be determined.

Parasites. The screen for some parasites (e.g., *Giardia* and Cryptosporidium) may be performed effectively with commercially available immunoassays. Otherwise, characterization of parasites is by morphologic study. Culture of parasites is uncommonly performed.

Viruses. As obligate intracellular pathogens, viruses require a very different diagnostic approach. Although traditionally cell culture techniques were used to detect viruses, these are being replaced by more sensitive molecular methods. Characterization of antigens and nucleic acids are the predominant techniques used for viral diagnosis.

BOX 1-11

Factors Involved in Susceptibility Testing

Factor	Criteria
Isolate	Potential pathogen from a valid specimen (i.e., not likely to represent colonizing microbiota)
Susceptibility	Not predictable for infectious agent and antimicrobial
Clinical situation	Antimicrobial therapy indicated
Interpretive standards	Validated criteria available for determining the clinical significance of the result

Testing of Susceptibility to Antimicrobial Agents. In many respects, determining the susceptibility of pathogens to appropriate antimicrobial agents is the most important task performed in diagnostic microbiology laboratories. Some of the factors that must be evaluated when susceptibility testing is considered are summarized in Box 1-11.

Antimicrobial susceptibility testing is most often used for guidance in therapy of bacterial and mycobacterial infections (Chapters 17 and 19). The clinical microbiologists must ensure that testing is done on appropriate isolates by valid methods. The easiest course of action is to give in to an insistent clinician who demands testing of an isolate for which there are no interpretive standards, but the microbiologist must resist that temptation, using evidence-based approaches and recruiting knowledgeable colleagues for support, if necessary.

Testing of anaerobic bacteria, fungi, and viruses may be required in certain situations, but is not necessary routinely. If these agents are tested, it is important that a sophisticated clinician, who will be able to interpret the results properly, be involved in care of the patient.

Use of Antimicrobial Susceptibility Results for Quality Control of Identification Results. Some bacteria have predictable susceptibility to certain agents and need not be tested. Many more have characteristic susceptibility patterns that are not sufficiently absolute to obviate testing. These patterns can be used, however, as a check on the taxonomic characterization. For instance, *K. pneumoniae* is typically resistant to ampicillin, but susceptible to first-generation cephalosporins. In contrast,

Enterobacter spp. are usually resistant to both groups of antibiotics. If an isolate is identified as *Enterobacter*, but is susceptible to these agents, the results should be questioned. Tests both for identification and for susceptibility should be repeated, as something is likely in error. Optimally, a different method should be used for the repeat test from that used initially.

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Cost-Effective Approaches in the Analytic Phase. In the analytical phase resources can be utilized efficiently by concentrating on several areas. It is possible to use abbreviated protocols for identifying certain organisms (Table 1-15). The CLSI has provided consensus recommendations for certain approaches.[26] Other means for optimizing resources are summarized in Table 1-16. The philosophical underpinning of these approaches is the focus on results that can be interpreted

TABLE 1-15 Abbreviated Identification of Bacteria and Yeast

Situation	Abbreviated Test Protocol	Identification
Gram-negative, nonswarming, oxidase-negative, β-hemolytic on sheep blood agar[a]	No additional tests	*Escherichia coli*
Gram-negative, nonswarming, oxidase-negative, nonhemolytic on sheep blood agar, lactose-fermenter[a]	PYR negative	*Escherichia coli*
Gram-negative, nonswarming, oxidase-negative, nonhemolytic on sheep blood agar, lactose-nonfermenter[a]	MUG positive	*Escherichia coli*
Small gram-negative bacilli or coccobacilli from respiratory or cerebrospinal fluid specimens; growth in 5% CO_2 on chocolate agar, but not sheep blood agar[a]	Rapid test for porphyrin synthesis negative	Haemophilus influenzae (cannot differentiate fromHaemophilus hemolyticus, an uncommon human pathogen)
Gram-negative diplococci, oxidase-positive, growth on	Rapid butyrate esterase or	*Moraxella*

both chocolate and sheep blood agars[a]	DNAse positive	*catarrhalis*
Gram-negative bacilli, oxidase-negative, lactose-nonfermenter, swarming growth on chocolate or sheep blood agar[a]	Indole positive	*Proteus vulgaris*
Gram-negative bacilli, oxidase-negative, lactose-nonfermenter, swarming growth on chocolate or sheep blood agar[a]	Indole negative; ampicillin susceptible	*Proteus mirabilis*
Gram-negative bacilli, oxidase-negative, lactose-nonfermenter, swarming growth on chocolate or sheep blood agar[a]	Indole negative; ampicillin resistant; maltose negative; ornithine positive	*Proteus mirabilis*
Gram-negative bacilli, oxidase-negative, lactose-nonfermenter, swarming growth on chocolate or sheep blood agar[a]	Indole negative; ampicillin resistant; maltose positive; ornithine negative	*Proteus penneri*
Gram-negative bacilli, oxidase-positive, typical smell of Concord grapes, typical colonial morphology (metallic sheen, green/reddish/black, mucoid)[a]	No additional tests	Pseudomonas aeruginosa (rare isolates of Aeromonas may be similar but are spot indole positive)
Gram-positive cocci in clusters; catalase positive; creamy, opaque, yellowish colonies on sheep blood agar (color accentuated on chocolate agar)[a]	Slide coagulase and/or 4-hour tube coagulase positive	Staphylococcus aureus (coagulase-positive Staphylococcus); rarely other staphylococci may be slide

		coagulase positive.
Gram-positive cocci in pairs and short chains, catalase negative or occasionally weakly positive; non-β-hemolytic on sheep blood agar[a]	PYR positive	Enterococcus spp.; failure to grow well for susceptibility tests suggests that the isolate may be another genus.
Gram-positive cocci in pairs and chains; catalase negative; usually narrow zone of β-hemolysis[a]	Rapid hippurate hydrolysis positive; spot CAMP test positive; or latex agglutination positive with specific antiserum	Streptococcus agalactiae (group B); β-hemolytic enterococci may be hippurate positive, but are also PYR positive.
Gram-positive cocci in pairs and short chains; catalase negative;α-hemolytic on sheep blood agar; colonies typically either mucoid or "checker" shaped	Spot bile or tube bile solubility positive	*Streptococcus pneumoniae*
Gram-positive cocci in pairs and chains; catalase negative; β-hemolytic on sheep blood agar with a wide zone of hemolysis; colonies usually dry and small relative to hemolysis[a]	PYR positive or latex agglutination positive with specific antiserum	Streptococcus pyogenes (group A); occasional strains of β-hemolytic enterococci have different colony morphology and are agglutination negative.
Small gram-negative bacilli; oxidase-positive; a "dew-drop" colony morphology on sheep blood agar with pitting of the agar; odor of bleach	No additional tests	*Eikenella corrodens*
	No additional	

Gram-negative regular bacilli or coccobacilli with large (>1 mm) colonies on anaerobic blood agar and similar size on LKV and BBE agars; no growth on chocolate agar in 5% CO_2[a]	tests essential; disk pattern may be used (penicillin resistant; kanamycin resistant; rifampin susceptible) as additional evidence.	Bacterioides fragilis group

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Gram-negative, thin, pointed, fusiform bacilli; breadcrumb or opalescent colonies on anaerobic blood agar; no growth on BBE agar; no growth on chocolate agar in 5% CO_2[a]	Spot indole positive	*Fusobacterium nucleatum*
Gram-negative bacilli; small (<1 mm) colonies on anaerobic blood agar and BBE agar after at least 48 hours incubation; no growth on chocolate agar in 5% CO_2; black dot in colony center from H_2S production[a]	Catalase strongly positive	*Bilophila wadsworthia*
Small coccobacillary gram-negative bacilli; black colonies (or brick-red fluorescence under long-wavelength UV light) on LKV agar; small translucent or opaque colonies on anaerobic BAP agar; no growth on chocolate agar in 5% CO_2[a]	No additional tests needed	Prevotella spp.; Prevotella interme dia if spot indole positive
Small coccobacillary gram-negative bacilli; small translucent or opaque colonies on anaerobic BAP agar with brick-red fluorescence under long-wavelength UV light; no growth on chocolate agar in 5% CO_2[a]	Spot indole positive	Porphyromonas spp.
Thin gram-negative bacilli; flat, transparent colonies that pit the agar on anaerobic BAP agar; no growth on LKV agar; no growth on chocolate agar incubated in	No additional tests needed	*Bacteroides ureolyticus*

5% CO2; catalase negative; urease positive[a]		
Tiny gram-negative diplococci; small (<1 mm) transparent-to-opaque colonies on anaerobic BAP agar with red fluorescence under long-wavelength UV light; no growth on BBE agar; no growth on chocolate agar in 5% CO_2[a]	No additional tests needed	Veillonella spp.
Large, boxcar-shaped, blunt-ended gram-positive or gram-variable bacteria; large (>2 mm) irregular colonies with a double zone of β-hemolysis on anaerobic BAP; no growth on LKV or BBE agars; no growth on chocolate agar in 5% CO2; catalase negative[a]	No additional tests needed	*Clostridium perfringens*
Thin gram-positive bacilli with swollen, subterminal spores; smoothly swarming growth on anaerobic BAP agar; no growth on LKV or BBE agars; no growth on chocolate agar in 5% CO2; catalase negative; spot indole negative[a]	No additional tests needed	*Clostridium septicum*
Thin gram-positive bacilli with subterminal spores; equivalent growth on anaerobic BAP agar and on chocolate agar in 5% CO2; catalase negative	No additional tests needed	*Clostridium tertium*
Pleomorphic, coryneform gram-positive bacilli; small (1–2 mm) enamel-white opaque colonies on anaerobic BAP agar; catalase positive; spot indole positive[a]	No additional tests needed	*Propionibacterium acnes*
Budding yeast cells[a]	Germ-tube test positive in <3 hours; or mycelial projections from colonies on blood-containing media at <24 hours incubation (*C. dubliniensis* is	*Candida albicans*

	another germ-tube positive yeast).	
Spherical budding yeast cells; often mucoid colonies[a]	Rapid phenol oxidase test positive	Cryptococcus neoformans (Cryptococcus can be excluded on rapid urease-negative colonies)

[a]From consensus recommendations in reference 26.

correctly and that will lead to the best treatment for the patient. One conceptual principle is to think about specimens in two broad categories: (1) those from which isolated agents are highly likely to be pathogens and (2) those from which isolated agents cannot be interpreted with confidence. If a microbiologist gives equal attention to these two categories, it is entirely possible that so many resources will have been squandered on specimens of questionable significance that the most important specimens will be shortchanged.

Each clinical microbiologist must make decisions for the local laboratory, depending on the local environment. A prime consideration is to avoid providing results that may

TABLE 1-16 Some Suggested Approaches to Optimizing Laboratory Identifications

Category	Proposed Policy[a]
Repeat specimens from a nonsterile site within a defined time period	Refer subsequent specimens to the previous evaluation; if the subsequent specimen has been inoculated to media and substantial differences from the original specimen are present, consult clinician about further action
Cultures that contain mixed microbiota (i.e., mixed flora) from a nonsterile site, especially if submitted on a swab; inflammation minimal or no Gram's smear available	Report as mixed gram-positive/gram-negative microbiota (or flora)

Cultures that contain mixed microbiota from a nonsterile site, especially if submitted on a swab; moderate to heavy inflammation present	Report as mixed gram-positive/gram-negative microbiota (or flora); append a comment to consult the laboratory within X days for further processing
Cultures that contain mixed microbiota from a nonsterile site, especially if submitted on a swab; moderate to heavy inflammation present; single predominant potential pathogen present	Report identification of predominant pathogen and presence of mixed microbiota (or flora); either perform antimicrobial susceptibility tests on predominant pathogen or append a comment to consult the laboratory for susceptibility testing
Yeast isolated in mixed culture with bacteria from a nonsterile or potentially contaminated site, such as a wound or peritoneal fluid	Report presence of yeast; consider appending a comment to consult the laboratory for further processing
Mixed microbiota from urine specimens obtained by clean catch or indwelling catheter	Report as mixed gram-positive/gram-negative microbiota (or flora); append comment suggesting (1) repeat collection with an in-and-out catheter if clinically indicated and the patient does not have an indwelling catheter or (2) notifying the laboratory if the patient has an indwelling catheter and requires antimicrobial therapy
Mixed microbiota with a single predominant pathogen from urine specimens obtained by clean catch or indwelling catheter	Evaluate predominant pathogen; append a comment indicating presence of mixed microbiota (or flora)
Mixed vaginal microbiota present	Report only presence or absence of documented vaginal pathogens that are best documented by culture; i.e., Listeria monocytogenes, Streptococcus agalactiae (in women of childbearing age), Streptococcus pyogenes, Staphylococcus aureus (if toxic-shock syndrome suspected), and yeast. Susceptibility testing rarely indicated.
Mixed microbiota from an intravascular catheter	Report mixed gram-positive/gram-negative microbiota (or flora) and consider appending a comment to consult the laboratory for further processing

Recovery of bacteria that resemble Haemophilus influenzae or Moraxella catarrhalisfrom expectorated sputum	Proceed with identification and report the result only if the organism is found in a concomitant Gram's stain associated with polymorphonuclear neutrophils or is the predominant organism present.

[a] It is good policy to preserve specimens that are not evaluated completely for at least 24 hours. If possible, retain the residual specimen for at least 7 days. Preserve agar plates that have not been evaluated completely for a period of time. Seven days is a convenient period to preserve the plates, because all plates from a day can be stored together and discarded 1 week later.

be misinterpreted. A report of "Mixed microbiota (or flora); no definitive pathogen identified" is likely to stimulate collection of an additional, and hopefully better, specimen or treatment of the patient based on the pathogens that are likely causes of the particular infection. Putting a name on an isolated organism that is, in fact, colonizing or contaminating microbiota gives that organism more apparent credibility than it deserves. This will often result in a request for susceptibility testing. The adding of antimicrobial susceptibility results compounds the difficulty, and implies the need for therapy.

The question becomes "What is mixed bacterial microbiota or flora?" Knowledge of the types of bacteria that colonize human body surfaces and a solid grounding in the principles of diagnostic microbiology are essential to this decision. For instance, *S. pyogenes* is important to recognize and is a possible pathogen, no matter how many other species are present; any β-hemolytic colonies should be evaluated for the presence of the group A streptococcal antigen. As a guideline, Schreckenberger and Miller have suggested the "Rule of Three."[81,103] They suggest that one or two potential pathogens should be evaluated even in the presence of commensal flora, but three or more potential pathogens should not be evaluated.

The "Rule of Three" guideline is a useful aid, but sound judgment should be used. For instance, the rule should not be applied automatically to biopsied tissue or aspirated fluids and pus. In the urinary tract, a mixture of three or more organisms of any variety suggests contamination with vaginal or perineal flora; even if a potential pathogen is a part of the mixture and does not predominate, it is difficult to be confident that the analysis will provide valid information. Sometimes, the quantitative relationship of the organisms in the mixture can provide a clue. If one or possibly two organisms clearly

predominate, it is often reasonable to evaluate those organisms and report these, as well as the presence of mixed microbiota.

It should be noted that when a microbiologist examines an agar plate and determines that mixed bacterial types are present, it is actually mixed bacterial colonial types (morphotypes) that are being observed. These visually distinctive colonies often represent different species (or even genera), but they may also represent phenotypic variants of a single genotypic organism. The only way to be sure that multiple bacterial species have been isolated is to identify all of them, by which time, of course, all the work will have been done. In practice, we must use judgment as to whether the colonies are sufficiently different to justify characterizing the growth as mixed and recognize that we may be wrong on occasion.

One practical approach to these situations is to offer the clinician a chance to request that the isolates be evaluated or to initiate a dialogue preemptively, either through a verbal phone report or a written communication on the laboratory record. It is a simple matter to store plates, rather that identifying all the bacteria in a mixture, in the event that a request will be made to identify a particular subset. If the plates are taped together each day and stored for 7 days, the outdated specimens can be discarded easily. Should a dispute arise as to further evaluation of a specimen, it is a better position for the microbiologist if the specimen has been retained than if it has been discarded.

The techniques and interpretations of the foregoing procedures are discussed in greater detail in subsequent chapters. In an era of stringent cost containment, it is imperative that microbiologists apply their skills of observation and use a few select characteristics to identify bacterial species presumptively whenever possible. Developing this microbiologic "sixth sense" can also be helpful in verifying the results obtained from packaged kit systems or from automated instruments. On occasion, the biotype numbers issued by these systems may be in conflict with colonial characteristics, Gram-stain morphology, and/or biochemical "spot tests." In such instances, additional studies may be in order to prevent issuing an erroneous report.

Postanalytic Phase

Reporting Results. Reports of microbiology culture results should be issued as soon as useful information becomes available. Each laboratory director must establish those results that will be considered "urgent values." The reporting of these and the action taken by clinicians needs to be monitored, as these will be reviewed during a Joint Commission inspection for hospital accreditation. The next tier consists of results that are considered "important," but not necessarily "urgent." There is not always agreement regarding what should be characterized as urgent versus important; this is a local healthcare delivery decision. Some examples of "urgent" and "important" results are listed in Table 1-17. The construction of such a list will vary greatly, depending on

the patient population served, the capability of the institutional information system and the practice patterns of clinicians who use the laboratory. In addition to the defined categories, the clinician should have the opportunity to request a telephoned result on any culture at the time the examination is requested.

"Urgent" results must always be telephoned to the caregiver. Having the person receiving this information repeat back (i.e., read back) the reported information and documenting to whom it was reported should be part of the standard procedure for reporting urgent results. "Important" results are often also telephoned to the healthcare team, but occasionally may be delivered by an electronic means. The danger of the latter approach in assuring that someone who can act on the information actually received it. The most important consideration is that all parties understand the communication policy and the responsibility of each participant. Confidentiality of patient data must be assured anytime a report might be viewed by unauthorized personnel (e.g., faxing reports only to office machines). It is good practice to have a protocol for telephone reports that specifies backup recipients if the primary caregiver is not available; this protocol should be acceptable to both clinicians and laboratory workers.

TABLE 1-17 Suggested List of "Urgent" and "Important" Reports[a]

"Urgent" Reports	"Important" Reports
Positive blood cultures	Positive tissues and fluids other than blood and cerebrospinal fluid
Positive cerebrospinal fluid cultures	Stool pathogens
Positive acid-fast smears	Positive parasites other than Plasmodium spp.
Mycobacterium tuberculosis	Positive sexually transmitted pathogens
Plasmodium spp., especially P. falciparum	Streptococcus agalactiae if recovered via predelivery culture protocol
Streptococcus pyogenes from a normally sterile site or genital site	Streptococcus pyogenes from throat cultures
Streptococcus agalactiae from a genital site of a woman at term	Positive viral pathogens
Herpes simplex virus from a genital site of a woman at term	Positive dimorphic fungi

	Positive antigen or nucleic acid tests performed directly on clinical specimens
	Results that are likely clinically unsuspected, but may be medically important (e.g., the recovery of an unusual or unexpected pathogen that may not be covered by empiric therapy)

[a] These are recommendations; this list should be modified as appropriate for local conditions.

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Timely preliminary and interim reports should be issued as a matter of policy. For example, preliminary reports on negative bacterial cultures should be issued within 24 to 48 hours. The timing of initial reports on other types of agents will vary with the speed of growth (e.g., weekly for mycobacteria and twice the first week followed by weekly for fungi). An interim report of "negative culture" may be helpful, because the physician may wish to reevaluate the patient pending the final report. Final reports should be issued as soon as possible; for most bacterial cultures, 48 hours is a reasonable goal. In most instances, the preliminary and final results are maintained in the LIS, which is interfaced with the hospital information system (HIS). As results are updated or finalized in the LIS, they should near-instantly appear in the HIS and be available for the provider. It is important to assure that the results successfully cross this interface and that the expected format has remained intact. This assurance can be incorporated in the checklist that is used when introducing new tests or validating a new computer system.

Interactions With Epidemiologists. Microbiologists play an important role in safeguarding the health of patients and the public at large.[52,89] Certain infectious agents must be reported to public health authorities by law; the list of such agents varies by state and should be available in the laboratory. The reports are now mainly electronic. Within the institution, similar relationships must be cultivated with hospital or healthcare system epidemiologists. These individuals are often called Infection Control or Infection Prevention specialists. The epidemiologists will review certain laboratory results on a regular basis. In addition, microbiologists should remain alert for unusual patterns of isolates that deserve mention to the epidemiologists for consideration of further investigation. For example, if a particular bacterium (e.g., *Serratia marcescens*) is isolated at an increased frequency from a particularly location (e.g., the neonatal intensive care unit), then the microbiologist should alert the

epidemiologist to this fact, as an outbreak is suspected and an intervention is likely needed. On occasion, molecular strain typing may be used to increase the quality of epidemiologic investigations. The additional testing may be performed locally or in a referral laboratory, depending on available expertise.

Analysis of Results. It is incumbent on the laboratory director to provide feedback to clinicians on some important parameters of laboratory performance (Box 1-12). If local information systems have the capability, feedback should be made optimally to individual clinicians or groups of clinicians (such as practice groups). The ultimate quality control tool for caregivers is to receive a report card on their use of the laboratory and results on their patients (such as frequency and rate of positive examinations) compared to other clinicians. Such reports are challenging to design because of the difficulty in being sure that the practitioners being compared serve patient groups that are truly equivalent. Studies of turnaround time (TAT) are more problematic in microbiology than in other areas of the laboratory because of the delays inherent in generating microbiologic results. However, certain examinations, such as direct examinations of clinical specimens, do lend themselves to TAT analysis. Summaries of antimicrobial susceptibility results should be provided on a yearly basis. Knowledge of the frequency of antimicrobial resistance in commonly recovered bacteria at a local level is important for guiding empiric therapy.

BOX 1-12

Parameters of Laboratory Performance and Clinical Results

Activity	Parameters
Blood cultures	Frequency and rate of recovery of "skin organisms"/"contaminants"
	Frequency and rate of submission of single cultures
Direct examination of clinical specimens	Analysis of TAT
Antimicrobial susceptibility testing	Summaries of % susceptibility of important bacterial pathogens
Mycobacterial cultures	Frequency of the recovery of *Mycobacterium gordonae.*
	Correlation of positive cultures with AFB smear results and review of the smears from

positive cultures

Maintenance of Samples and Records. Local and national guidelines for storage of requisitions and reports must be followed. They differ by specimen type and clinical situation.

After specimen processing and media inoculation, the residual specimen should refrigerated at least until the bacterial culture is complete. Often specimens are maintained for a set number of days after submission, in the event that the clinician would like to have additional tests performed. Although this is useful in that another specimen is not required, it should be noted that refrigeration diminishes the recovery of some microorganisms (e.g., *N. meningitidis* recovery is diminished at 4°C). Ideally, tissue should be frozen at −70°C for potential future use, but this is impractical for most laboratories. The retention of clinical specimens is importance, since subsequent analysis (e.g., histologic examination) may disclose the presence of an unsuspected pathogen (e.g., mycobacteria, viruses, or fungi) and a culture for that pathogen can be attempted. If successful, the retention of the specimen prevented the need for obtaining another specimen, which may be from an invasive procedure.

Blood culture isolates should be maintained for at least 30 days. Ideally, all positive cultures should be retained for a period (e.g., 7 days) to allow further evaluation, such as additional susceptibility tests, if clinically indicated. Some institutions stock clinically-significant blood culture isolates at −70°C for extended periods. A variety of methods have been described for storage of microbial isolates.[95] We have found the simple expedient of a loopful of bacteria into trypticase soy broth with 15% to 20% glycerol to be an effective

method of preserving most bacterial and yeast isolates. Fungi can be maintained as "water cultures" at room temperature or on slants under glycerol. Viruses must be frozen at −70°C.

Administrative Aspects of the Microbiology Laboratory

The focus of this book is on the science of microbiology as applied to diagnosis and treatment of infectious diseases. In daily practice, however, it is impossible to separate the business of microbiology from the science. One of the strengths of clinical microbiology laboratories is the sophistication of support systems for the scientific process. Research laboratories have much to learn from the planned maintenance and rigorous quality control of their diagnostic counterparts. The reader is referred to an

extensive exploration of laboratory management in theory and action.[51]

Government Regulations

In the United States, the government is involved in virtually every aspect of laboratory testing. Although some issues are directly related to specific sets of regulations, all aspects of laboratory management derive from governmental imperatives in some manner. In many cases, leaders in the private and academic laboratory industry developed the framework and defined the standards, which were then adopted by the government and made applicable to all laboratories. The federal agencies and other groups involved in laboratory and medical regulation are summarized in Box 1-13.

The authority for regulatory involvement in laboratory medicine derives from the Clinical Laboratory Improvement Act of 1967 (CLIA '67). This legislation authorized the regulation of hospital and commercial clinical laboratories. For 20 years, laboratories in other settings were exempt. The Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) extended the mandate to include governmental laboratories, public health laboratories, and physicians' office laboratories that perform laboratory testing for human disease. Testing for research purposes (results not used in the care of patients) and veterinary medicine do not fall under governmental purview.

Several categories of testing were established in the CLIA '88 regulations (Table 1-18), and many other regulations depend on the category in which a test is placed. The Centers for Disease Control and Prevention (CDC) is charged with the task of assigning each test to a complexity category. An advisory group, the Clinical Laboratory Improvement Advisory Committee (CLIAC), composed of individuals from government, industry, medical groups, clinical and public health laboratories, and consumers advises the government on these decisions.

BOX 1-13

Agencies Involved in Laboratory and Medical Practice

Agency	Area of Responsibility
Center for Medicare and Medicaid Services (CMS)	Establishes the rules for accreditation, licensure, and inspection of laboratories; sets reimbursement rates for services to Medicare
Centers for Disease Control and Prevention (CDC)	Participates in categorization of test complexity; provides recommendations on a variety of issues, including laboratory techniques and bioterrorism preparedness
National Institute of Occupational	Responsible for regulations on protection from chemical

Health and Safety (NIOSH); a division of CDC	Responsible for regulations on protection from chemical and biologic hazards
Occupational Health and Safety Administration (OSHA)	Responsible for regulations on workplace safety
Food and Drug Administration (FDA)	Responsible for approving medicines and medical devices; FDA "clears" medical devices, but does not "approve" them; and is determining how to regulate laboratory-developed tests.
Veterans Affairs Department	Responsible for the health and welfare of eligible veterans, including a national network of hospitals and clinics
International Air Transport Association (IATA)	An international organization of air carriers; involved in promulgation of rules on safe shipment of infectious agents by air
Clinical and Laboratory Standards Institute (CLSI)	A voluntary organization of academia, industry, and government; its goal is to improve laboratory performance; although advisory, the recommendations often become standards of laboratory practice
American Medical Association (AMA)	Largest organization of physicians in the United States; responsible for developing coding of laboratory tests and medical procedures, which serve as the basis for reimbursement
The Joint Commission	Inspects and accredits hospitals and hospital laboratories, home healthcare agencies, long-term healthcare facilities, among others.
College of American Pathologists (CAP)	Provides laboratory accreditation and proficiency testing services.

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TABLE 1-18 Complexity Categories of Laboratory Testing Under CLIA '88

Category	Description	Personnel Requirements	Proficiency Testing	Quality Control	Comments
High complexity	Tests requiring the greatest analytical skill and judgment	Most stringent; requires equivalent of Associates Degree	Required	Required	High and moderate complexity testing are jointly considered nonwaived testing.
Moderate complexity	Tests requiring analytical skill and judgment but at a somewhat lower level	Less stringent; requires training which may be on the job	Required	Required	There is a complex formula for categorizing tests as to level of complexity.
Provider performed microscopy	Microscopic examinations by certain groups of clinicians	Defined provider groups; cannot be delegated to other personnel	Required if applicable	Required if applicable	This is a subcategory of moderate complexity testing.
Waived testing	Simple tests that are not likely to have adverse consequences if performed incorrectly	None	Not required	Must follow manufacturer's instructions	Most laboratory workers have a hard time understanding how a test could be worth doing if a wrong answer causes no harm.

For more detailed information, see the CMS website: http://www.cms.hhs.gov/clia/appendc.asp

Detailed specifications for personnel qualifications, proficiency testing, and quality control are delineated in the regulations for all laboratories that perform nonwaived testing. Several consequences of the CLIA '88 regulations have devolved.

1. Most physicians have stopped performing all but the most basic testing because the regulatory requirements are considered too burdensome.
2. There is constant pressure from physician groups (other than pathologists) to loosen the regulations and allow performance of more complex tests without stringent controls.
3. Manufacturers strive to have their instruments and products placed into the waived category, so they can market them to physicians as simple tests without rigorous requirements for control and documentation.

Accreditation and Laboratory Inspection. The CLIA '88 regulations provide for licensure of laboratories after inspection by state inspectors or by another organization that has been "deemed" by Centers for Medicare and Medicaid Services (CMS) to provide equivalent—or stricter—standards. The two organizations that are most commonly used by clinical laboratories for accreditation and inspection are Joint Commission on Accreditation of Healthcare Organizations (JCAHO) and College of American Pathologists (CAP). In every case, the laboratory must be evaluated biennially. Laboratories that utilize JCAHO are usually located in hospitals, and the inspection of the laboratory is performed at the same time as the hospital inspection. In this case, the inspectors are "professional inspectors," usually with a medical or nursing background rather than training in laboratory medicine specifically. CAP was the first organization to evaluate laboratories; before the entry of the government into the process. Initially, accreditation was voluntary and viewed as an educational means

to self-improvement. The CAP philosophy is based on peer evaluation. Each laboratory that is inspected must provide a team to inspect another similar laboratory in turn. The inspectors are, therefore, "volunteers" who are practicing laboratory professionals.

Each organization that inspects laboratories has a set of standards for evaluation; these standards must be submitted to CMS for approval. In some states, accreditation by a state agency must also be performed if a laboratory does business in that state (e.g., New York State).

Proficiency Testing. An integral part of continuing accreditation is participation in proficiency testing. Specimens are sent to participating laboratories as unknowns on a periodic basis. The laboratory evaluates the specimens and returns the answers to the accrediting agency, after which the results are evaluated and a grade provided. Once again, CAP initiated this protocol many years before CLIA '67. The rules are now set by the government, although there is some freedom to work within them. The best programs provide an educational experience as part of the exercises. CLSI has provided guidance for improving laboratory performance through proficiency testing.[25] If an external source for proficiency testing samples is not available for an analyte, then another method for assessing performance must be developed in the laboratory. A variety of methods exists, including exchanging samples between laboratories and constructing a panel of "unknown" challenges within the laboratory.[27]

In CLIA '88, some seemingly obvious rules were codified: the protocol for proficiency tests must be as close to that used for clinical specimens as feasible (including the use of the same testing personnel) and laboratory personnel cannot

compare results before submitting the answers—in other words, no cheating!

Personnel Qualifications. Detailed specifications for personnel at all levels are provided in the CLIA '88 regulations for laboratories that perform nonwaived testing.

Procedure Manuals. Although there is not a rigid prescription for preparation of written policies and procedures, some general principles must be followed.[34] Policies must be clearly stated—and followed. Procedures should include the principle of the test, instructions for performance, and references. The best procedures are so well written that a qualified individual who has never before performed the test could read the detailed procedure, follow it and perform the test. Manuals must be available to all workers who will need to refer to them.

Space Requirements. The regulations do not specify detailed space requirements. Rather they indicate that the space must be adequate to perform the work satisfactorily. Informal guidelines for general laboratories[24] and for microbiology laboratories[120]have been described, however; they can serve as a useful guide when

constructing a new facility or evaluating the adequacy of an existing laboratory.

Reference or Referral Laboratories. It is a rare laboratory that will be able to fulfill all the requests made by clinicians. Some specimens must be sent to a specialized laboratory for further testing.[33] Selection of the referral laboratory or laboratories should not be done casually. The available choices should be evaluated carefully in conjunction with the medical staff, as appropriate. Price is not the only determinant, or even the most important factor. The selection should be reviewed on a regular basis.

Patient Confidentiality. The Health Insurance Portability and Accountability Act of 1996 (HIPAA) provides safeguards for the confidentiality of information and allows patients to have access to their medical records. This now includes laboratory test results. Upon request, the test results must be provided to the patient within a reasonable timeframe. Safeguards must be in place to assure the identity of the patient is accurate. In most institutions, there will be similar policies regarding the release of medical records to patients upon request. It may be preferred to simply use these same policies and procedures for laboratory derived test results.

The FDA and Laboratory-Developed Tests. The FDA has oversight of the production of medically-important manufactured products. This agency has declared that this oversight responsibility extends to include laboratory-developed tests. This poses great concern to the medical community, given the number of laboratory-developed tests that are in daily operation throughout this country, and have become the standard of care in medical practice. To immediately "cease and desist" using such test, which fortunately has not been mandated to date, would be detrimental to patient care and would set medical practice back decades. The challenge of "how" the FDA will implement oversight, given an already strained infrastructure remains to be determined.

Risk Management and Patient Safety

Most healthcare facilities are currently involved in risk management. In fact, many hospitals and clinics have established formal risk management offices, fully funded and staffed, to help reduce to a minimum the chances for accidents and high-risk practices that might cause harm to patients and employees. The accreditation of hospitals has emphasized the need for continuous improvement in the area of safety. It is expected that patients should receive the latest from medical care without being harmed.[97] These initiatives also emphasize a safe work environment for employees. In addition to improving the healthcare environment for altruistic reasons, these initiatives also reduce costly workers' compensation claims and malpractice suits.

Working in conjunction with the quality assurance committee, a risk manager is assigned to investigate cases in which quality management is below the established thresholds or situations wherein employees or patients may be at undue risk. After

reviewing the details of the situation with the appropriate representatives of the department involved and after gathering the necessary data, the risk manager submits a condensed report to the quality assurance committee along with recommendations for corrective actions. A dialogue continues between the risk manager and the committee chairman until an appropriate plan of action is agreed upon. The compliance of the departmental personnel with the corrective action plans is then monitored.

Although the major focus of risk management is directed toward patient care, the clinical laboratory participates in seeing that all laboratory operations are in compliance with the overall practices and policies of the institution. If equipment or instruments are damaged because of fire or electrical accidents, if personnel are injured or if workers contract serious laboratory-acquired infections, then work flow may be disrupted and laboratory reports may be delayed. Thus, for the most part, laboratory risk management is related to implementing and monitoring laboratory safety practices, directed more to employees than to patients. Liability for injury clearly rests with the employer, even though the negligence leading to the injury may be that of a fellow worker.[64] For this reason, risk managers are insistent on conducting safety-oriented education courses and assuring that all rules and regulations pertaining to laboratory safety are both implemented and followed.

The other major area that is of increasing concern to risk managers is financial. There are strict regulations covering conflicts of interest, fraudulent billing, and unlawful enticements for business (i.e., kickbacks). Billing of government programs (Medicare and Medicaid) must utilize a set of test codes, called Current Procedural Terminology (CPT) codes. Most other insurers also utilize these codes. They are revised and published yearly by the American Medical Association (AMA), which has been charged by the government with this task. Input into the construction of the codes can be provided by any person or organization, but the operative committee of the AMA is drawn from its constituent societies. In the field of laboratory medicine, these are CAP and American Society for Clinical Pathology (ASCP).

Laboratory Safety

Although it is the legal responsibility of hospital and laboratory managers to provide for a safe working environment, employees must also bear the responsibility for adhering to safety

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standards outlined in the Laboratory Safety Manual, to bring to the attention of the supervisor any hazards or potential hazards that may be encountered during work activities and to seek immediate medical attention for any job-related injury.[64]

There should be a designed safety officer in every laboratory or department, whose duties are to assure that safety standards and guidelines are written and published and that employees are informed of these standards through regularly scheduled laboratory safety courses and meetings. They also work to maintain a system to monitor compliance. The safety officer will work closely with the hospital risk manager to reconcile and correct any breaches of conduct or any irregularities discovered.

General Safety Rules and Regulations. Laboratory workers are advised not to take unnecessary risks. Carelessness, negligence, and unsafe practices may result in serious injuries, not only to the individual, but to coworkers and patients as well. The following are general considerations that will make working in microbiology laboratories less of a risk.[31] Each laboratory director is responsible for ensuring that laboratory policies and procedures follow current legal requirements (federal, state, and local) and standards of good laboratory practice.

1. Each employee should be instructed on the location and operation of all safety equipment and facilities, such as fire blankets, fire extinguishers, showers and eye wash fountains. Each of these must be readily accessible in the laboratory.
2. Personal protective equipment (surgical gloves, lab coats, etc.) should be worn when indicated. Laboratory coats should be worn (with buttons closed) at all times while in the laboratory, and removed when leaving the laboratory. Masks that are individually fitted for each individual must be used for some manipulations that might result in the generation of infectious aerosols with important pathogens, such as *M. tuberculosis*.
3. Personal habits and grooming must be put in perspective. Long hair must be tied such that it will not interfere with equipment or reagents. Application of cosmetics in the work area is prohibited. Sandals and open-style shoes do not afford proper foot protection and are not acceptable. Fingers, pencils, and other implements should not be placed in the mouth. Horseplay and practical jokes are inappropriate in this environment and should not be tolerated.
4. Contact lenses, especially the soft type, absorb certain solvents and may be a hazard after splashes and spills. Employees are strongly advised not to wear contact lenses in the laboratory, or to wear safety glasses when working with caustic or infective materials.
5. Eating or storing food and beverages in the laboratory or in refrigerators used for specimens or laboratory materials is not permitted. A refrigerator physically located outside of the laboratory should be designated specifically to store food and drink.
6. Pipetting by mouth of any material is absolutely prohibited. A variety of suitable pipetting aids are available.
7. Laboratory personnel with current skin infections, acute respiratory infections, or other contagious diseases should avoid patient contact. If such infections

(e.g., influenza) place coworkers at risk, then the affected employee should not come to work.

8. It is important that laboratory workers know the characteristics of all materials in use, so appropriate precautions can be taken during use and disposal. The manufacturer is required to provide this information in material safety data sheets (MSDS). These sheets should be maintained in one location in the laboratory and readily available to everyone in the laboratory.
9. Appropriate labels and signs must be placed on all specimens or instruments and in all areas of the laboratory where they are necessary for maintenance of a safe work environment.
10. Types and levels of decontamination are summarized in Table 1-19. It is important to match the type of disinfection or sterilization to the biohazard.[19,20,119]

Routine Safety Precautions

Centrifugation

1. Before centrifuging any item, check tubes, vials, or bottles for cracks. Periodically replace the rubber cushions in the bottoms of the trunnions and remove any material that should not be present (e.g., broken glass).
2. Make sure that the centrifuge is properly balanced before use. Check the trunnion rings and tube carriers to be sure the weights match.
3. Wait for the centrifuge to come to a complete stop before opening the lid to remove samples. Use only the braking device to bring the rotation to a more rapid and complete stop.
4. Should breakage of a tube occur in the centrifuge, first turn off the instrument, wait at least 20 minutes before opening the lid, and, after donning mask and gloves, thoroughly clean and disinfect the inside of the centrifuge.
5. As part of the routine maintenance program, each centrifuge should be thoroughly cleaned with a suitable disinfectant on each day of use. Preventive maintenance of all working parts should be on a regular schedule as appropriate.
6. If potentially infectious material is centrifuged, which is always the case with biologic specimens, the specimen must be placed in a closed carrier (sealed dome centrifuge tubes).

Needles and Glassware

1. Discard all chipped or cracked glassware in appropriate containers.
2. Pick up broken glass with a brush and pan; do not use hands.
3. Glass articles should not be discarded in the sink or loosely in a wastebasket where paper articles are being discarded. They can cut the fingers and hands of individuals removing the refuge. They should be placed in a container designed

for sharp objects.

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TABLE 1-19 Types of Decontamination, Disinfection, and Sterilization[a]

Level[a]	EPA/FDA Category	CDC Category	Example	Type of Organisms	Examples
1	Hospital disinfectant	Low-level disinfectant	Quaternary ammonium compounds	Vegetative bacteria	*Staphylococcus* spp. *Pseudomonas* spp.
				Enveloped or midsized viruses	Human immunodeficiency virus Herpes simplex virus Hepatitis viruses B and C Coronavirus
2	Hospital disinfectant with tuberculocidal claim	Intermediate-level disinfectant	Quaternary ammonium compounds with alcohol; phenolics; iodophors; chlorine-containing products	Fungi	*Aspergillus* spp. *Candida* spp.
				Nonlipid or small viruses	Enteroviruses Rhinoviruses
				Mycobacteria	*Mycobacterium tuberculosis*
3	Sterilant/high-level disinfectant	High-level disinfectant	Glutaraldehyde; hydrogen peroxide	Bacterial spores	*Bacillus* spp.
4	Sterilization	Sterilization	Ethylene oxide; autoclave	All agents	

[a]Agents at each level are also effective against organisms killed by agents at higher levels. Prions require separate consideration; consult reference 99. Adapted from references 19 and 20.

4. Used needles and lancets (sharps) must be placed into appropriate used-needle containers for safe disposal. These sharps containers should be replaced periodically, when approximately three-fourths full; they should be monitored to prevent overfilling.
5. Avoid removing needles or exchanging needles on syringes as much as possible. There are devices that may be used to help protect employees if needles need to be removed. The practice of changing needles before discharging venepuncture blood into blood culture bottles has been abandoned in most hospitals.

Electrical Safety

1. All personnel should know the location of master switches and circuit breaker boards. Do not attempt to repair any instrument while it is still plugged in.
2. Plugs or cords that are broken, frayed, or worn should not be used.
3. Outlets must not be overloaded. Never use gang-type plugs.
4. All cord and plug-type electrical equipment should have grounded power cords and plugs. All shocks, including small tingles, must be immediately investigated.
5. Extension cords should be used only in compliance with the overall hospital

policies and procedures.

Corridor Cautions

1. Open doors into corridors with caution. Watch out for swinging doors. If there is a window in the door, look out to be certain the way is clear before opening the doors.
2. Keep to the right when approaching corridor intersections and when using stairways. Walk, never run, in halls, rooms, and stairwells. Appropriately placed mirrors provide images of potential traffic on crossing corridors.
3. Watch for hall hazards such as beds, carts, or tables. Watch out for articles on the floor, such as paper clips, electrical cords, loose tiles, and spilled liquids. Corridors should not be used for the storage of equipment, as this represents a hazard, particularly to egress in the event of a fire.

Lifting

1. Back injuries are among the most frequent causes of debilitating illness among hospital personnel. Avoid lifting heavy objects when possible. Always get help.
2. If you must lift objects alone, exercise the following precautions:

a. Have a good footing. Keep feet about 10 inches apart.
b. Bend at the knees to grasp the object.
c. Keep the object close to you and get a firm hold.
d. Keep the arms and back as straight as possible and lift gradually upward by straightening the legs.

Handling Specimens and Spills

1. Specimens should be collected in approved, sturdy containers with adequate closure to prevent spillage or leakage. All specimens must be considered a potential hazard.
2. Cuts on hands should be adequately covered with adhesive bandages. Wear disposable gloves if the

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work activity involves contact with any body fluid or tissues.
3. If a sample shows evidence of breakage, leakage, or soiling biohazard bag that contains the specimen, then follow the institutional guidelines regarding specimen leakage. This may necessitate containing the physician for the procurement of another specimen.
4. Blood-contaminated specimen requisitions should be rejected. Handle such a requisition only with gloves, if processing is necessary in an emergency. Notify

the requestor that such contaminated materials present a health hazard. Fortunately, the widespread use of electronic order entry is making this less of an issue.

5. Wash hands thoroughly with soap and water several times a day and, particularly, after handling specimens before leaving for a coffee or lunch break.
6. Spills must be handled according to the nature of the material involved and should follow local policy.

Handling of Wastes and Hazardous Material

1. Set aside certain sinks in the laboratory to dispose urine and other body fluid specimens. Hand washing should not be allowed in these sinks.
2. Biohazard bags (so-labeled) must be used to dispose of all potentially contaminated samples—blood tubes, specimen containers, pipettes, pipette tips, and reaction vessels, among others. Leave sufficient room at the top so that the bag can be easily closed and secured. It is good practice to double bag hazardous waste.
3. Dispose of glassware and sharps in appropriate hard-walled containers. When three-fourths filled, such containers should be placed in the appropriate labeled waste boxes for proper disposal.
4. Remove filled biohazard bags to designated waste areas as frequently during the day as necessary to avoid buildup.
5. Immerse contaminated reusable glassware into disinfectant solution. Thoroughly rinse with water and autoclave before reusing.
6. At the end of each day or after a spill, all work surfaces must be disinfected with an agent that is effective against the agents expected at that site. All equipment that is removed from the laboratory should be decontaminated first. In addition, laminar flow safety cabinets must be decontaminated (preferably by a technician certified in care of this equipment) before any maintenance is done or filters changed.

Biologic Agents

Classification of Biologic Agents. The CDC and National Institutes of Health have categorized infectious organisms into risk groups,[99] which are summarized in Table 1-20 The document can be ordered from the government printing office or downloaded free of charge from the Internet: (http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf).

Physical Containment of Biohazards. The physical barriers to infection in the laboratory are either personal or institutional. The personal barriers include good hygiene (e.g., separate facilities for food and for hand washing) and protective equipment (e.g., splash shields, goggles, gloves, gowns, and masks). Institutional barriers are structural (e.g., isolated facilities, doors, and locks) and technological (e.g.,

air handling and filtration). BSCs are a critical component of protection for workers. It is important to recognize that BSC and chemical hoods serve very different purposes. It is possible to construct a BSC that can be used as a chemical hood, but only certain types of BSC should be used for chemicals and chemical hoods should not be used for infectious agents.

The classification of BSCs is summarized in Table 1-21 and depicted in Figures 1-21 through 1-25. Most clinical laboratories use Type II A BSCs for processing of specimens and for work with isolates, especially those that present an aerosol hazard. Type I hoods are rarely used today; Type III hoods are not commonly employed in diagnostic laboratories. In the class II and III cabinets, air is directed across the work surface from the top of the cabinet in a laminar fashion, minimizing contamination of the samples from aerosolized agents. These devices are sometimes referred to as laminar flow safety cabinets. The downward laminar flow also protects the work from unfiltered air that is drawn in through the front portal in the class II cabinets; the front intake air is directed by the laminar flow into the sump below the work surface where it is filtered before eventually joining the downward laminar flow.

The velocity of the airflow in Class I and II BSCs must be validated by a certified technician at least yearly or whenever repair work is done. The cabinet must be decontaminated by a certified technician before any manipulations that require compromise of the contaminated area are undertaken (e.g., changing filters, replacing or repairing motors, or moving the cabinet).

Common infectious hazards in diagnostic laboratories: Although most agents encountered in a clinical laboratory have the potential for causing diseases in laboratory workers, a relatively small number fulfill that potential. Miller and associates[80]describe a 25-year experience of laboratory-acquired human infections at the National Animal Disease Center (NADC) in Ames, Iowa, an institution where research on domestic diseases of livestock and poultry is conducted. The level of risk among workers is probably somewhat greater than in the average clinical laboratory. From 1960 to 1985, 128 laboratory exposures to zoonotic organisms were reported at NADC. Thirty-four laboratory-associated infections resulted. Brucellosis accounted for 47% of the cases, leptospirosis for 27%, and mycobacteriosis for 9%. *Salmonella* spp. and *Chlamydophila* spp., Newcastle disease virus (not a human pathogen) and *Trichophyton* spp. accounted for the other laboratory-acquired infections at NADC.

The 10 most frequent laboratory-acquired infections from accumulated studies are (1) brucellosis, (2) Q-fever, (3) typhoid fever, (4) tularemia, (5) tuberculosis, (6) typhus, (7) viral hepatitis, (8) Venezuelan equine encephalitis, (9) coccidioidomycosis, and (10) psittacosis.[90–92,118] Some of these infections are difficult to prevent, although zero infections should always be the goal. In some circumstances, however, a means to prevent the infection is obvious, at least in retrospect. For instance, a 22-year-old medical technology student contracted typhoid fever with complications after working

with *Salmonella typhi* as an unknown organism.[58]

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TABLE 1-20 Classification of Biologic Agents by Risk[a]

Biosafety Level (BSL)	Class of Agents	Examples of Agents	Practices	Safety Equipment (Primary Barriers)	Facilities (Secondary Barriers)
1	Not known to cause disease consistently in healthy adults		Standard microbiologic practices	None required	Open bench top sink required
2	Associated with human disease Hazard = percutaneous injury, ingestion, or mucous membrane exposure	*Enterobacteriaceae* *Candida* spp. *Mycobacterium avium* complex Herpes simplex virus	BSL-1 practice plus: • Limited access • Biohazard warning signs • "Sharps" precautions • Biosafety manual defining any waste decontamination or medical surveillance practices	Class I or II BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials PPEs: laboratory coats, gloves, face protection as needed	BSL-1 plus: • Autoclave available
3	Indigenous or exotic agents with potential for aerosol transmission Disease may have serious or lethal consequences	*Mycobacterium tuberculosis* *Franciscella tularensis* West Nile virus	BSL-2 practices plus: • Controlled access • Decontamination of all waste • Decontamination of lab clothing before laundering • Baseline serum	Class I or II BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials PPEs: laboratory coats, gloves, face protection as needed	BSL-2 plus: • Physical separation from access corridors • Self-closing, double-door access • Exhausted air not recirculated • Negative airflow into laboratory
4	Dangerous or exotic agents which pose high risk of life-threatening diseases, aerosol-transmitted laboratory infections, or related agents with unknown risk of transmission	Arenaviruses that produce hemorrhagic fever (e.g., Lassa, Junin, Machupo) Filoviruses that produce hemorrhagic fever (e.g., Marburg, Ebola)	BSL-3 practices plus: • Clothing change on entering • Shower on exit • All material decontaminated on exit from facility	All procedures conducted in Class III BSCs or Class I or II BSCs in combination with full-body, air-supplied, positive-pressure personnel suit	BSL-3 plus: • Separate building or isolated zone • Dedicated supply and exhaust, vacuum, and decontamination systems • Other requirements listed in Reference[99]

[a]The safety level of many agents is increased when manipulations that might reasonably be expected to generate aerosols are performed or when large volumes of materials are employed. For some Level 2 agents, a higher level is indicated when cultures known to contain the agent are manipulated. A separate classification is used for agents in naturally or experimentally infected animals.
BSL, Biosafety level; BSC, Biologic safety cabinet; PPE, Personal protective equipment.
Adapted from reference 99.

TABLE 1-21 Comparison of Biologic Safety Cabinets

Type	Face Velocity (Lfpm)	Airflow Pattern	Radionuclides/ Toxic Chemicals	Biosafety Levels	Product (Specimen) Protection
Class 1 Open front	75	In at front; rear and top through HEPA filter (see Fig. 1-21)	No	2, 3	No
Class II Type A	75	70% recirculated through HEPA filter; exhaust through HEPA filter (see Fig. 1-22)	No	2, 3	Yes
Class II Type B1	100	40% recirculated through HEPA filter; exhaust via HEPA filter and hard ducted (see Fig. 1-23)	Yes (low levels/ volatility)	2, 3	Yes
Class II Type B2	100	No recirculation; total exhaust via HEPA filter and hard ducted (see Fig. 1-24)	Yes	2, 3	Yes
Class II Type B3	100	Same as IIA, but plena under negative pressure to room and exhaust air is ducted	Yes	2, 3	Yes
Class III	NA	Supply air inlets and exhaust through 2 HEPA filters (see Fig. 1-25)	Yes	3, 4	Yes

Lfpm, linear feet per minute; HEPA, high-energy particulate.
From reference 99.

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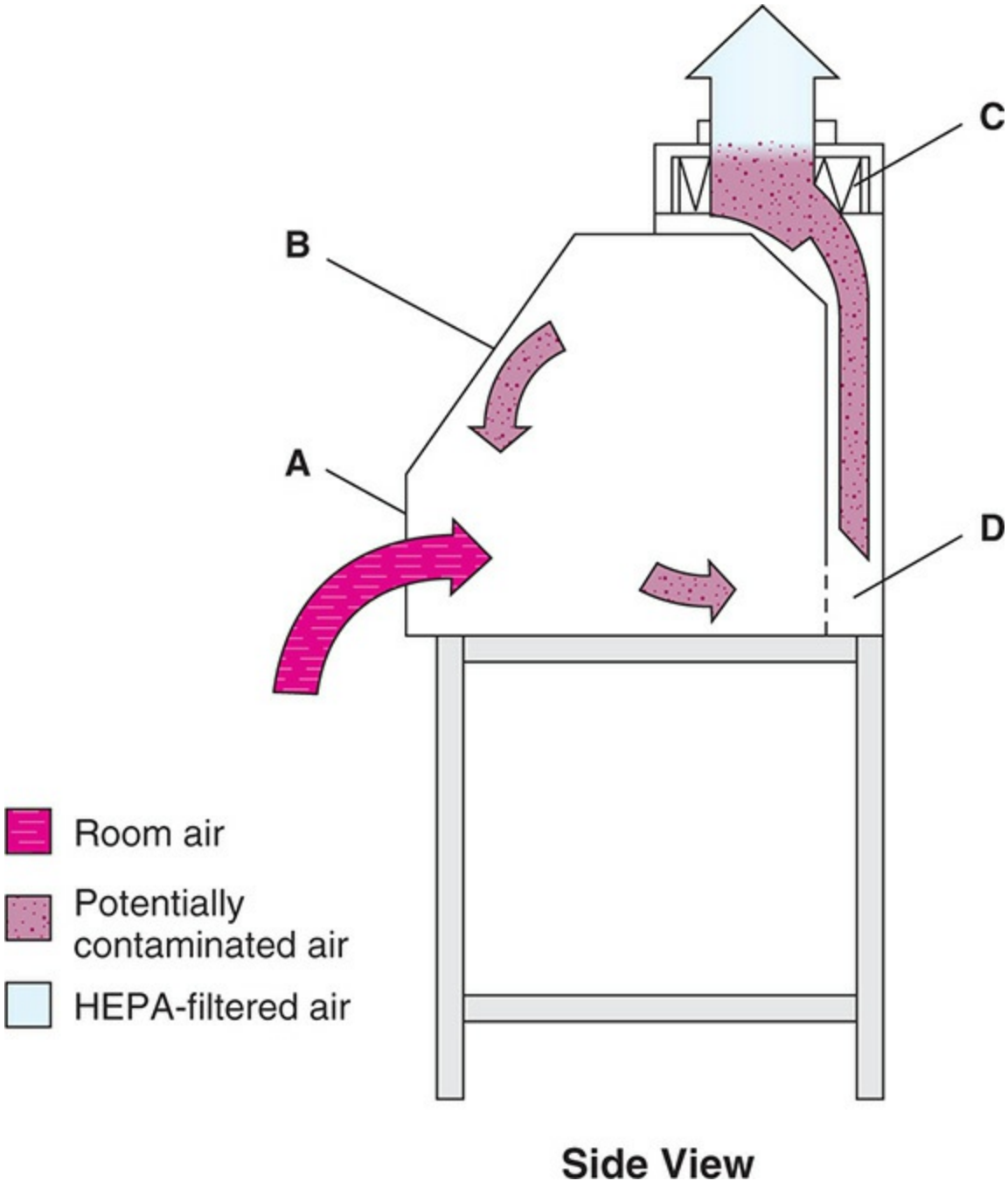
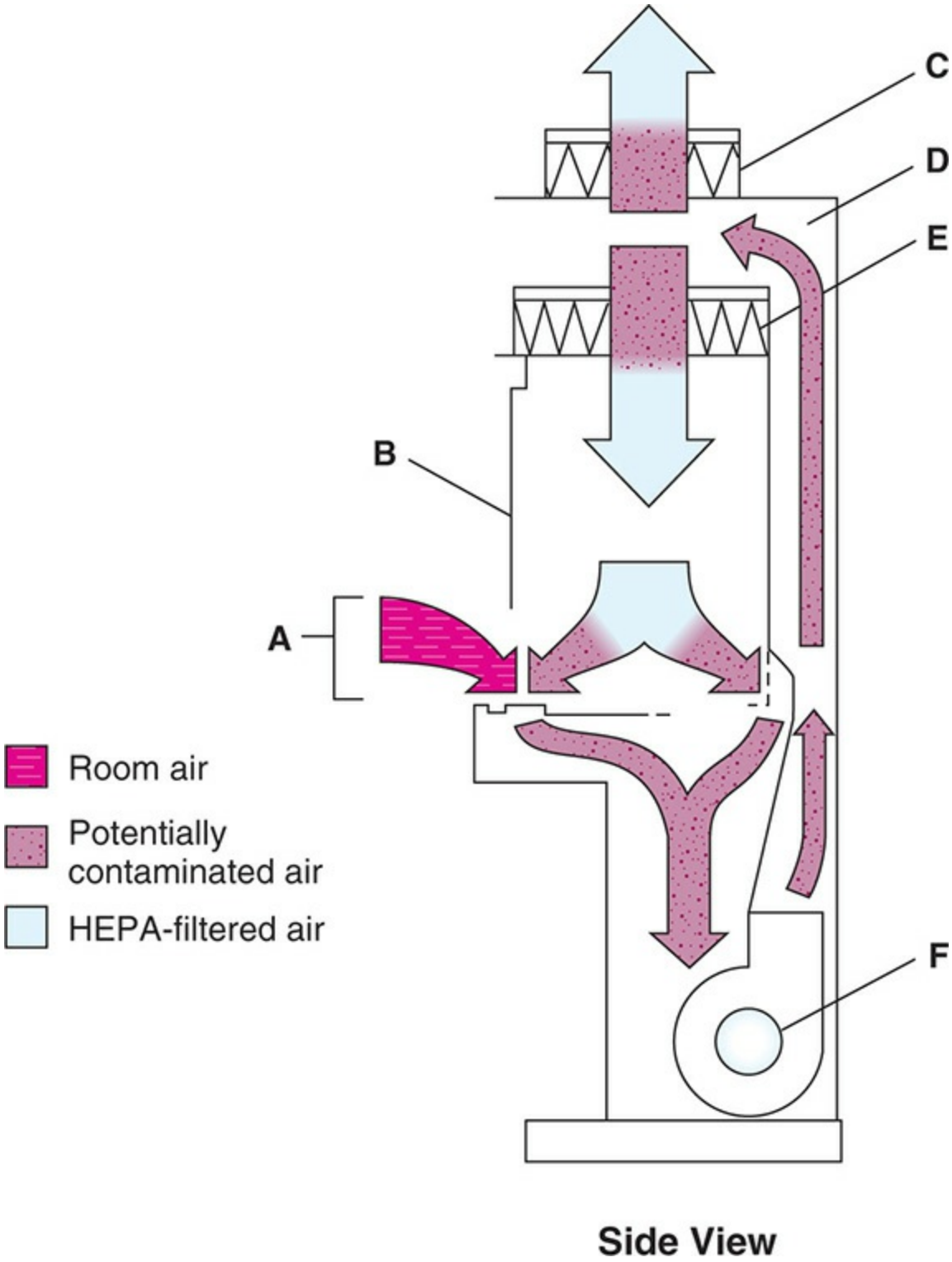
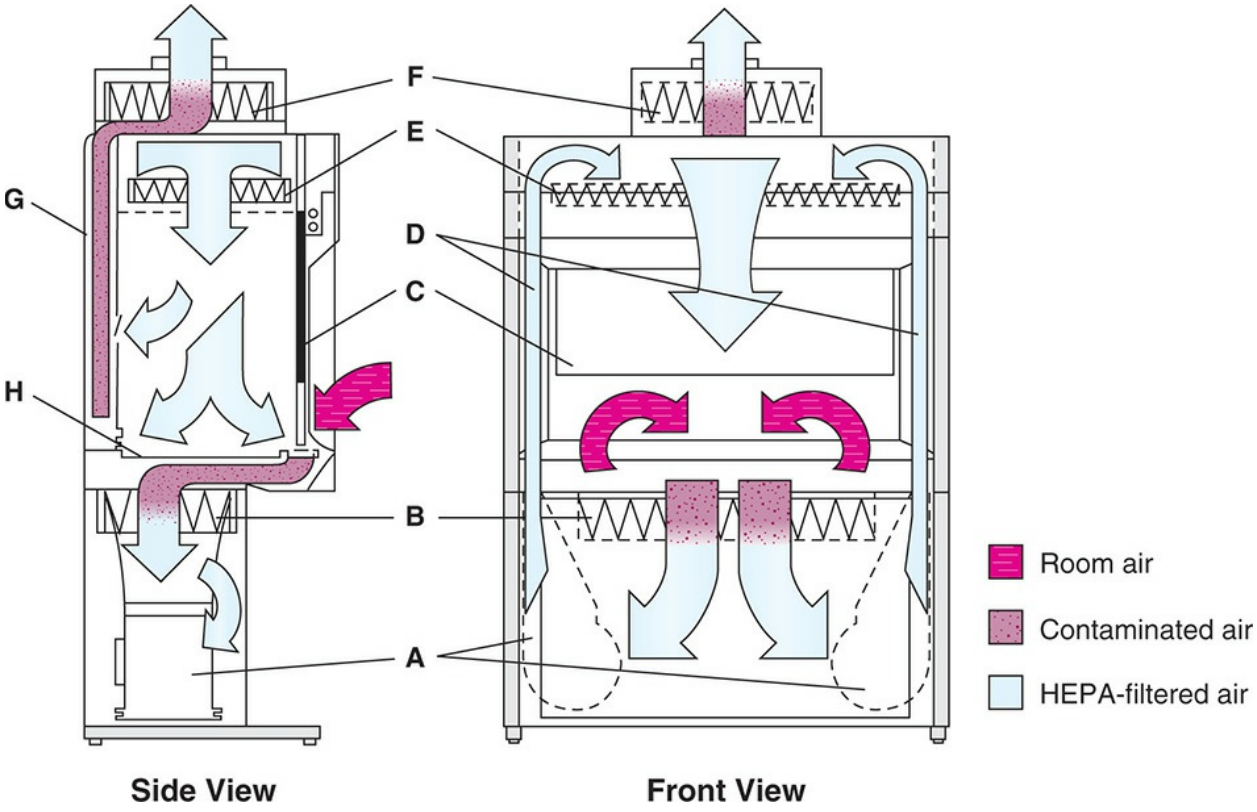


■ **FIGURE 1-21** Design of class I biologic safety cabinet (BSC). (**A**) These negative-pressure cabinets draw air from the room into the cabinet at a velocity of 75 fpm and exhaust it through a HEPA filter to the room or to the outside through a duct. It does not protect the specimen or sample from contamination with material present in room air. Because the specimen is not protected, these are uncommonly used in clinical microbiology, and have largely been replaced by class II units. (Adapted from reference 99.)



■ **FIGURE 1-22** Design of class II A biologic safety cabinet. Air is drawn from the room into the cabinet through the front opening (**A**), typically at a velocity of 75 fpm. It is then recirculated through a plenum (**D**) and a HEPA filter (**E**); a portion of the air, typically 70%, is recirculated into the workspace; the remainder is exhausted through the HEPA filter (**C**)

to the room. If the remainder is exhausted through a negative pressure plenum to the outside of the building, the cabinet is classified as class III B3. The work can be viewed through a glass sash (**B**). The blower is designated by (**F**). (Adapted from reference 99.)



■ **FIGURE 1-23** Design of Class II B1 biologic safety cabinet. Air enters the cabinet from the room through the front opening at a velocity of 100 fpm. It is then recirculated (typically 40%) through a sump (**A**) and recirculating plenum (**D**), passing through HEPA filters (**B** and **E**) before being exhausted (**G**) through a HEPA filter (**F**) into negative pressure ducts to the outside. Because of minimal recirculation, limited quantities of low-level chemicals can be used in addition to biologic agents, but this should not be considered a chemical fume hood. The work space (**H**) can be viewed through a glass sash (**C**). (Adapted from reference 99.)

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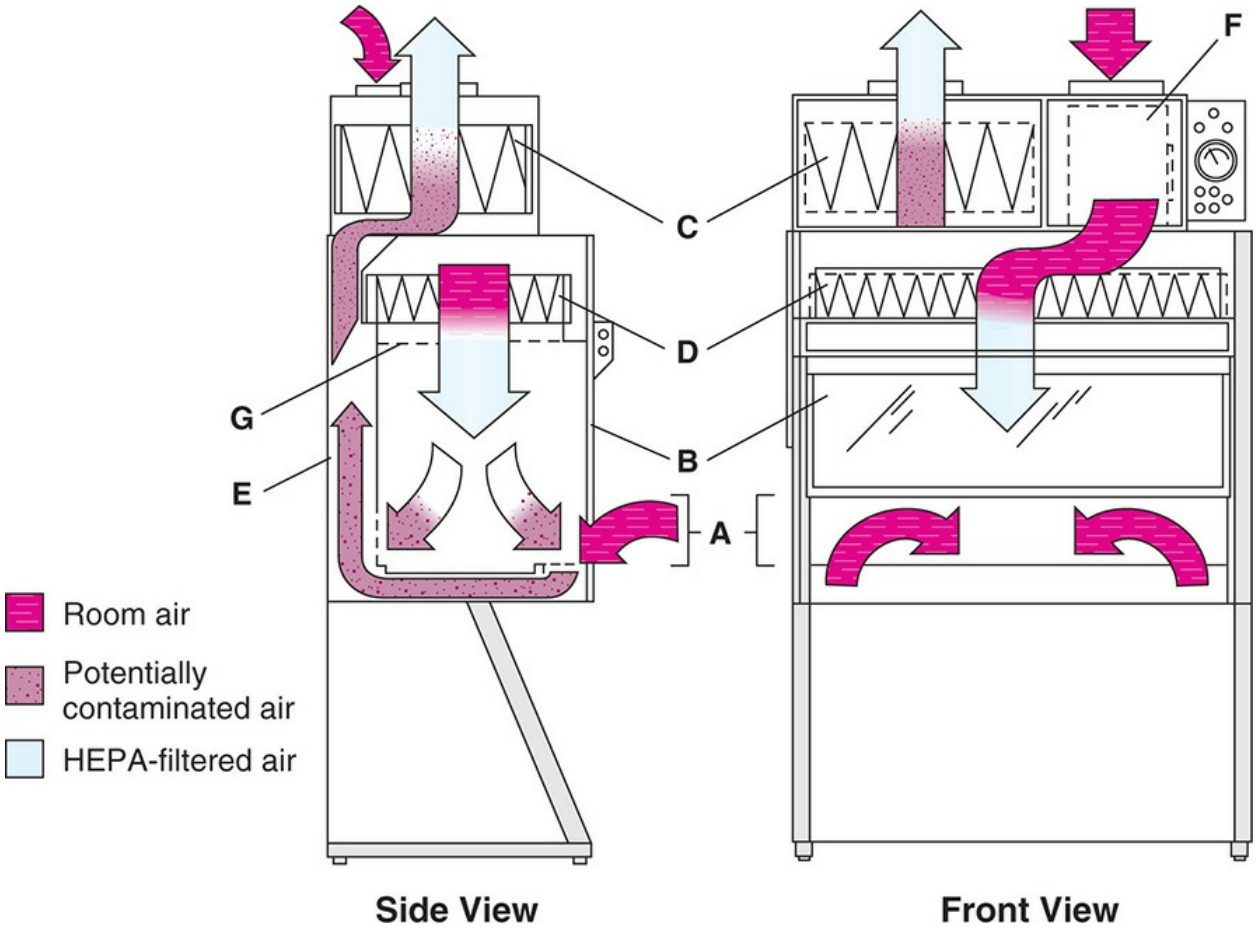
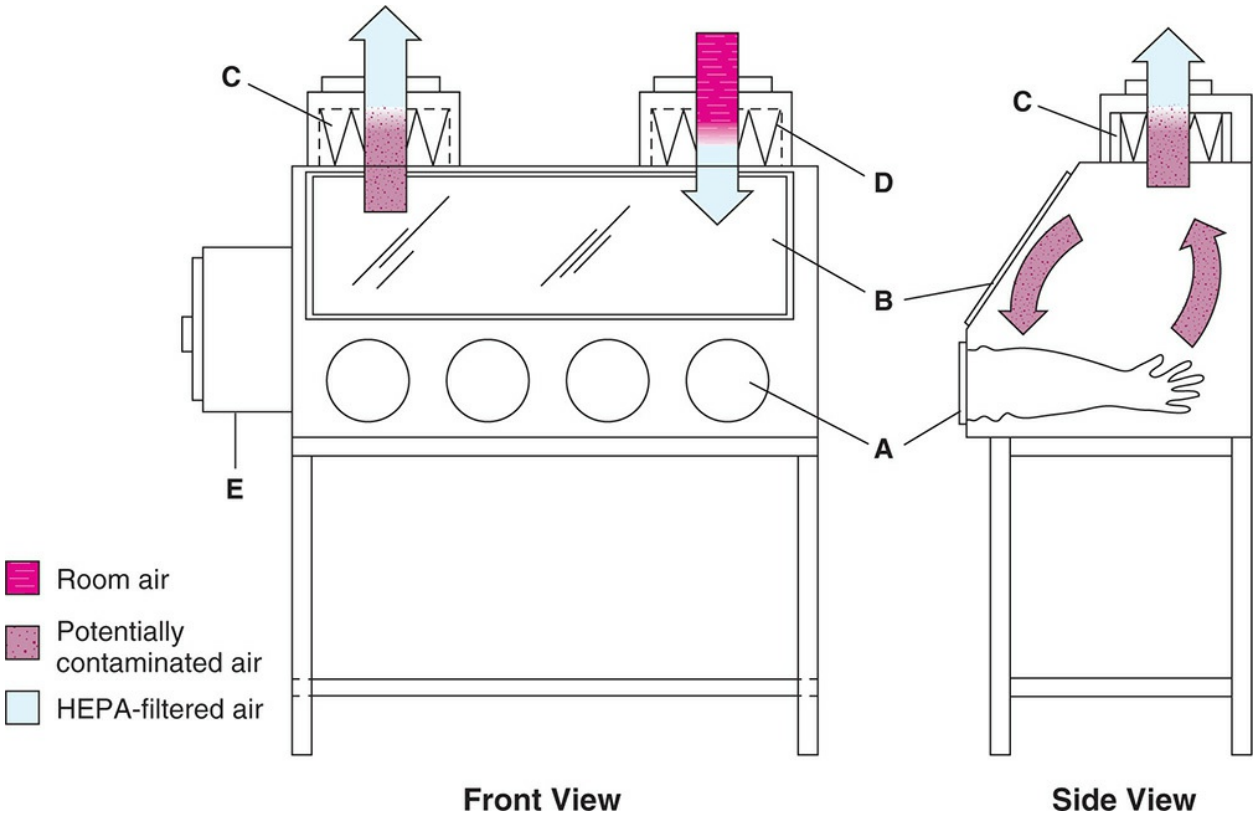


■ **FIGURE 1-24** Design of Class II B2 biologic safety cabinet. Air enters the cabinet from the room through the front opening (**A**) and is drawn into a cabinet plenum (**E**) before being exhausted to the outside through a HEPA filter (**C**) and a negative pressure duct system. Simultaneously, room air enters the cabinet through a second portal (**F**) and a HEPA filter (**D**), after which it passes down across the work area and then joins the outflow stream in the cabinet plenum (**E**). The work can be viewed through a glass sash (**B**). Toxic chemicals can be used in this cabinet that recirculates no air. (Adapted from reference 99.)



■ **FIGURE 1-25** Design of Class III biologic safety cabinet. This cabinet functions as a totally enclosed glove box with recirculating air. The hazardous agents are totally contained within the box, so the worker is not exposed. The same effect can be achieved if a Class II cabinet is used in conjunction with a biologic suit connected to an air supply for the worker, essentially isolating the worker and their air supply. Air enters through a portal and HEPA filter (**D**) before exhausting out through a HEPA filter (**C**) into a negative pressure duct system and the outside environment. The worker manipulates the samples through sealed gloves (**A**) and views the work through a glass shield (**B**). Samples are passed into the box through an interlock (**E**). The disadvantages of this approach include the difficulty of performing fine manipulations through thick rubber gloves. (Adapted from reference 99.)

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Even worse, 22% of the technologists in a laboratory-developed gastroenteritis from *Shigella sonnei*. Typing of the isolates from the infected technologists revealed that these were all identical to one that had been given to a student as an unknown.[78] Some agents that would not be encountered in a clinical laboratory may cause infection in research workers,[5,37,57] sometimes when manipulations that produce aerosols are performed without adequate containment.

Universal Precautions. Ironically, laboratory workers are at considerably less risk of

acquiring infections than are their clinical colleagues. There are several reasons for this phenomenon. Patients sneeze and cough, whereas culture plates and clinical specimens can be handled in a safe, standardized manner that prevents infection. Unless procedures that produce large aerosols are performed in the laboratory, the risk for laboratory workers is relatively low. Physicians and nurses are much more likely to use sharp objects, such as scalpels and needles than are laboratory personnel. In modern medicine the greatest infectious risks are blood-borne pathogens. Technologists who work in microbiology are at lower risk than colleagues in other areas of the laboratory, such as chemistry and hematology, where large numbers of blood specimens are handled daily. The most serious laboratory-acquired infections are HIV, hepatitis B virus, and hepatitis C virus. Of these agents, hepatitis C virus is the most prevalent in most hospitals, because an effective vaccine is available for hepatitis B virus and the incidence of HIV in the general population is not as high as HCV. It is fortunate that an effective vaccine for hepatitis B virus exists, because the risk of an unimmunized worker contracting this infection is far greater than infection with hepatitis C virus or HIV.

Recommendations for minimizing blood-borne infections have been promulgated by CDC,[15–18] by the CLSI,[35] and by laboratory workers.[9]

The risks of contracting the major blood-borne pathogens are summarized in Table 1-22.[18]

It is usually not possible to predict those individuals who may harbor blood-borne pathogens, so the concept of **universal precautions** has arisen. That is, every specimen is considered a risk. Specifying some specimens as risky increases the possibility that a false sense of security will be engendered when dealing with the other specimens, which may in fact contain blood-borne pathogens.

The CDC and Occupational Health and Safety Administration (OSHA) have established the following universal precautions[13]:

1. Blood and body fluids from all patients must be handled as infectious material. These specimens from all patients should be assumed to be infectious (i.e., contain blood-borne pathogens).
2. All specimens of blood and body fluids should be put in a well-constructed container, with a secure lid, to prevent leaking during transport.
3. All persons who process blood and body fluid specimens (e.g., removing tops from vacuum tubes) should wear gloves—plus a face shield (or a mask with glasses or goggles); alternatively, they may work in a BSC that has adequate protection against splashes (i.e., a glass barrier between the specimen and the technologist).
4. Workers must change gloves and wash hands when finished processing specimens.

5. Workers should never pipette by mouth; they must use mechanical devices.
6. The use of needles and syringes should be limited to situations in which there is no alternative; needle-handling safety devices should be used.
7. Laboratory work surfaces should be decontaminated with an appropriate chemical germicide after a spill of blood or other body fluids and when work activities are completed.
8. Contaminated materials used in laboratory tests should be decontaminated before reprocessing, or, if disposable, these should be placed in bags and disposed of in accordance with institutional policies.
9. All persons should wash their hands after completing laboratory activities and should remove protective clothing before leaving the laboratory.

The recommendations for dealing with exposures vary by agent; recommendations are reviewed regularly by CDC and altered as necessary. In general, the source patient should be tested to determine if a risk exists and the injured worker should be followed to determine if infection has resulted. Postexposure immunization and/or prophylactic antiviral chemotherapy are appropriate in certain situations. These should be guided by infectious diseases physicians or other experienced clinicians. It must be emphasized strongly that primary attention should be given to prevention, so that the

problem of an accident never presents itself. Prevention can take the form of immunizing at-risk personnel and minimizing the exposure to "sharps" (needles, scalpel blades, broken glass, etc.), which are the most common sources of injury.

TABLE 1-22 Risks of Contracting Certain Blood-Borne Viral Infections After Needlestick Exposure to Blood[a]

Virus	Status of Source Patient	Outcome	Risk (%)
Hepatitis B	Positive for HBsAg and HBeAg	Clinical hepatitis	22–31
	Positive for HBsAg and negative for HBeAg	Clinical hepatitis	1–6
		Seroconversion	23–37
Hepatitis C	Seropositive	Seroconversion	0–7
HIV	Seropositive	Seroconversion	0.30

[a]The risk after contact of blood with mucous membranes is less well defined, but less than after needlestick exposure.
Data from reference 18.

Cleaning Spills of Infectious Materials. The recommended protocol for cleaning up skills of infectious material is[35]:

1. Wear gloves (preferably heavy-weight, puncture-resistant utility gloves), gowns, and masks.
2. If there are fragments of glass or other objects, they should be removed without touching them directly before proceeding.
3. Wear shoe covers that are impermeable to water if the spill is large.
4. Cover the spill with absorbent material and add concentrated disinfectant. After waiting 10 minutes, proceed with cleanup.
5. If aerosols may have been generated, as in a broken centrifuge tube, turn the centrifuge off but leave it closed for at least 30 minutes to allow any aerosols to settle.
6. Absorb the bulk of the spill with disposable materials before proceeding with disinfection.
7. Clean the spill site of all visible contaminating material with an aqueous detergent solution or a 10% solution of household bleach.
8. Decontaminate the site with an appropriate disinfectant (Table 1-19).
9. Absorb the disinfectant material and rinse the site with water. Dry the site a final time to prevent slipping.
10. Dispose of all materials in a biohazard container.

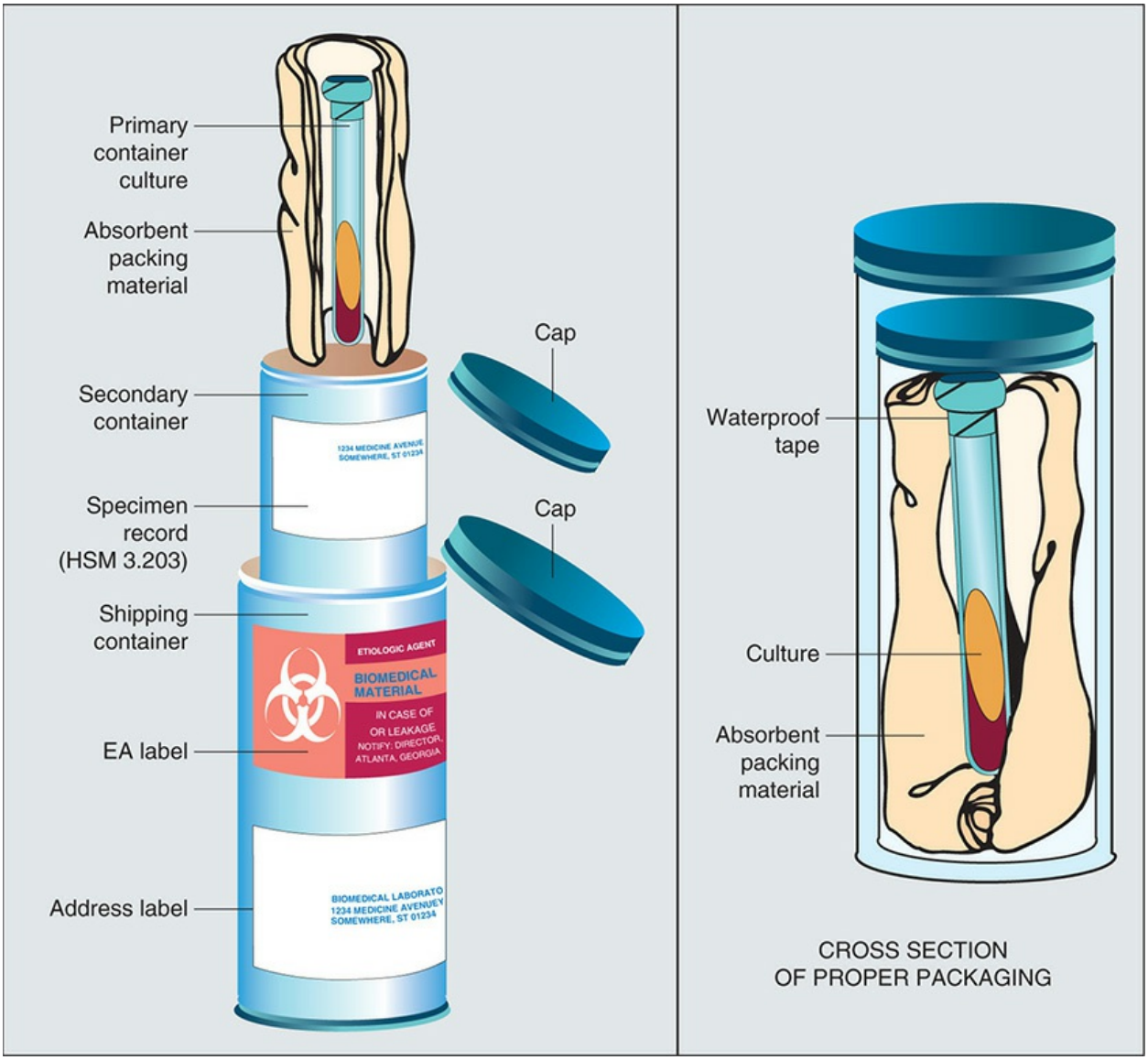
If a BSL-3 agent is spilled outside of a BSC, evacuate the area for at least 60 minutes, and notify the appropriate authorities according to your local policies.

Shipping of Specimens and Etiologic Agents. All microbiology specimens to be transported through the United States mail or commercial carriers must be packaged under strict regulations specified by the Department of Transportation (DOT) and the International Air Transport Association (IATA). Isolated organisms (etiologic agents) other than biosafety level 1 (Table 1-20) and diagnostic specimens reasonably expected to contain those etiologic agents must be appropriately packaged and labeled.

Specimens must be prepared to withstand shocks or pressure changes that may occur during handling and cause the contents to leak. A leaking container not only predisposes the specimen to potential contamination, but may also expose handlers or personnel at the receiving site to pathogenic agents. Figure 1-26 illustrates the proper packaging and labeling of etiologic agents. The primary container (test tube, vial) must be fitted with a watertight cap and surrounded by sufficient packing material to absorb the fluid contents should a leak occur. This container is then placed

in a watertight secondary container, preferably constructed of metal or rigid plastic, fitted with a screw-cap lid. The primary and secondary containers are then enclosed in an outer shipping carton constructed of corrugated fiberboard, cardboard or

Styrofoam.



■ **FIGURE 1-26** Proper technique for packaging of biologically hazardous materials.

Dry ice is considered a hazardous material. A shipping carton containing dry ice as a refrigerant for a specimen must be marked **"DRY ICE FROZEN MEDICAL SPECIMEN."** The packaging should be such that carbon dioxide gas can escape, preventing a buildup of pressure that could rupture the container. The dry ice should be placed outside the secondary container along with shock-absorbent material in such a manner that the secondary container does not become loose inside the outer container as the dry ice sublimates.

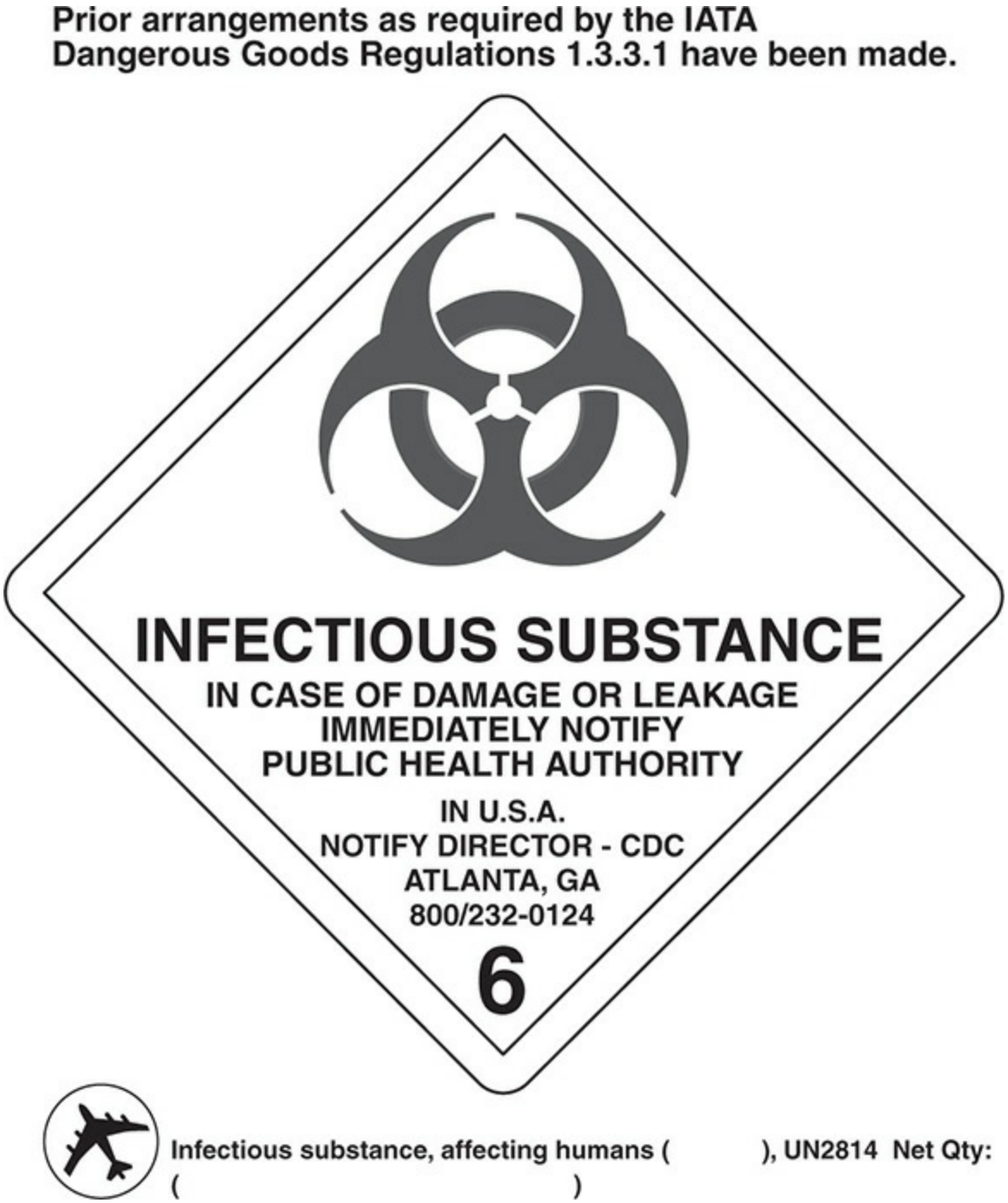
In addition to the address label, the outer container must also have the etiologic agents/biomedical material label (with its red logo against a white background) affixed as well as a notice to the carrier, as illustrated in Figure 1-27.

Individuals who package isolates and clinical specimen should have specialized training with respect to current regulations. The record of their certification should be maintained in their personnel files. Noncertified individuals should not ship microbial isolates or clinical specimens.

Nonbiologic Hazards

Chemicals

1. A chemical hygiene plan should be constructed for the laboratory.[31] Guidelines for managing laboratory waste are available.[29]



■ **FIGURE 1-27** Etiologic agents logo and "notice to carrier" that must be affixed to the outside of any package containing potentially hazardous or infectious materials.

2. Volatile combustible substances give off vapors along the surface of the liquid. The flash point is the lowest possible temperature at which a sufficient concentration of vapors is produced for a flame to occur. Volatile substances are collectively referred to as ignitables. The classification based on flash points and

boiling points is:

a. Flammables
   1) Class IA: Flash point, <73°F (22°C); boiling point, <100°F (38°C)
   2) Class IB: Flash point, <73°F (22°C); boiling point, >100°F (38°C)
   3) Class IC: Flash point, >72°F (21°C) and <100°F (38°C)

b. Combustibles:
   1) Class IIIA: Flash point, >140°F (60°C) and <200°F (94°C)
   2) Class IIIB: Flash point, >200°F (94°C)

A number of combustible materials may be found in the microbiology laboratory, although not in the quantities that are used in some other areas. A particular hazard is diethyl ether, which can form explosive peroxides after exposure to air. Ether has been used in some parasitology concentration procedures. Other alternatives that eliminate this hazard are now available and should be considered. If it is necessary to use ether, the smallest quantity possible should be stored appropriately.

3. Corrosive chemicals are defined as agents that have a pH <2.1 or >12.5 or that can corrode steel (SAE 1020) more than 0.25 inches per year at a temperature of 130°F. In the laboratory, the most common corrosives are strong acids, such as hydrochloric acid. Acid bottle carriers must be used for concentrated acids in quantities greater than 500 mL.

4. Incompatible chemicals (identified from MSDS) must not be used or stored together.

5. Storage safety cans and cabinets must be located away from sources of heat, flame, sparks, and exits. Storage areas should be adequately ventilated and of limited access to personnel. All flammable and corrosive chemicals should be stored in a fire-proof chemical storage cabinet according to local and national regulations.

6. All containers should be clearly labeled with

a. Content
b. Hazard warnings
c. Special precautions
d. Date received/prepared
e. Date opened/put in use
f. Expiration date
g. Manufacturer

7. In case of a liquid chemical spill[29,31]:

a. Determine the nature of the hazard by consulting the MSDS, if necessary. If the spill is an emergency, evacuate the area. If the material is a fire hazard,

eliminate all sources of ignition and take step to neutralize the chemical.

b. Notify the appropriate personnel and obtain additional help, if necessary. Identify any need for personal protective devices.

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c. Confine the spill to as small an area as possible.

d. Neutralize acids with disodium carbonate. Neutralize alkalis with 1% boric acid. For larger amounts of acids or bases, flush with large amounts of water after neutralization.

e. Clean any areas that have been splashed by the spill.

f. For flammable and toxic liquid spills, use an absorbent to reduce the vapor pressure and prevent possible ignition of the liquid.

8. Disposal of chemicals:

a. Wear rubber gloves, a rubber apron, and goggles.

b. Remove all items from the sink designated for disposal. Start a nonsplashing stream of cold water into the sink.

c. Slowly pour the liquid as close to the drain as possible without splashing. Only quantities less than 500 mL can be disposed of in the sink drain.

d. Continue to run cold water for several minutes after completion.

e. Dispose of water-soluble organic solvents (methanol, acetone) as described in the foregoing. For water-insoluble organic liquids, only quantities less than 100 mL can be disposed of as just described. For quantities over 100 mL, consult with the hospital safety officer or the local Environmental Health and Safety Office.

9. Radiologic hazards: Formerly, clinical laboratories used radiochemicals, primarily for immunoassays. These procedures have been replaced by enzyme immunoassays or molecular methods. However, if any radioactive materials are used in the laboratory, appropriate regulations must be strictly followed. There is usually an institutional safety officer for radioactive materials, often in the Department of Radiology.

10. Carcinogens: Careful attention to safety issues is necessary for those chemicals that have been shown to have some neoplastic potential. In the microbiology laboratory, the most likely compound to be encountered in this category is formaldehyde. Formalin is a commercial stabilized solution of formaldehyde, usually used as a 10% buffered solution (4% formaldehyde). Formaldehyde is combustible as well as a potential carcinogen. Local regulations for storage, use, and disposal differ. In general, if formaldehyde is used, then the work area should be well ventilated, preferably a chemical exhaust hood should be used.

CAP requires monitoring of formaldehyde vapors in the workspace.

11. Mercury: Elemental mercury is a significant health hazard. In the microbiology laboratory, it is most often encountered in nonelectronic thermometers and in some fixatives in parasitology. Although only small quantities are involved, many institutions have elected, either voluntarily or because of local laws, to eliminate mercury entirely.

Fire

1. Every hospital employee is responsible for preventing fires and assisting in minimizing losses should fire occur.
2. Keep work areas free of trash accumulation and excess flammable materials. Corridors, passageways, aisles, and stairs must be kept free of obstructions that may inhibit exit or add fuel to a fire.
3. Be aware of ignition sources, open flames, heating elements, and spark gaps (motors, light switches, friction, and static). More than 22% of hospital fires are caused by faulty wiring.
4. Personnel should be instructed on the differences in, and use of, fire extinguishers for the four classes of fire:

a. Class A: Fires involving ordinary combustible materials such as wood, paper, cloth, and plastics. The use of a pressurized water extinguisher (type A) is acceptable.
b. Class B: Fires involving flammable liquids, such as alcohol, gasoline, kerosene, and grease; use a carbon dioxide extinguisher (type B).
c. Class C: Fires involving energized electrical equipment in which a resulting shock hazard owing to electrical conductivity may exist. De-energize the circuit and use a nonconductive extinguishing agent (i.e., never use a water extinguisher; use a dry chemical type extinguisher [type C]).
d. Class D: Fires involving combustible metals, such as magnesium and potassium. Special techniques are required. Never use water; use a dry power agent. Immediately call the local fire station.

5. Fire blankets are used to smother clothing by wrapping the victim in the blanket. If clothing should catch fire, drop to the ground and roll to smother the flame against the floor. Do not run for the blanket—airflow will only fan the flames and result in more serious injury. Similarly, a fire blanket should be used on the floor; wrapping the blanket around a standing person only creates a chimney for the flames.
6. Comply with all local fire regulations. Participate in periodic fire drills as conducted by the hospital. Each employee should know the fire drill procedure and the evacuation route for their area of the laboratory.

Biodefense

The possibility of terrorists using chemical, biologic, or radiologic agents has been real for many years, but the possibility took on new significance after (1) the tragedy at the World Trade Center and (2) the discovery of letters that contained anthrax spores and had been sent through the postal service soon thereafter. All laboratories are now required to limit certain agents to absolutely essential use and to provide the government with an inventory of those agents. In addition, each laboratory must prepare a biodefense plan for dealing with bioterrorism. Very few diagnostic laboratories will have one of the select agents on their premises (Table 1-23). The primary responsibility for dealing with most of these organisms falls to reference laboratories, public health laboratories, and other government laboratories. The primary responsibility of the diagnostic laboratory is to recognize the possibility of one of the listed agents in the normal course of doing assessing clinical specimens. Once the possibility of a select agent is recognized, then the agent is referred to a designated support facility, the proper authorities are notified, and residual specimens from which the agent was isolated must be destroyed. If terrorism is suspected, the investigation will quickly assume a criminal component. The categories of potential bioterrorism agents are summarized in Table 1-23.[14]

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TABLE 1-23 Classification of Biologic and Chemical Agents With Potential for Terrorism

Category of Agent	Description of Category	Examples
Category A	• Easily disseminated or transmitted person-to-person • Cause high mortality with potential for major public health impact • Might cause public panic and social disruption AND • Require special action for public health preparedness	• Variola major (smallpox virus) • Bacillus anthracis (anthrax) • Yersinia pestis (plague) • Clostridium botulinum toxin (botulism) • Francisella tularensis (tularemia) • Filoviruses (Marburg and Ebola viruses) • Arenaviruses causing hemorrhagic fever
		• Coxiella burnetii (Q fever) • Brucella spp. (brucellosis) • Burkholderia mallei (glanders)

Category B	• Moderately easy to disseminate • Moderate morbidity and low mortality • Require enhanced disease surveillance	• Alphaviruses (e.g., eastern and western encephalomyelitis viruses) • Ricin toxin from Ricinus communis (castor beans) • ε-toxin of Clostridium perfringens • Staphylococcal enterotoxin B • Salmonella spp. • Shigella dysenteriae • Escherichia coli (O157:H7) • Vibrio cholerae • Cryptosporidium parvum
Category C	Emerging agents with: • Availability • Ease of production and dissemination • Potential for high morbidity, mortality, and public health impact	• Nipah virus • Hantaviruses • Tick-borne hemorrhagic fever viruses • Tick-borne encephalitis viruses • Yellow fever virus • Multidrug-resistant Mycobacterium tuberculosis

Adapted from reference 14.

The national preparedness plan for terrorism envisions a multilevel categorization of laboratories with a graded responsibility for evaluating potential threats. The classification of laboratories is detailed in Table 1-24. The original four categories have been compressed to three.[14]

Quality Assurance

Constant attention to the quality of work is a hallmark of good clinical laboratories. Quality assurance is the broad umbrella that covers several activities. These activities are sometimes known by slightly different terms at different institutions. Quality improvement or total quality improvement has given way to quality assurance, which now may be giving way to "continuous improvement." The basic message, however, is that microbiologists must constantly examine their methods and processes in an effort to improve performance, accuracy, and quality. Quality control is a traditional element that will be discussed separately.

Quality assurance can be accomplished entirely internally or may be done as part of

an external program. National consensus guidelines are available.[32,30] These programs should encompass all phases of the diagnostic cycle.[32]

Many of the features of a quality assurance program were discussed in the section on laboratory accreditation, which includes quality programs as a critical feature. Internal activities include documentation of the validation/verification for each new test introduced into the laboratory. The documentation of the utilization of the laboratory by clinicians is important, and may be part of a broader test utilization program. The performance of technical personnel in each task for which they are responsible must be documented. The comparability of morphologic observations among technologists performing the same tasks must be evaluated. Formal evaluation of the competencies of all personnel who perform laboratory tests (including nurses and physicians) is an accreditation requirement for all nonwaived tests. National consensus guidelines for setting up the program have been published.[28]

Evaluating laboratory performance can be improved by comparing your performance to that of your peers, using an external quality assurance program. CAP offers two such programs.[122,102] A parameter that is chosen for evaluation is referred to as an indicator.

The first program, called Q-Probes, consists of a one-time study on a single issue, such as the frequency of skin organisms in blood cultures (i.e., blood culture contamination rates). Each participating laboratory submits the results of a study that has been performed according to a defined protocol. The results of all participants are analyzed carefully and a critique is provided to all who have submitted results.

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TABLE 1-24 Classification of Laboratories Involved in Investigation of Biologic or Chemical Terrorism

Level	Description	Examples
A	• Early detection of intentional dissemination of biologic or chemical agents • Initial processing of clinical specimens	• Hospital or public health laboratories • Low-level biosafety facilities
	• Core capacity for isolation and characterization of select bioterrorism agents • Serve to minimize higher-level laboratories from being	• Local and state public health

B (originally B and C)	overwhelmed with specimens • Advanced capacity for rapid identification of select agents, using culture and molecular techniques • Participate in test development and evaluation	laboratories • High-level federal laboratories • Academic medical centers
C (originally D)	• Highest level of containment and sophistication • Ability to detect engineered agents	• Select group of federal laboratories • Academic laboratories operating at highest containment level under federal contract

Adapted from reference 14.

In contrast, the Q-tracks program is a repetitive monitor of a limited number of indicators that have been judged to be particularly useful for assessment of laboratory performance. For instance, laboratory TAT for a particular analyte might be studied over time. It is then possible to plot performance over time in relation to peers.

A laboratory can also judge its performance against peers by participating in proficiency testing, as discussed previously.[25,109] Proficiency testing was initially provided by CAP as an educational tool for improving laboratory performance. After the introduction of the two CLIA laws, proficiency testing became a mandated part of doing business in laboratory medicine.

Quality Control

Quality control in the narrow sense has consisted of an ongoing, systematic assessment of work to ensure that the final product conforms to previously established tolerance limits of precision and accuracy.[3] Laboratory directors and supervisors must now realize that quality control is only one facet in the larger arena of quality assurance. Interested readers can access the current requirements of CAP for quality control (as well as safety and other important management issues) by downloading the Laboratory General Checklist from the College Web site (http://www.cap.org).

In a broad sense, quality control in microbiology is as much an art as it is a science. It involves intangible items such as common sense, good judgment, and constant attention to detail. Quality control programs should be organized, with well-defined objectives in mind and metrics should be documented to monitor, and if necessary improve, performance.

Components of a Quality Control Program. A basic microbiology quality control program contains several specific items that must be considered when implementing the various phases of the program. Bartlett[6] developed a quality control program and discusses different levels of activity ranging from basic to most advanced. Using his outline, a supervisor can select the level of activity that is appropriate for the personnel and volume of work in any given laboratory. These have been used and improved upon in the CLSI documents referenced throughout this text.

CAP has established standards for accreditation of medical laboratories, including an inspection checklist for microbiology laboratories. This checklist provides microbiology supervisors with valuable guidelines for making a point-by-point assessment of the quality control needs in their laboratories. Many of the requirements are embedded in the federal regulations of CLIA '88.

Regulations are constantly under review and may be revised. Some of the changes are more stringent than those of some deemed accreditation agencies, such as CAP; others are less stringent. By law, the deemed agencies must have standards that are at least as stringent as CMS, but their standards may be more stringent.

A significant and ongoing change is occurring during the writing of this chapter. This is the mandating of a new quality control plan by the CMS called the Individualized Quality Control Plan (IQCP). Guidance regarding this plan is available on the CMS website, www.cms.gov. Also look for guidance from CAP, CLSI, and the American Society for Microbiology.

At the onset, a quality control coordinator must be selected. The duties of the coordinator must be clearly established and appropriate authority conferred to the extent that problems can be efficiently handled when they arise. It is the responsibility of this coordinator to establish the minimal standards for quality control that are to be met by the laboratory and to outline the several steps to be taken for daily monitoring and surveillance of all facets of the program.

The coordinator should see that all activities are clearly described in a quality control manual. The quality control manual should also clearly outline the following:

1. The details of all quality control practices, such as the procedures and schedules for monitoring equipment function

2. The process for monitoring of all media and reagents for reactivity, expiration dates, and reaction patterns of appropriate challenge organisms
3. All proficiency testing results

Appropriate forms must be designed to collect data in the form of columns of numbers, graphs, or diagrams so that any item that is out of control can be detected

quickly. The coordinator also must review all control records and verify that all measurements that are out of control. There must be documentation of the corrective actions for all measurements that were out of control. A brief review of the several components of a quality control program follows.

Monitoring Laboratory Equipment. A preventive maintenance program should be established in all microbiology laboratories to ensure proper functioning of all electrical and mechanical equipment. Equipment should be checked at prescribed time intervals; certain working parts should be replaced after a specified period of use, even though they may not appear worn-out. A brief list of some of the equipment, the monitoring procedures to be carried out, and the frequency and tolerance limits is shown in Table 1-25. Assignments should be made among laboratory personnel to ensure that all inspections are carried out and all data are recorded accurately onto charts or in maintenance manuals. It is important to detect upward or downward trends immediately, so appropriate corrective action can be taken before serious errors result. The temperature of incubators, refrigerators, freezers, water baths, and heating blocks must be determined and recorded daily with a thermometer calibrated by the Bureau of Standards or with one that has been checked against a calibrated thermometer. The concentration of CO_2 in all CO_2 incubators must also be determined daily. For any reading that is outside of the established quality control range, the cause must be determined and the defect quickly corrected.

Monitoring Culture Media, Reagents, and Supplies. All media and reagents must be checked against appropriate controls for the proper reactivity. It has been recognized that many modern commercial media perform with a high degree or reliability. Consensus recommendations have been developed for the necessity of local quality control.[23] The few media with occasional quality control problems (e.g., chocolate agar, media for *C. jejuni,* and Thayer–Martin agar) should be subjected to control tests in each laboratory. Many others need not be tested if the manufacturer of the media provides documentation that the appropriate reactivity has been observed.

TABLE 1-25 Quality Control Surveillance Procedures of Commonly Used Microbiology Equipment

Equipment	Procedure	Schedule	Tolerance Limits
Refrigerators Freezers	Recording of temperature[a] Recording of temperature[a]	Daily or continuous Daily or continuous	2°C to –8°C –8°C to –20°C –60°C to –75°C
Incubators	Recording of temperature[a]	Daily or continuous	35.5°C ± 1°C
Incubators (CO_2)	Measuring of CO_2 content Use blood–gas analyzer or Fyrite[b] device	Daily or twice daily	5%–10%
Water baths	Recording of temperature[a]	Daily	36°C–38°C 55°C–57°C
Heating blocks	Recording of temperature[a]	Daily	±1°C of setting
Autoclaves	Test with spore strip *(Bacillus stearothermophilus)*	At least weekly	No growth of spores in subculture indicates sterile run
pH meter	Test with pH-calibrating solutions	With each use	±0.1 pH units of standard being used
Anaerobic jars	Methylene blue indicator strip	With each use	Conversion of strip from blue to white indicates low O_2 tension
Anaerobic glove box	*Clostridium novyi* type B culture	Run periodically	Growth indicates very low O_2 tension. It is used only where extremely low O_2 tension is required.
	Methylene blue indicator solution	Continuously or daily	Solution remains colorless if O_2 tension is low.
Serology rotator	Count revolutions per minute	With each use	180 rpm ± 10 rpm
Centrifuges	Check revolutions with tachometer	Monthly	Within 5% of dial indicator setting
Safety hoods	Measure air velocity[c] across face opening	Semiannually or quarterly	50 ft of airflow per minute ± 5 ft/min

[a]Each monitoring thermometer must be calibrated against a standard thermometer.
[b]Bacharach Instrument Co., Pittsburgh, PA.
[c]Velometer Jr., Alnor Instrument Co., Chicago, IL.

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A list of suggested organisms and acceptable results for the culture media most commonly used in clinical laboratories is found in Table 1-26. Quality control stock organisms may be maintained in the laboratory by subculturing bacterial isolates recovered as part of the routine work. Alternatively, and more conveniently but also more costly, dried stock organisms may be purchased from culture collections, such as ATCC (American Type Culture Collection, 12301 Parklawn Dr., Rockville MD) or from commercial vendors. Each batch of media should be checked for reactivity and for appropriate support of microbial growth, either by the manufacturer or in the local laboratory.

Culture tubes, plates of media, and reagents must bear a label that clearly indicates the content and the dates of preparation and expiration. “Coded” culture tubes, plated media, and reagents should be referenced in such a way that even nonlaboratory personnel would be able to interpret the code. Clearly defined quality control rules apply to antimicrobial susceptibility testing.

Each batch of tubed or plated media must also undergo sterility testing, particularly media in which components are added after sterilization. Sterility checks must be done

both visually and by subculture. Certain selective media, for example, may sufficiently suppress the visible growth of bacteria; however, viable organisms may appear on subculture. Prepared media should also be observed for other signs of deterioration, such as discoloration, turbidity, color changes, evidence of freeze/thawing, and status of hydration.

The frequency with which quality control testing of media and reagents (including serologic reagents) is performed is clearly defined by various accrediting agencies. Some of the rules for quality control of media and reagents are displayed in Table 1-27. Recommendations for quality control are not static. It is important to follow current recommendations, at least in part, because changes are as likely to result in less work as in more work.

TABLE 1-26 Quality Control of Commonly Used Media: Suggested Control Organisms and Expected Reactions

Medium	Control Organisms	Expected Reactions
Blood agar	Group A Streptococcus	Good growth, β-hemolysis
	S. pneumoniae	Good growth, α-hemolysis
Bile–esculin agar	Enterococcus species	Good growth, black
	α-Hemolytic	No growth; no discoloration of media
	Streptococcus, not group D	
Chocolate agar	*Haemophilus influenzae*	Good growth
	Neisseria gonorrhoeae	Good growth
Christensen urea agar	*Proteus mirabilis*	Pink throughout (positive)
	Klebsiella pneumoniae	Pink slant (partial positive)
	Escherichia coli	Yellow (negative)
Simmons citrate agar	*K. pneumoniae*	Growth or blue color (positive)
	E. coli	No growth, remains green (negative)
Cystine trypticase (CTA) agar Dextrose	*N. gonorrhoeae* *Moraxella catarrhalis*	Yellow (positive) No color change (negative)

Sucrose	*E. coli*	Yellow (positive)
	N. gonorrhoeae	No color change (negative)
Maltose	Salmonella species, or N. meningitidis	Yellow (positive)
	N. gonorrhoeae	No color change (negative)
Lactose	*N. lactamicus*	Yellow (positive)
	N. gonorrhoeae	No color change (negative)
Decarboxylases Lysine	*K. pneumoniae*	Bluish (positive)
	Cronobacter sakazakii	Yellow (negative)
Arginine (dihydrolase)	*E. cloacae*	Bluish (positive)
	Proteus mirabilis	Yellow (negative)
Ornithine	*P. mirabilis*	Bluish (positive)
	K. pneumoniae	Yellow (negative)
Deoxyribonuclease (DNase)	*Serratia marcescens*	Zone of clearing (add 1 N HCl)
	E. cloacae	No zone of clearing

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Eosin–methylene blue agar	*E. coli*	Good growth, green metallic sheen
	K. pneumoniae	Good growth, purple colonies, no sheen
	Shigella flexneri	Good growth, transparent colonies (lactose negative)
Hektoen enteric agar	*Salmonella typhimurium*	Green colonies with black centers
	S. flexneri	Green transparent colonies
	E. coli	Growth slightly inhibited, orange colonies

Indole (Kovac's)	*E. coli*	Red (positive)
	K. pneumoniae	No red color (negative)
Kligler iron agar	*E. coli*	Acid slant/acid deep
	Shigella flexneri	Alkaline slant/acid deep
	Pseudomonas aeruginosa	Alkaline slant/alkaline deep
	Salmonella typhimurium	Alkaline slant/black deep
Lysine iron agar	*S. typhimurium*	Purple deep and slant, + H^2S
	Shigella flexneri	Purple slant, yellow deep
	P. mirabilis	Red slant, yellow deep
MacConkey agar	*E. coli*	Pink colonies (lactose positive)
	P. mirabilis	Colorless colonies, no spreading
	Enterococcus species	No growth
Malonate	*E. coli*	No growth
	K. pneumoniae	Good growth, blue (positive)
Motility (semisolid agar)	*P. mirabilis*	Media cloudy (positive)
	K. pneumoniae	No feather edge on streak line (negative)
Nitrate broth or agar	*E. coli*	Red on adding reagents
	Acinetobacter lwoffi	No red (negative)
Phenylethyl alcohol blood agar	Streptococcus species	Good growth
	E. coli	No growth
o-Nitrophenol-β-D-galactopyranoside (ONPG)	*Serratia marcescens*	Yellow (positive)
	Salmonella typhimurium	Colorless (negative)
Phenylalanine deaminase	*P. mirabilis*	Green (add 10% FeCl3)

	E. coli	No green (negative)
Salmonella–Shigella (SS) agar	*S. typhimurium*	Colorless colonies, black centers
	E. coli	No growth
Voges–Proskauer	*K. pneumoniae*	Red (add reagents)
	E. coli	No development (negative)
Xylose–lysine–dextrose (XLD) agar	Salmonella species	Red colonies (positive lysine)
	E. coli	Yellow colonies (positive sugars)
	Shigella species	Transparent colonies (negative)

[a]From Microbiology Checklist, College of American Pathologists, Revised 2014.

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TABLE 1-27 Quality Control of Selected Reagents and Media[a]

Media or Reagents	Frequency	Controls
Gram's stain	Each new batch of stains and at least weekly	Gram-positive and gram-negative organism
Other nonimmunologic, nonfluorescent stains	Each day of use and each new batch, lot number, and shipment	Appropriate reactivity
Fluorescent stains	Each time of use	Appropriate reactivity
Catalase, coagulase, oxidase, bacitracin, optochin, ONPG, X or V or XV disks, identification systems	Each new batch, lot number, or shipment	Positive and negative controls
Antisera (Salmonella and	Each new batch, lot number, and shipment when prepared or	Positive and negative controls

Shigella)	opened and once every 6 months thereafter	Positive and negative controls
β-Lactamase (other than Cefinase)	Each day of use	Positive and negative controls
β-Lactamase (Cefinase)	Each new batch, lot number, or shipment	Positive and negative controls
Nucleic acid probes	Each day of use	Positive and negative controls
AFB stains	Each day of use	Positive and negative controls
Antimicrobial susceptibility tests	Daily or weekly if criteria met (see Chapter 17)	Appropriate organisms

Note: This will change with the implementation of IQCP in the future, but functions as a guide for quality control.

[a] From Microbiology Checklist, College of American Pathologists, Revised 2014

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CHAPTER 2

Introduction to Microbiology

Part II: Guidelines for the Collection, Transport, Processing, Analysis, and Reporting of Cultures From Specific Specimen Sources

thePoint For additional ancillary materials related to this chapter, please visit the point.

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In this chapter, we focus on the steps necessary for the diagnosis of infections. In each of the sections that follow, signs and symptoms of patients with infections that involve the major organ systems will be presented. Procedures for collection, transport, and processing of specimens are described. This information is summarized in Table 2-1. Otherwise stated, this chapter will predominantly consider the preanalytical phase of the diagnostic cycle. Later phases will be considered only briefly because they are the subject of most of the succeeding chapters. Joint guidelines concerning the utilization of the microbiology

TABLE 2-1 The Diagnosis of Bacterial Infections at Different Body Sites

Site of Infection	Presenting Signs and Symptoms	Specimens to Culture	Bacterial Species Potentially Associated with Infections
Respiratory tract	Upper tract—nose sinuses: Headache Pain and redness over malar area Rhinitis Radiograph: Sinus consolidation, fluid levels, or membrane thickening	Acute: Nasopharyngeal swab Sinus washings Chronic: Sinus washings Surgical biopsy specimen	*Streptococcus pneumoniae* *Streptococcus*, β-hemolytic group A *Staphylococcus aureus* *Haemophilus influenzae* *Klebsiella* spp. and other *Enterobacteriaceae* *Bacteroides* spp. and other anaerobes (sinus)
	Upper tract—throat and pharynx: Redness and edema of mucosa Exudation of tonsils Pseudomembrane formation Edema of uvula Gray coating of tongue/"strawberry tongue" Enlargement of cervical nodes	Swab of posterior pharynx Swab of tonsils (abscess) Nasopharyngeal swab	*Streptococcus*, β-hemolytic group A *Corynebacterium diphtheriae* *Neisseria gonorrhoeae* *Bordetella pertussis*
	Lower tract—lungs and bronchi: Cough: bloody or profuse Chest pain Dyspnea Consolidation of lungs: Rales and rhonchi Diminished breath sounds Dullness to percussion Radiographic infiltrates Cavity lesions Empyema	Sputum (poor return) Blood Bronchoscopy secretions Transtracheal aspirate Lung aspirate or biopsy	*Streptococcus pneumoniae* *Haemophilus influenzae* *Staphylococcus aureus* *Klebsiella pneumoniae* and other *Enterobacteriaceae* *Moraxella catarrhalis* *Legionella* spp. *Mycobacterium* spp. *Fusobacterium nucleatum, Prevotella melaninogenicus* and other anaerobes *Bordetella* species
Middle ear	Serous or purulent drainage Deep pain in ear and jaw Throbbing headache Red bulging tympanic membrane	Acute: No culture Nasopharyngeal swab Tympanic membrane aspirate Chronic: Drainage of external meatus	Acute: *Streptococcus pneumoniae* and other streptococci *Haemophilus influenzae* Chronic: *Pseudomonas aeruginosa* *Proteus* spp. Anaerobic bacteria
Gastrointestinal tract	Upper—stomach and duodenum: Gastritis and peptic ulcer disease	Gastric or duodenal biopsy	*Helicobacter pylori*
	Lower—small and large intestine: Diarrhea Dysentery Purulent Mucous Bloody Cramping abdominal pain	Stool specimen Rectal swab or rectal mucus Blood culture (typhoid fever)	*Campylobacter jejuni* and other *Campylobacter* spp. *Salmonella* spp. *Shigella* spp. *Escherichia coli* (toxigenic strains) *Vibrio cholerae* and other *Vibrio* spp. *Yersinia* species *Clostridium difficile* (demonstration of toxin)
Urinary tract	Urinary bladder infection: Pyuria Dysuria Hematuria Pain and tenderness; suprapubic or lower abdomen Kidney infection: Back pain Tenderness: costovertebral angle (CVA)	Clean-catch midstream urine Catheterized urine Suprapubic aspiration of urine	*Enterobacteriaceae* *Escherichia coli* *Klebsiella* spp. *Proteus* spp. *Enterococcus* spp. *Pseudomonas aeruginosa* *Staphylococcus aureus, S. epidermidis,* and *S. saprophyticus*

Genital tract	Males: Urethral discharge: serous or purulent Burning on urination Terminal hematuria	Urethral discharge Prostatic secretions	*Neisseria gonorrhoeae* *Haemophilus ducreyi* *Treponema pallidum* (syphilis) *Mobiluncus* spp. and other anaerobes *Gardnerella vaginalis*
	Females: Purulent vaginal discharge Burning on urination Lower abdominal pain, spasm, and tenderness Mucous membrane chancre or chancroid	Uterine cervix Rectum (anal sphincter swab) Urethral swab Darkfield examination	Nonbacterial: *Trichomonas vaginalis* *Candida albicans* *Mycoplasma* spp. *Chlamydia trachomatis* Herpes simplex virus
Central nervous system	Headache Pain in neck and back Stiff neck Straight-leg raising: positive Kernig sign Nausea and vomiting Stupor to coma Petechial rash	Spinal fluid Subdural aspirate Blood culture Throat or sputum culture	*Neisseria meningitidis* *Haemophilus influenzae* *Streptococcus pneumoniae* Streptococcus, β-hemolytic groups A and B (group B in infants) *Enterobacteriaceae:* debilitated patients, infants, and postcraniotomy *Listeria monocytogenes*
Eye	Conjunctival discharge: serous or purulent Conjunctival redness (hyperemia): pink eye Ocular pain and tenderness	Purulent discharge Lower cul-de-sac Inner canthus	*Haemophilus* spp. *Moraxella* supp. *Neisseria gonorrhoeae* *Staphylococcus aureus* *Streptococcus pneumoniae* *Streptococcus pyogenes* *Pseudomonas aeruginosa* (report immediately)
Blood	Spiking fever Chills Cardiac murmur (endocarditis) Petechiae: skin and mucous membranes "Splinter hemorrhages" of nails Malaise	Blood: two to three cultures; repeat as needed Any suspected primary site of infection: Cerebrospinal fluid Respiratory tract Skin—umbilicus Skin—ear Wounds Urinary tract	*Streptococcus* spp. Group A—all ages Viridans group streptococci (endocarditis) Groups A, B, D—neonates *S. pneumoniae* *Staphylococcus aureus* *Listeria monocytogenes* *Corynebacterium jeikeium* *Haemophilus influenzae* "HACEK" (*Haemophilus* species, *Aggergatibacter aphrophilus, Aggregatibacter actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens*, and *Kingella* species) Group *Escherichia coli* and other "coliforms" *Salmonella enterica* serogroup Typhi *Pseudomonas aeruginosa* *Bacteroides fragilis* and other anaerobic bacteria
Wounds	Discharge: serous or purulent Abscess: subcutaneous or sub-mucosal Redness and edema Crepitation (gas formation) Pain Ulceration or sinus formation	Aspirate of drainage Deep swab of purulent drainage Tissue biopsy	*Staphylococcus aureus* *Streptococcus pyogenes* *Clostridium* spp., *Bacteroides* spp., and other anaerobic bacteria *Enterobacteriaceae* *Pseudomonas aeruginosa* *Enterococcus* species
Bones and joints	Joint swelling Redness and heat Pain on motion Tenderness on palpation Radiograph: synovitis or osteomyelitis	Joint aspirate Synovial biopsy Bone spicules or bone marrow aspirate	*Staphylococcus aureus* *Haemophilus influenzae* *Streptococcus pyogenes* *Neisseria gonorrhoeae* *Streptococcus pneumoniae* *Enterobacteriaceae* *Mycobacterium* species

laboratory were published in 2013 from the American Society for Microbiology (ASM) and Infectious Diseases Society of America (IDSA).[7]This is a guideline to which every student of clinical microbiology should have ready access.

Infections of the Respiratory Tract

The respiratory tract is divided into the upper and lower respiratory tract. The upper respiratory tract consists of the nose, throat, oropharynx, and nasopharynx. The lower tract includes the larynx, trachea, bronchi, bronchioles, and the terminal airways or alveoli of the lungs. The middle ear, which is connected to the posterior pharynx through the eustachian tube, the salivary glands, which are connected to the oral cavity by ducts, and the paranasal sinuses, which are connected to the nasal cavity by ostea, will also be considered here.

Infections of the Upper Respiratory Tract

Indigenous Microbiota. Diagnosis of upper respiratory infections is complicated in that microorganisms that may be pathogens are also often present in the uninfected individual as part of their normal microbiota. Even *Streptococcus pyogenes* may be found, usually in small numbers, in the throats of asymptomatic individuals, which is the reason that following a streptococcal pharyngitis culture or other testing is not recommended as a "test of cure." There are certain organisms, such as *Neisseria gonorrhoeae*, that are never considered part of the normal microbiota.

The oropharyngeal microbiota of normal individuals is composed mainly of viridans streptococci, β-hemolytic streptococci, *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, many anaerobic bacteria including *Fusobacterium* spp. and *Actinomyces israelii*, and yeast including *Candida albicans.* These microorganisms inhabit the upper respiratory tract without causing disease. In patients who are sick and have been admitted to the hospital, the indigenous microbiota switches from predominantly gram-positive (mostly streptococci) to predominantly gram-negative (*Enterobacteriaceae* and *Pseudomonas* spp.).[134] The reason for this alteration may be related to the loss of fibronectin, which enhances binding of gram-positive bacteria, from the surfaces of oropharyngeal epithelial cells.[286,338] This increased quantity of gram-negative bacteria in the nasopharynx of hospitalized patients is problematic for these patients should an aspiration event occur, as once these bacteria are displaced from the mucosal surface to the lung, pneumonia and/or lung abscess often ensues.

Pharyngitis. Acute pharyngitis is the most common infection of the upper respiratory

tract. By far the most important pathogen is *S. pyogenes* (group A β-hemolytic *Streptococcus*). Viral infection is the other common cause of pharyngitis.[25]

Streptococcal Pharyngitis. *S. pyogenes* pharyngitis may be suggested clinically by observing an inflamed and edematous pharyngeal mucosa in a patient who reports throat pain, difficulty swallowing, and secondary symptoms such as fever, headache, and tender anterior cervical lymph nodes. Occasionally, these patients may develop a scarlatiniform rash. Purulent exudates over the posterior pharynx and tonsillar area are commonly observed. The clinical presentations of streptococcal and nonstreptococcal pharyngitis overlap broadly.[26] Although diagnosis by clinical criteria alone has been advocated, the prevailing opinion is that it is impossible to determine the cause from clinical observations and that diagnostic support from the laboratory is required.[27]

The presence of a tough, fibrinous, gray membrane ("pseudomembrane"), pus exuding from abscesses or from draining sinuses, and mucosal ulcerations suggests an infectious disease other than acute streptococcal pharyngitis or advanced, invasive disease. In such instances, a diagnostic approach that addresses other pathogens, in addition to group A *Streptococcus*, is necessary.

β-Hemolytic streptococci other than group A (groups C and G) produce symptoms similar to, but milder than, those of group A strains.[25] They are not associated with rheumatic fever as a noninfectious sequela. Although group A strains may be found in the oropharynx occasionally in small numbers, groups C and G strains are regular colonizers. It is, therefore, more difficult to interpret isolation of small quantities of these organisms. If they are recovered in pure culture or as the predominant organism, it is reasonable to report their presence with a note about their clinical significance and interpretation.

The traditional standard for diagnosis of streptococcal pharyngitis has been bacterial culture on sheep blood agar, which remains an acceptable method for detection. Rapid methods for detection of group A streptococcal antigen are widely used in laboratories and at the point of care in offices and clinics. Most of the assays have high specificity, but the sensitivity is not good enough to depend on a negative result.[25] Newer methods that employ automated readers, and in some instances immunofluorescence, have increased the sensitivity of these assays. It has been the common practice that a negative rapid test should be followed by a bacterial culture, given the limited sensitivity of these assays. However, practice guidelines from the IDSA no longer recommend the routine use of throat culture as a backup for adults with usual pharyngitis and a negative antigen-detection test.[267] There have been no changes with respect to backup throat cultures for children with pharyngitis. Additionally, it is important to review the package insert of the rapid antigen-detection test used, as backup testing may be necessary for accreditation compliance purposes.

The primary advantages of antigen-detection tests are that they allow expedited treatment of patients who are detected, thus reducing the period of discomfort and

allowing the patient to return to school or work sooner. The frequency of noninfectious sequelae (rheumatic fever and glomerulonephritis) is not increased by using conventional culture for diagnosis. The rapid antigen tests add expense, and although many are waived, many of them are classified as moderately complex (see Chapter 1)—with attendant investment of time and effort by clinical staff. There is little justification for performing "rapid" tests in a central laboratory, because the patient will already have gone home by the time the result is available. Each clinician must make a determination as to the utility of performing the antigen tests.

There are molecular alternatives to culture for the detection of group A *Streptococcus.* A genetic probe for group A *Streptococcus* is available (GASDirect, Hologic, San Diego, CA) as an acceptable alternative to culture, although some have demonstrated it to be slightly less sensitive than

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enhanced culture.[50] In contrast, there have been a large number of studies that demonstrate the improved sensitivity of polymerase chain reaction (PCR) for group A *Streptococcus* over culture. Although the detection rates are improved with PCR, one must always consider the possibility of detecting *S. pyogenes* that is part of the normal microbiota in some individuals. A highly sensitive assay, such as a PCR assay, could give a positive result for group A *Streptococcus* in a setting wherein this organism was part of the normal microbiota of the patient, but the pharyngitis was due to a viral etiology. Currently, there is no way to determine whether the positive group A *Streptococcus* result is from an etiologic agent or bystander microbiota; quantitative information available from real-time PCR assays could be potentially useful in this regard. There are at least two rapid molecular diagnostic assays that have or are seeking "waived" status for use in the point-of-care setting at the time of this writing. Group A *Streptococcus* assays may be detected by these methods in the future, which would obviate the need for the reflex testing.

Diphtheria. Diphtheria is now extremely rare in the United States.[25] It is primarily an infection of children, in whom it occurs in sporadic outbreaks, but adults may become infected, particularly those in lower socioeconomic groups.[115] The thick blue-white or gray membrane that covers the posterior pharynx, with marked edema of the underlying and surrounding tissues, can usually be differentiated from the bright-red throat of acute streptococcal pharyngitis.

The diagnosis is made by culture of the membrane on Loeffler's medium or tellurite selective medium. This organism can be differentiated from other *Corynebacterium* species by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Once *Corynebacterium diphtheriae* is detected, it must be determined if the organism produces toxin. Specimens for culture of *C. diphtheriae*

are best referred to a reference or public health laboratory (see Chapter 14). These laboratories may also offer PCR-based assays for this organism and/or the lysogenic-bacteriophage that encodes the toxin.

***Arcanobacterium haemolyticum* Pharyngitis.** This bacterium causes acute pharyngitis that closely mimics streptococcal pharyngitis, including a scarlatiniform rash in many patients. In fact, this organism is more likely to produce a scarlatiniform rash than group A *Streptococcus.* It usually affects adolescents and young adults, in contrast to β-hemolytic streptococci, which typically cause disease in young children.[25] *A. haemolyticum* is better isolated on human blood agar than on sheep blood agar, but can be recovered in the clinical laboratory. It may inadvertently be dismissed as a non–group A *Streptococcus*, if Gram's stain and/or a catalase test are not performed (see Chapter 14).

Gonococcal Pharyngitis. Oropharyngeal infection by *N. gonorrhoeae* may be asymptomatic, produce pharyngitis that is indistinguishable from group A *Streptococcus*, or may be associated with disseminated disease.[25] It should be suspected in women and homosexual men who practice fellatio. Clinicians must indicate this possibility so that media for recovery of gonococcus can be inoculated. Culture on media that support the growth of *N. gonorrhoeae* is recommended, but some have validated oropharyngeal swabs for nucleic acid amplification tests.

Viral Pharyngitis. A variety of viruses may cause pharyngitis, including adenovirus, rhinovirus, coronavirus, parainfluenza virus, influenza virus, and the Epstein–Barr virus (EBV). Infection with EBV deserves special mention. After a nonspecific, febrile prodromal phase of infection by EBV, an acute pharyngitis with tonsillar enlargement, white exudates and cervical lymphadenopathy ensues. Palatal petechiae may be present.[25] Subsequently, systemic signs and symptoms of infectious mononucleosis, including hematologic abnormalities, hepatosplenomegaly, and generalized lymphadenopathy, make the diagnosis more obvious. In the early stages, the pharyngitis may suggest streptococcal disease, but adolescents and young adults are most commonly symptomatically infected. The diagnosis is made on a serologic basis. A similar illness may occur as part of the acute retroviral syndrome, caused by human immunodeficiency virus, but the onset is more acute and exudates are not seen. A disseminated maculopapular rash is common in the acute retroviral syndrome, but it is rarely seen in infectious mononucleosis unless ampicillin has been administered.

Adenoviruses produce an acute pharyngitis that resembles streptococcal pharyngitis closely. Conjunctivitis often is present in addition, a clue to the etiology (pharyngoconjunctival fever). Some enteroviruses, (e.g., serotypes of Coxsackie A) virus produce acute pharyngitis with vesicles in the posterior pharynx as part of hand, foot, and mouth disease. The infection typically occurs in children and is readily diagnosed clinically by the occurrence of cutaneous and mucosal vesicles. Herpes

simplex virus pharyngitis has been described in young adults, but this virus is found in asymptomatic individuals, as is adenovirus. More details about the viral infections are found in Chapter 23.

Other Infectious Causes of Pharyngitis. *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* have been described as causes of pharyngitis, but more commonly cause disease in the lower respiratory tract. *Candida* spp., especially *C. albicans*, produces a tenacious, creamy exudate when it infects the oropharynx (i.e., thrush), but does not produce prominent pharyngitis. Cytomegalovirus may cause the infectious mononucleosis syndrome, as part of the primary infection with this virus. As noted, a number of respiratory viruses may cause pharyngeal discomfort as part of an acute respiratory illness.

The joint IDSA and ASM guidelines state that *H. influenzae, S. aureus, Neisseria meningitidis,* and *S. pneumoniae* are not etiologic agents of pharyngitis and should not be sought in throat cultures.[7] For this reason the request of "Full Throat Culture," wherein every bacterium that grows is identified, should be discouraged. The default test should be a "Streptococcal Culture" or "Streptococcal Screen."

Collection of Throat Cultures. The proper method for obtaining a throat swab specimen is shown in Figure 2-1. A bright light from over the shoulder of the specimen collector should be focused into the oral cavity so that the swab can be guided to the posterior pharynx. The patient is instructed to tilt the head back and breathe deeply. The tongue is gently depressed with a tongue blade to visualize the tonsillar fossae and posterior pharynx. The swab is extended between the tonsillar pillars and behind the uvula. Care should be taken not to touch the lateral walls of the buccal cavity or the tongue to minimize contamination with commensal bacteria. Having the patient phonate a long "ah" serves to lift the uvula and helps prevent gagging. The tonsillar areas and the posterior pharynx should be firmly rubbed with the swab. Any purulent exudate should also be sampled.

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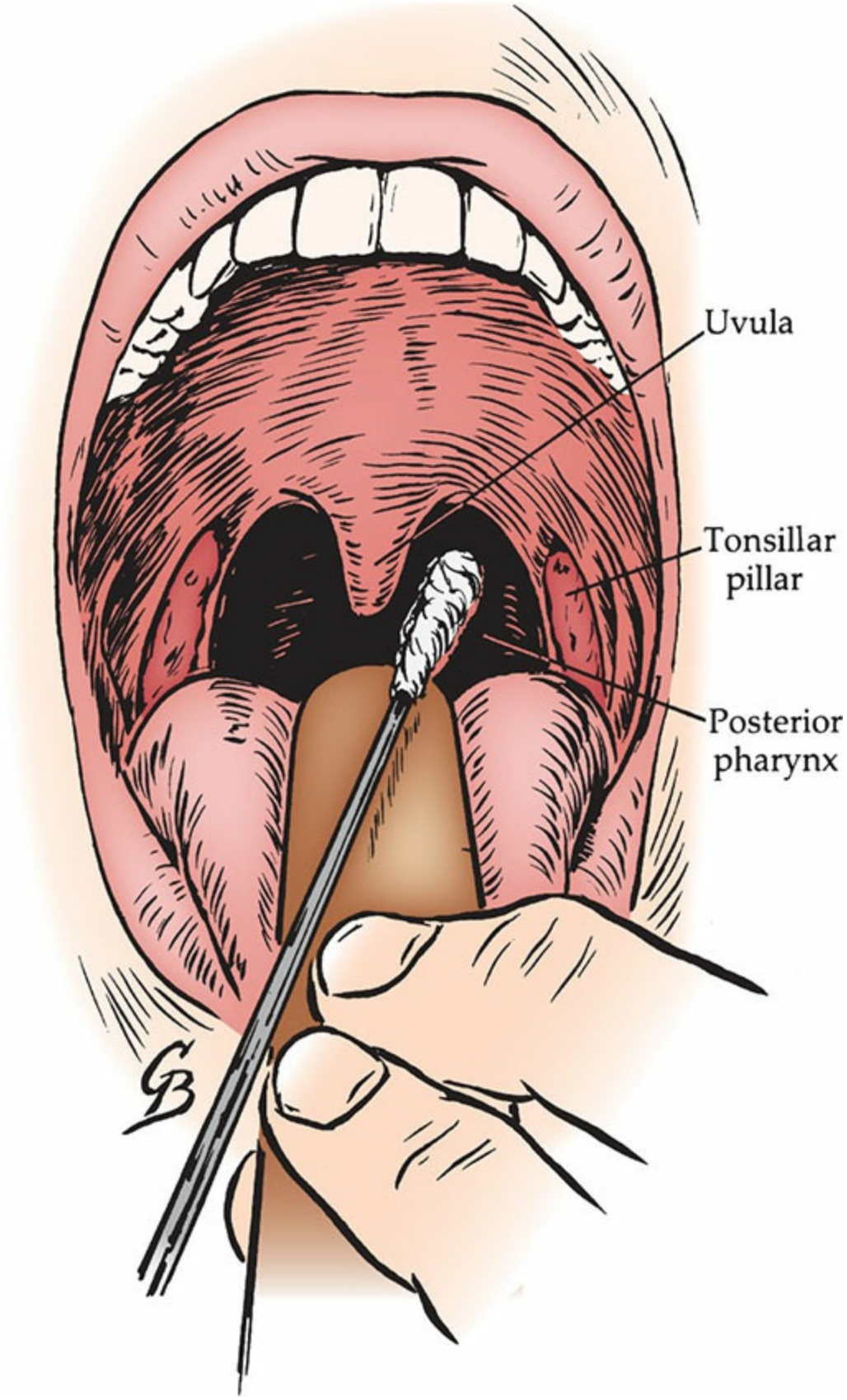
Uvula
Tonsillar pillar
Posterior pharynx
GB

■ **FIGURE 2-1** Throat culture technique. The patient is asked to open the mouth widely and phonate an "ah." The tongue is gently depressed with a tongue blade and a swab is guided over the tongue into the posterior pharynx. The mucosa behind the uvula and between the tonsillar pillars is swabbed with a gentle sweeping motion.

After collection, the swab should be placed immediately into a sterile tube or other suitable container for transport to the laboratory. If the recovery of only group A β-hemolytic streptococci is desired (i.e., a "strep screen"), swabs may be allowed to dry during transport without compromising the recovery of viable organisms. Some reference laboratories recommend that swab tips be placed in a desiccant, such as silica gel, to suppress survival of commensal organisms and improve the recovery of *S. pyogenes*. Swabs for the recovery of viral agents should be placed in a special transport medium (see Chapter 23), although some microbiologists have found swabs in transport medium designed for bacteria to be acceptable for viral culture.[274]

Infections of the Oral Cavity Other Than Pharyngitis. Gingivitis and dental caries are caused by bacteria, but are primarily a concern for dentists, rather than physicians. In actuality, bacteria cause very few infections of the nasal and oral cavities, other than pharyngitis. Given the impossibility of collecting a surface culture of these spaces without including the abundant indigenous microbiota, cultures of lesions for bacteria will not produce interpretable results.

Necrotizing, ulcerative gingivostomatitis (Vincent's infection, Vincent's angina, or "trench mouth") is a synergistic infection caused by multiple oral anaerobic bacteria. It is rarely encountered today. Culture is not indicated. The condition may be accompanied by sepsis and metastatic infection, however, so blood cultures should be obtained in patients with this disease. Observing gram-negative fusiform bacilli and spirochetes in Gram-stained smears prepared from an ulcerative buccal or gingival ulcer is helpful for presumptive diagnosis of Vincent's angina. Cultures of the mouth and oral cavity are rarely helpful because of the presence of many species of commensal anaerobes.

Capnocytophaga spp., fusiform bacteria that are normally present in the oropharynx, have also been associated with ulcerations of the oral mucosa and positive blood cultures, particularly in patients with severe neutropenia.[318] These organisms may be recovered on selective media for pathogenic *Neisseria* because of their resistance to vancomycin, colistin, and trimethoprim. By using a selective medium similar in formulation to selective *Neisseria* medium, Rummens and associates[244] recovered *Capnocytophaga* spp. from 96% of oropharyngeal cultures compared with only a 6% recovery on chocolate agar plates inoculated in parallel.

Fungi may also cause infections of the oral cavity. The most common fungal infection in this location is thrush. White patches on the oral mucosa or the more

extensive involvement of the oral cavity with production of a thick, curd-like exudate is caused by *Candida* spp., especially *C. albicans*. A diagnosis that can be made by observing pseudohyphae and budding blastoconidia in a Gram-stained smear of the exudate. If the clinical history reveals a long-standing, nonhealing mucosal ulcer in the oral cavity, the possibility of the cutaneous extension of a systemic fungal disease must be considered. A biopsy of tissue for histologic study and direct examination of exudative material and culture should be performed in such instances. A black eschar on the hard palate in a neutropenic patient suggests the possibility of invasive zygomycosis, which is a medical emergency. In this scenario, an emergent frozen section should be performed, as well as a direct examination and culture. The differentiation of the mucoraceous hyphae of a zygomycete from the hyaline septate hyphae of *Aspergillus* or a similarly appearing mold is of critical importance since voriconazole, a commonly used antifungal drug, is of no use in the treatment of zygomycosis.

Herpes simplex virus, predominantly serotype 1, may produce an acute gingivostomatitis as a part of primary infection. Most individuals are exposed as children and young adults. The vesicular lesions occur on the skin of the face and the mucosa of the lips, but they may extend into the anterior portions of the mouth. Traditionally the diagnosis may be made by demonstrating infected cells in a stained smear (Tzanck preparation), but this is no longer the preferred method. More commonly, the detection of the virus is made by direct fluorescent antibody (DFA) testing or culture. Nucleic acid amplification assays could also be used in this setting following appropriate validation.

Infections of the Nasopharynx and Nasopharyngeal Cultures. The most frequent infection of the nasopharynx is the "common cold," usually caused by one of more than 100 serotypes of rhinovirus. This infection is rarely life-threatening, although we now recognize that the lower respiratory tract and even other organ systems may be affected.[79] The common cold is, however, a major cause of

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morbidity and the large numbers of lost workdays makes it a potent economic force. The ubiquity of this infection is illustrated by the fact that, although very few of us will have had tuberculosis or hemorrhagic fever, we will all have experienced the discomfort of the common cold. The predominant symptom is coryza (i.e., runny nose). Fever is not a part of the infection. Laboratory studies are not necessary, because the illness is self-limited and specific therapy is not yet available. However, the detection of rhinovirus and/or coronaviruses is now possible using some of the commercially available multiplex respiratory viral panels. The detection of one of these viruses as the etiologic agent may be useful, particularly when conjoined with the

exclusion of other respiratory viral pathogens, such as influenza and respiratory syncytial virus (RSV).

Obtaining nasopharyngeal specimens is of little practical value, except in a few defined situations. Nasopharyngeal swabs are of limited value for establishing the diagnosis of acute otitis media[256] or bacterial sinusitis,[85] but they are the specimen of choice for the isolation of *Bordetella pertussis*, the etiologic agent of whooping cough.[124] These specimens are also useful for molecular testing for *M. pneumoniae* and *C. pnuemoniae.* Nasopharyngeal swabs and aspirates are equally effective for the diagnosis of viral respiratory infections,[95] although some virologists prefer aspirates for recovery of RSV.

Nasopharyngeal specimens are obtained under direct vision using over-the-shoulder illumination. With the thumb of one hand, gently elevate the tip of the nose. Moisten the tip of a small flexible nasopharyngeal wire swab with sterile water or saline and gently insert it into one of the nares. Guide the swab backward and upward along the nasal septum until a distinct feel of resistance indicates that the posterior pharynx has been reached. Gently remove the swab. If while guiding the swab undue resistance is met, attempt the procedure through the opposite nares. Leave the swab in contact with the posterior nasopharynx for 15 to 30 seconds if the patient can tolerate the discomfort. Newer flocked swabs with a higher surface area are becoming favored for the delivery of a higher quality specimen.

Otitis Media and Sinusitis. The middle ear and the paranasal sinuses are connected to the upper airway by ducts. Infections, primarily caused by viruses and bacteria, result when pathogens that populate the nose and throat make their way into the normally sterile middle ear and sinus.[89,109,314] Acute infections are caused by respiratory viruses and by certain bacteria—*S. pneumoniae*, *M. catarrhalis* and *H. influenzae*.[118] When the infection becomes chronic, facultatively aerobic gram-negative bacilli, anaerobic bacteria and fungi assume a prominent role in a polymicrobial infection.[70,89] It is virtually impossible to obtain an interpretable specimen by sampling the airway.

Sampling these structures for culture is difficult. Fortunately, the pathogens in acute disease are predictable, so antimicrobial therapy can be initiated without the necessity of invasive procedures. In chronic diseases, invasive procedures and surgical approaches may be required.

Epiglottitis. Acute bacterial epiglottitis, which was most commonly caused by *H. influenzae* type B,[186] is a life-threatening infection, but fortunately the incidence of this disease has been substantially reduced by immunization. Acute epiglottitis is a medical emergency. Swelling of the epiglottis can block the airway unless relieved by tracheostomy or expert directly visualized intubation. Most historians believe that

George Washington died from acute epiglottitis, which was treated by repeated bleeding.[198] The youngest of his three consulting physicians wanted to try the newly developed technique of tracheostomy on our first President, but he was overruled by his "wiser" superiors. The diagnosis of epiglottitis must be made clinically, because touching the epiglottis to obtain a sample can aggravate the obstruction. In *Haemophilus,* epiglottitis blood cultures were frequently positive.

Laryngitis. Acute laryngitis and (more commonly) laryngotracheobronchitis (croup) are most commonly caused by respiratory viruses. The parainfluenza viruses are likely the most common of laryngotracheobronchitis, but it may be a component of disease caused by a variety of respiratory viruses. *M. pneumoniae* is another common cause.[69] Rarely, the herpes simplex virus may cause tracheitis.

Other Infections of the Upper Respiratory Tract. Infection of the soft tissues of the head and neck usually originate in the oral cavity and are caused by organisms that reside there normally. Retropharyngeal abscesses and infection of the retropharyngeal space (quinsy) are infectious complications of streptococcal tonsillitis and pharyngitis. Compression of the airway can result; this also represents a medical emergency.[106] Sampling of peritonsillar abscesses must be done by percutaneous aspiration to avoid contamination with oropharyngeal microbiota.

Similarly, dental infections can extend into the adjacent soft tissues, resulting in abscesses, or into bone, causing osteomyelitis. Chronic draining infections of the neck may be caused by *A. israelii* (actinomycosis),[35,321] a component of the oropharyngeal microbiota in a minority of individuals. Material from the draining sinus must be collected by aspiration or curetting; a swab is not adequate to recover the concretions (sulfur granules) in which the bacteria are concentrated. Alternatively, gauze may be placed over the draining sinus to collect the granules that become enmeshed in the interstices of the cloth. Draining material can be placed in a Petri dish, diluted with sterile water, and examined for the concretions. If the granules are crushed, demonstration of branching gram-positive bacilli establishes the diagnosis. The crushed granule can then be cultured anaerobically to determine the specific cause.

Historically, mycobacterial infection also caused cervical lymphadenopathy, and the lymph nodes often drained to the surface of the skin (scrofula). It was commonly caused by *Mycobacterium bovis*, which entered the body through the oropharynx after being ingested in contaminated milk. Pasteurization eradicated this form of infection. Tuberculous lymphadenopathy usually follows primary pulmonary disease. Today, in resource-rich countries with a low incidence of tuberculosis, the most common mycobacterial infection of cervical lymph nodes is caused by members of the *Mycobacterium avium* complex (MAC) and primarily affects young children.[21] Lemierre's syndrome is a life-threatening infection of the oropharynx with extension

into the cavernous sinus and other important anatomic structures in the head and neck. It is characteristically associated with *Fusobacterium necrophorum,* but other bacteria may also be

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involved. This commonly presents as a sore throat followed by neck pain and a neck mass. The extension of this infection causes into the cavernous sinus results in internal jugular vein thrombosis. Overall, 5% of the patients with this disease will die.[140]

The most common infection of the salivary glands in past years was mumps, but effective immunization has essentially eliminated the infection. Bacterial infection of the parotid glands is uncommon, but when it occurs is often caused by *S. aureus*.

Infections of the Lower Respiratory Tract

The lower respiratory tract consists of all structures below the larynx. The structures and infections considered here include the trachea (tracheitis), bronchi and bronchioles (bronchitis and bronchiolitis) and the distal air spaces (pneumonia).

Tracheobronchitis. Acute infections can be caused by bacteria or viruses. In children, viral and mycoplasmal agents predominate.[69] Symptoms include cough, fever, and varying degrees of sputum production. A distinctive illness, characterized by an inspiratory whoop and peripheral lymphocytosis, is caused by *B. pertussis* or *B. parapertussis*, which usually produces a milder illness.[31] The characteristic whoop of whooping cough is not present in the initial phases of illness, however. Apneic spells (absence of breathing) are common, but not lethal. Adenoviruses may produce a similar syndrome, although their etiologic role is controversial. Herpes simplex virus may cause disease, particularly in the infected neonate and those with other underlying severe illness. *C. pneumonia* is a recognized pathogen that causes acute bronchitis as well as pneumonia.

Chronic tracheobronchitis is symptomatically similar to acute disease, but more prolonged and less intense. Bacteria causes predominate, particularly *S. pneumoniae*, nonencapsulated *H. influenzae* and *M. catarrhalis*.

Prolonged acute disease may overlap with chronic bronchitis. *M. pneumoniae* has classically been associated with a lingering cough, but it is now recognized that *B. pertussis* can produce chronic illness in older children, adolescents, and adults[260] and *C. pneumoniae* may result in a lingering illness.[112]

Viral infections are usually not diagnosed with laboratory support unless the illness is sufficiently severe to require hospitalization. Whooping cough, *M. pneumoniae,* and *Chlamydophila* infection are diagnosed by culture or, increasingly, by molecular

methods. Serologic studies, consisting of either the detection of immunoglobulin M (IgM) or a rise in the immunoglobulin G (IgG) titer are often sought in patients suspected to be infected by *M. pneumoniae* and *C. pneumophila.* There are now Food and Drug Administration (FDA)-approved molecular assays for the detection of *Bordetella,* the sensitivity of which exceeds that of culture. Chronic bronchitis is usually evaluated by sputum culture if the illness is sufficiently severe. Special requests must be made if *Bordetella*, *Mycoplasma,* or *Chlamydophila* is considered.

Bronchiolitis. Infection of the smallest airways before the distal air spaces is dominated by viruses and *M. pneumoniae*.[117,336] Bronchiolitis affects infants and young children primarily and it occurs most commonly during the winter months. After a prodromal period of upper respiratory infections, primarily coryza, the predominant symptoms are cough, wheezing, and stridor (i.e., difficulty breathing). The differential diagnosis may include asthma and physical obstructions, such as foreign bodies. The illness is self-limited and laboratory diagnosis is required only if the patient is sufficiently ill to require hospitalization.

Pneumonia. The most serious infection of the respiratory tract is pneumonia, which is centered on the distal air spaces from the alveolar ducts to the alveolar sacs. The symptoms of pneumonia include fever, cough, varying degrees of sputum production, dyspnea (i.e., shortness of breath; difficulty breathing), and chest pain. Chest pain may be diffuse, vague and constant, or localized and intermittent, accentuated by deep respiration, if pleuritis is present. Shortness of breath and dyspnea usually indicate involvement of the terminal bronchioles and alveoli in a more diffuse pneumonic process. Physical signs supportive of a lower respiratory tract infection include rales and rhonchi, diminished breath sounds, and localized dullness to percussion in patients with lobar pneumonia.[73]

Pneumonia has been categorized several ways. For simplicity, we will consider: atypical pneumonia, acute pneumonia and chronic pneumonia. In addition, aspiration pneumonia, lung abscess and empyema require special consideration. Finally, from a management perspective pneumonia may be classified epidemiologically: outpatient pneumonia ("walking pneumonia") and pneumonia requiring hospitalization; community-acquired pneumonia versus hospital-acquired (i.e., nosocomial) pneumonia; pneumonia in immunosuppressed patients; pneumonia in cystic fibrosis; and pneumonia at the extremes of life. The distinction between lobar pneumonia, classically caused by *S. pneumoniae* (pneumococcus) and *Klebsiella pneumoniae* serotype 1 (Friedlander's pneumonia), and multifocal pneumonia, caused by other bacteria, is less useful clinically, because there is extensive overlap between the two patterns.

The type of pneumonia produced is a combination of microbial factors and the status of host defense mechanisms. Most pneumonia results from inhalation of

respiratory pathogens or aspiration, which may be microscopic and subclinical, of contents of the upper respiratory tract. As the colonizing microbiota of the oropharynx change, for example during prolonged hospitalization, so will the nature of organisms that infect the lung.

Atypical Pneumonia. Atypical pneumonia was defined in the 1930s as lower respiratory infection that did not resemble the classic lesions that had been described. The major difference is that sputum production is minimal in atypical pneumonia. Infection is often milder than in classic pneumonia, but not necessarily so. The primary pathogens responsible for atypical pneumonia are *M. pneumoniae*, *C. pneumoniae*, and *Legionella* spp.

Acute Pneumonia. Acute pneumonia in the outpatient setting (i.e., community-acquired pneumonia) is a combination of atypical pneumonia and classic pneumonia caused by oropharyngeal bacteria. The most important "classic" pathogen remains pneumococcus; other contributors are *H. influenzae* (now predominantly nontypeable strains), and *M. catarrhalis*.[11,177,191]

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Viral pneumonia is rare in immunocompetent adults, or may be under recognized. Viral pneumonia will develop in a small percentage of previously healthy adults with influenza. In this situation, secondary bacterial infection (superinfection) is the major threat. Acute viral pneumonia is a more important consideration in young children, in whom it overlaps with bronchiolitis, caused by RSV and parainfluenza virus type 3. The most common cause of viral pneumonia in the immunosuppressed population is cytomegalovirus.

Acute pneumonia in the hospital is caused most commonly by *Enterobacteriaceae*, *Pseudomonas* spp., and *S. aureus*.[38] The shift in etiologic agents reflects changes in the nature of colonizing microbiota in the upper airways (see Chapter 1).

Aspiration Pneumonia. Although most pneumonia is caused by aspiration of oropharyngeal contents, massive aspiration causes a distinctive picture of focal pneumonia, affecting the dependent portions of the lung when standing or lying down (i.e., lower lobes and superior segment of the upper lobe). The infecting organisms in the outpatient are a mixture of aerobic (primarily gram-positive) and anaerobic bacteria.[8] In the hospitalized patient, aspiration pneumonia reflects the newly acquired gram-negative microbiota of the upper airways.

Chronic Pneumonia. As the name implies, the course of chronic pneumonia is prolonged. Symptoms are usually less dramatic than in acute pneumonia. As a result, diagnosis may be delayed for weeks or months, because nonspecific signs and symptoms such as low-grade fever, malaise (i.e., feeling "poorly"), and weight loss may be the only manifestations of infection. The etiologic agents in chronic pneumonia

are mycobacterial and fungal, but smoldering bacterial infection is also be a cause. *Candida* spp. are notably absent from the list of pulmonary pathogens, except for rare exceptions, most of which are part of disseminated candidiasis in immunosuppressed patients.

Lung Abscess. Closely related to aspiration pneumonia is lung abscess, sometimes called putrid lung abscess, because of the fecal odor of the infecting anaerobic bacteria.[8,9] The formation of abscesses is also a function of microbial virulence factors. *S. aureus*, *Enterobacteriaceae,* and *Pseudomonas* spp. often cause destructive lesions that destroy the architecture of the lung. In contrast, classic pneumococcal pneumonia preserves the architecture of the lungs. Certain fungi, primarily *Aspergillus* spp. and Zygomycetes, characteristically invade blood vessels. Thrombosis ensues and tissue death results when oxygenation is blocked, a process called infarction. *Pseudomonas aeruginosa* also has the capacity to produce vasculitis. When an infectious agent and thrombosis are present together, the process is called a septic infarct and is particularly destructive. The thrombosis can occur in situ or can result from a blood clot that travels through the bloodstream to the lung (i.e., an embolus).

Granulomas are another type of destructive lesion (see Chapter 1). They are typically produced by mycobacteria, especially *Mycobacterium tuberculosis* and by dimorphic fungi, particularly *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides* spp. These are considered further in the chapters covering mycobacteriology and mycology.

Empyema. Inflammation of the pleura (i.e., the mesothelial membrane that lines the lung and the chest wall) is common in pneumonia (pleurisy) and may produce an exudation of fluid (i.e., pleural effusion).[1] If the pleural effusion itself becomes infected, the filling of this fluid with a thick pus (i.e., polymorphonuclear leukocytes) results in empyema. The causes are those of bacterial pneumonia. A tuberculous empyema also occurs. Healing may result in obliteration of the pleural cavity. In many instances, the restrictive fibrous layer that results from the inflammation constricts the lung and must be surgically removed (i.e., decortication).

Pneumonia in Special Populations. Pneumonia in the elderly is more of a threat than in younger adults, but the list of pathogens is similar. *S. pneumoniae* is the most frequent agent.[168,206] This is why it is critically important for adults 65 years of age or older and certain higher risk individuals receive the pneumococcal vaccines. These same individuals should also receive the vaccination for influenza each year. It is now recognized that some agents such as RSV, originally thought to be etiologic agents in children, also infect adults and the elderly. Similarly, *C. pneumoniae* may also cause infections in this patient population.

Immunosuppressed patients are at risk for many pathogens that do not cause disease regularly in immunocompetent individuals. Among these opportunistic

pathogens are parasites, including *Toxoplasma gondii;* viruses, including cytomegalovirus; fungi, including *Aspergillus* spp., Zygomycetes, *Cryptococcus neoformans*, and *Pneumocystis jirovecii*; and mycobacteria, such as MAC.[261] Additionally, immunocompromised patients are less able to clear infections caused by more commonly encountered pathogens. Infections with adenovirus and even parainfluenza virus may result in serious, life-threatening infections.

A special category of compromised individuals is those with cystic fibrosis.[99] A progression of pathogens follows these patients throughout the course of their illness: *S. aureus*, *H. influenzae,* and *P. aeruginosa*. Strains of *P. aeruginosa* in cystic fibrosis have a characteristic mucoid character that is seen much less frequently in other patients. *Burkholderia cepacia* and *Stenotrophomonas maltophilia*, both previously categorized as species of *Pseudomonas*, and *Alcaligenes* spp. are also major concerns in cystic fibrosis because the infections are difficult to treat and the pathogens are often multidrug resistant and easily spread from one individual to another.

Allergic bronchopulmonary disease may be caused by *Aspergillus fumigatus*, other fungi and aerobic actinomycetes.[102] *A. fumigatus* is a versatile opportunistic pathogen that can produce invasive disease in immunosuppressed patients, a fungus ball in preexisting cavities of immunocompetent hosts, as well as an immunologic reaction in individuals with an atopic (allergic) predisposition.[94,97] In allergic aspergillosis, hyphae are found in the air spaces, where they produce an inflammatory reaction but do not invade tissue as in allergic aspergillosis.

Specimen Collection for the Diagnosis of Lower Respiratory Infection

Expectorated Sputum. The simplest and least expensive specimen for the diagnosis of lower respiratory infections is

expectorated sputum. The utility of this approach is the subject of considerable argument, because of the difficulty some patients have mobilizing lower respiratory secretions and the frequency with which the specimen is contaminated by oropharyngeal microbiota as it passes through the mouth.[233] When the specimen is collected carefully, however, it can provide useful information for initial therapy for the patient with pneumonia.[243]

The patient should be instructed carefully on the proper collection of sputum, rather than saliva. Having patients brush their teeth and gargle with water immediately before obtaining the specimen reduces the number of contaminating oropharyngeal bacteria. Spada and colleagues[278] showed a 1-log decrease in the mean concentration of contaminating bacteria from $3.6 \pm 7.5 \times 10^8$ to $3.7 \pm 7.2 \times 10^7$ in sputum samples

obtained from patients immediately following a simple mouth wash. Avoid using proprietary mouthwashes or gargles that may contain antibacterial substances. Once the specimen is collected, it should be submitted to the laboratory promptly rather than sitting at the bedside.

Early-morning sputum samples should be obtained because they contain pooled overnight secretions in which pathogenic bacteria are more likely to be concentrated. Twenty-four-hour collections should not be accepted because of the overgrowth of contaminating microbiota. When sputum production is scant, induction with nebulized saline may be effective in producing a specimen that is more representative of the lower respiratory tract. Avoid the use of "saline for injection," many preparations of which contain antibacterial substances.[235]

Special sputum collection devices are commercially available through laboratory supply companies, or a sterile wide-mouth jar with a tightly fit screw-cap lid can be used. To prevent contamination of the outside of the container, the patient should be instructed to press the rim of the container under the lower lip to catch all of the expectorated sputum specimen.[2]

Endotracheal Aspirate. The lower respiratory tract may be sampled by introducing a catheter through the larynx into the trachea. If an endotracheal tube is in place or there is a tracheotomy, aspirating tracheal secretions is simple. It is often assumed that endotracheal aspirates avoid some of the contamination problems of expectorated sputum. In fact, intubation introduces the possibility of contamination because oral secretions can seep down the endotracheal tube. These specimens must, therefore, be interpreted with the same care as that taken with expectorated sputum. It has been suggested that quantitative cultures of tracheal aspirates may provide better data (see the discussion of bronchoalveolar lavage below).[22] The quantitative technique involved performing a dilution of sputum that had been liquefied enzymatically before plating onto agar. A simpler semiquantitative approach, in which the sputum was washed twice with saline and struck onto agar as usual (see Chapter 1), correlated closely with the quantitative technique. This technique has not been widely adopted, as most laboratories use the semiquantitative technique.

Translaryngeal (Transtracheal) Aspiration. Translaryngeal aspiration is an invasive technique that was introduced to avoid contamination problems. Technical difficulties with proper performance and too frequent complications have nearly eliminated this approach to specimen collection.

Rigid Bronchoscopy. The rigid bronchoscope was capable of sampling only the central airways. Although it is adequate for mycobacterial cultures, it is not appropriate for organisms that colonize the oropharynx and infect the distal tract. It is seldom used today.

Flexible Bronchoscopy. Fiberoptic bronchoscopy is the technique frequently used for obtaining transbronchial biopsies, bronchial washings, bronchoalveolar lavage and

brushings, particularly in patients with lung abscesses or other suspected deep pulmonary infections. These techniques are also used in cancer diagnostics. The bronchial brush technique uses a telescoping double catheter plugged with polyethylene glycol at the distal end to protect a small bronchial brush. This technique has been recommended for the optimal recovery of aerobic and anaerobic bacteria, both facultative and obligate anaerobes, from deep-seated pulmonary lesions.[10] Discrete sampling of focal lesions may be accomplished after fluoroscopic localization of the tip of the bronchoscope. Although initially popularized, the rate of contamination of these specimens has limited the usefulness of cultures obtained by these methods. Protected brush specimens are not significantly better than those obtained by bronchoalveolar lavage.

Specimens that cannot be cultured immediately should be refrigerated. The success of this procedure depends on the following: (1) obtaining ample brush material from the distal bronchioles and alveoli to make several slides, (2) preparing a full set of special stains and multiple cultures, and (3) searching for more than one type of microorganism.

Bronchoalveolar Lavage. Bronchoalveolar lavage involves the injection of 30 to 50 mL of physiologic saline through a fiberoptic bronchoscope that has been threaded into the peripheral bronchiolar ramifications. The saline is then aspirated and submitted for smear preparation and culture. Semiquantitative or quantitative cultures of respiratory secretions obtained by protected bronchial brush and alveolar lavage techniques have been recommended for diagnosis of pneumonia in intubated patients undergoing ventilation.[52] Organisms that are present in concentrations greater than 10^3 to 10^4 colony-forming units (CFU)/mL and specimens that demonstrate intracellular bacteria in more than 25% of the inflammatory cells present are indicators of pneumonia that requires specific treatment. Unfortunately, the experience with quantitative microbiology of respiratory secretions has been highly variable.[300] One group of investigators found high specificity if a cutoff of 10^5 CFU/mL was used for fluid obtained by bronchoalveolar lavage, but the sensitivity was only 33%, largely because of prior antimicrobial therapy.[279] They concluded that at this bacterial concentration, pneumonia could be diagnosed reliably, but a negative result did not exclude the diagnosis.

Fine Needle Aspiration and Lung Biopsy. Fine needle aspiration is usually performed under radiologic guidance, particularly if a localized lesion is present.[301] This is an excellent means of obtaining material for cytology and culture; on-site cytologic assessment is useful to guide culture. For

example, the presence of granulomas would make one prioritize mycobacterial and fungal cultures, whereas all neutrophils would suggest a bacterial etiology. The transbronchial biopsy is a way of obtaining smaller amounts of intact tissue at the time of bronchoscopy. Although these biopsies are often diagnostic, they are subject to sampling error. When the diagnosis has not been achieved by the aforementioned techniques, then an open lung biopsy may be necessary. This is the most invasive approach and is reserved for situations in which other measures have failed. A small wedge is resected from the lung in which the disease process may be studied histopathologically and microbiologically.

Additional procedures may be helpful in determining the etiology of respiratory infections. Blood cultures should always be obtained during the acute phases of pneumonia. *S. pneumoniae* may be recovered from the blood in 25% to 30% of patients with pneumococcal pneumonia, often when sputum cultures are negative.[200] Although the blood culture is not sensitive for detecting the etiologic agent of pneumonia, it is highly specific.

The detection of antigens secreted in the urine is another important means of determining the etiologic agent of infection. The *Legionella pneumophila* serogroup 1 urinary antigen test and *S. pneumoniae* urinary antigen are ancillary diagnostics important for the diagnosis of pneumonia caused by these pathogens.[20,201] The antigen may be excreted for days, weeks, or even months, so a positive test does not absolutely document a recent or ongoing infection. Careful correlation with clinical information and culture and/or molecular diagnostic studies is required. The *Histoplasma* urinary antigen is also commonly ordered for patients with suspected fungal pneumonia due to the organism. This test is highly sensitive in disseminated disease, only moderately sensitive in infections limited to the lungs and insensitive for remote infections.

Laboratory Diagnosis of Pneumonia. The etiologic diagnosis of pneumonia is a cooperative enterprise that requires microbiologists, pathologists, radiologists and clinicians. The diagnosis of pneumonia is a clinical one, using history, the stethoscope, and chest radiographs. With a few important exceptions, such as recovery of *M. tuberculosis*, pneumonia is not diagnosed in the microbiology laboratory. Clinicians who send a sputum specimen to the laboratory without having a clinical diagnosis of pneumonia may receive confusing or even misleading test results. Once a diagnosis of pneumonia has been made, the laboratory can help a clinician define the etiology, through culture and other testing, and select appropriate therapy, through antimicrobial susceptibility testing.[43]

Sputum samples should be processed as soon after collection as possible. A significant decrease in recoverable organisms from sputum samples after 20 hours of refrigeration has been found,[218] although there was no compromise in the number or

quality of epithelial cells and segmented neutrophils. A decline in the number of viable tubercle bacilli that could be recovered from sputum after storage at room temperature for several days has also been found,[216] although the concentration of acid-fast bacilli seen in acid-fast stains was not reduced after 20 days.

The quality of sputum samples should also be assessed using one of the grading systems described in Chapter 1. There is controversy about the value of providing presumptive bacterial identifications based on morphologic criteria. Bartlett and coworkers[13] first suggested that categories of bacteria could be defined accurately by morphology in Gram-stained smears of sputum. For example, the morphologic identification of staphylococci, "*Bacteroides- Haemophilus*," and bacteria of mixed morphology were made with 75% accuracy if the sputum specimens were of high quality. It has been demonstrated[305] that semiquantitative enumeration of the bacteria in Gram-stained sputum cannot be reproduced from one technologist to another (or even with the same technologist examining smears on repeated occasions) and that such estimations should not be reported. One possible explanation for this observation is the variability of smears from one area to the next. Conversely, a more optimistic report[101] suggests that Gram's stains of high-quality sputum specimens, performed in a select population of adults with community-acquired pneumonia, may provide clinicians with enough information to initiate empiric antibiotic therapy. In either event, considerable experience on the part of the observers and regular correlation of laboratory results with clinical indicators is necessary before Gram's stain interpretations of sputum samples will be of value.

A useful quality assurance tool is the retrospective comparison of Gram's stain results and culture results. If organisms seen in the smears do not grow in culture or if organisms that grow in moderate to heavy quantities are not seen in the smear, the smear should be reviewed. Gram's stain is relatively insensitive (approximately 10^5 CFU/mL must be present to be visualized), so small numbers of bacteria in the culture may well not be seen in the smear. If the smear has been misinterpreted, it should be reviewed with the reader as an educational and process improvement tool. If the smear was correctly interpreted, this may be a clue that additional approaches to culture should be considered.

The grading systems for sputum samples do not apply in lower respiratory tract infections caused by *Legionella* spp., mycobacteria, fungi, *Mycoplasma, Chlamydophila,* and viruses. Purulent inflammatory cell responses are not necessarily elicited in these infections.

The semiquantitation of stainable tubercle bacilli in sequential examinations of sputum by acid-fast smears is of value for determining the efficacy of antituberculous drug therapy. A decrease from 4+ to 1+ to rare or even to an absence of bacilli over a 4- to 6-week course of therapy indicates good drug response and may be used to determine when it may be safe to remove the patient from respiratory isolation.

The importance of the microorganisms recovered from respiratory samples must always be evaluated in the context of the clinical information, as well as with the results from other studies. Interpretation of sputum cultures is particularly difficult because they are neither specific nor sensitive in the evaluation of lower respiratory infections. Lentino and Lucks[162] have succinctly stated the problem based on their experience in a study of 249 patients with suspected pneumonia (Box 2-1).

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BOX 2-1

Interpretation of Sputum Cultures: Observations of Lentino and Lucks[162]

1. Using the sputum quality-grading system of Bartlett and of Murray and Washington as outlined in Chapter 1, 48% of the sputum cultures submitted to their laboratory did not pass the quality grade and were more reflective of oral secretions.
2. In the above case, 26.5% of purulent sputum samples were from patients showing no radiologic or clinical evidence for pneumonia.
3. Overall, 40% of sputum samples obtained from patients with evidence of pneumonia were not deeply expectorated, again more reflective of oral secretions.
4. Only 10.8% of patients producing nonpurulent sputa had pneumonia.
5. Only 56.8% of patients with pneumonia produced purulent sputum.

Infections of the Gastrointestinal Tract

Establishing the bacterial etiology of acute and chronic bronchitis can also be difficult, because so many species of bacteria can be found as normal microbiota or commensals in the respiratory tract (Table 2-2). The recovery of *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *Moraxella* (*Branhamella*) *catarrhalis* as the predominant microorganism from respiratory secretions, particularly when Gram-stained smears support their presence and/or the organisms are also recovered from concomitant blood cultures, supports their role in the development of acute pneumonia.[73,343] Induced sputum samples, collected after inhalation of nebulized saline, may be required to increase the yield of detection of certain organisms,

particularly *P. jirovecii*.

If pulmonary infections caused by mycobacteria, fungi, human parasites, or viruses are suspected, special techniques must be used to recover the etiologic agents, as outlined in the chapters devoted to each of these groups of microbes. Although the recovery of certain fungi, such as the dimorphic pathogens, usually indicates disease, other fungi, such as *Aspergillus* species, must be recovered repeatedly from successive samples before the diagnosis can be confirmed. The presence of fungal hyphae is less likely to represent environmental contamination. Diagnosis of viral pneumonia will be attempted most frequently in immunosuppressed patients and in children who are sufficiently ill to require admission to the hospital.

The use of blood culture, urinary antigen detection, and quantitative culture techniques has been discussed above. Modern molecular techniques are now available for expedited diagnosis of viral and mycobacterial infections; the number of situations in which these approaches will be applicable is unlikely to expand in the future. Measurement of a soluble triggering receptor that is found on myeloid cells has been proposed as a useful approach,[98] but the promising results require confirmation.

TABLE 2-2 Selected Commensal Microbiota and Potential Pathogens in the Respiratory Tract

Commensal Microbiota	Potential Pathogens
α/γ-Hemolytic streptococci β-Hemolytic streptococci, other than group A *Candida* spp. Coagulase-negative staphylococci *Corynebacterium* spp. (diphtheroids) *Haemophilus parainfluenzae* *Neisseria* spp.	Adenovirus Anaerobes (as part of mixed infection) *Bordetella pertussis* *Chlamydophila pneumoniae* *Chlamydophila psittaci* *Corynebacterium diphtheriae* *Cryptococcus neoformans* Cytomegalovirus *Enterobacteriaceae* *Haemophilus influenzae* Herpes simplex virus *Legionella* spp. *Moraxella catarrhalis* *Mycobacterium* spp. Myxoviruses and paramyxoviruses *Neisseria gonorrhoeae* *Neisseria meningitidis* *Pneumocystis jirovecii* *Pseudomonas aeruginosa*

	Staphylococcus aureus *Streptococcus pneumoniae* *Streptococcus pyogenes* (group A)

Lower Intestinal Infections

Clinical Symptoms. The most common presenting symptom of lower intestinal tract infections is diarrhea.[297] Although diarrhea is difficult to define quantitatively, patients usually know when they have bowel movements in excess of normal and when the stool takes on a form that is softer or more liquid than usual. The diarrhea may be accompanied by cramping abdominal pain of varying severity. "Enterocolitis" is a term used to describe the several types of lower gastrointestinal infection.

"Dysentery" is a term used to describe the condition in which diarrhea is accompanied by cramping abdominal pain, tenesmus (painful straining when passing a stool) and purulence of the stool. Dysentery results from "enteroinvasive" microorganisms that penetrate the mucosa and cause inflammation of the intestinal wall. These stools contain inflammatory cells and often may be grossly visible blood. *Shigella* and *Entamoeba histolytica* are classically associated with dysentery, but both of these actually cause a spectrum of disease wherein the diarrhea ranges from watery to dysenteric.

At the opposite end of the spectrum are the nonpainful, profusely watery diarrheal syndromes caused by viruses, certain parasites and some bacteria. Most acute, watery diarrheal syndromes resolve spontaneously within a week. If symptoms persist without explanation, parasites, such as

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Giardia intestinalis (formerly *G. lamblia*), and noninfectious etiologies should be considered. The stools produced by *Giardia* are often foul-smelling, greasy, and float in the toilet bowl.

A unique, and fortunately uncommon category of enteritis in resource-rich countries, is enteric fever. This syndrome is classically caused by *Salmonella enterica* serotype Typhi, although it may also be caused by Serotype Paratyphi and rarely by other serotypes. Although there may be a diarrheal component to enteric fever, it is actually a systemic disease. Enteric fever is characterized by fever—first remitting and later constant, headache, abdominal pain, splenomegaly, relative bradycardia (low pulse rate), and leukopenia. Diarrhea is not prominent; in fact, constipation may be more common at the outset. This organism, after invading the intestine, becomes disseminated throughout the reticuloendothelial, such as the liver, spleen, and bone marrow. As demonstrated by the historically significant "Typhoid Mary," the

gallbladder may be colonized, particularly if calculi are present, and the organism may be shed into the environment for extended periods of time after the initial infection.

Certain infectious agents are associated with discrete risk factors or clinical features. Some agents, such as microsporidia, occur almost exclusively in severely immunosuppressed patients. *Clostridium difficile* produces disease predominantly in patients who have been treated with antimicrobial agents that alter the normal gastrointestinal microbiota. Many of the agents are transmitted through contaminated food and water.[49] The major syndromes of lower gastrointestinal disease are summarized in Table 2-3.

In many communities the most commonly identified agents of gastroenteritis are *Campylobacter jejuni*, *Salmonella* spp., *Giardia intestinalis*, rotaviruses and noroviruses.[66] Unfortunately, the diagnostic yield from stool cultures in most clinical laboratories is a disappointing 1.5% to 5.6%.[297] It is possible that this will be substantially improved with the implementation of commercially available molecular gastrointestinal panels, which include agents like norovirus and astrovirus, which have heretofore gone undetected in most laboratories.

Some agents of gastroenteritis require prompt treatment (e.g., *Shigella* spp.); for others specific therapy is not available (e.g., viruses); for still others therapy is indicated only if the patient is very young, immunosuppressed, or has complicated disease; enteric fever always requires antimicrobial therapy. Physicians are most likely to order stool cultures if one of the following conditions is present.[119]

1. The patient is immunocompromised.
2. The patient has traveled recently to a resource-poor country.
3. Presence of bloody stools.
4. Diarrhea has been present for more than 3 days.
5. Diarrhea has required intravenous rehydration.
6. Fever is present.

Laboratories differ greatly in the sophistication of their evaluation of fecal specimens. In a survey of 388 clinical laboratories during 1999 most microbiologists examined feces for the presence of *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. In contrast, approximately 50% of sites included *Escherichia coli* O157:H7, *Vibrio* spp., and *Yersinia* spp. in their diagnostic protocol.[308] Examination of stools for parasites was performed in only 59% of 455 laboratories surveyed; the remaining laboratories sent specimens to a reference laboratory for testing.[137] Examination of feces for *Cryptosporidium* spp., *Cyclospora cayetanensis*, and microsporidia was performed only on specific request in 89% of these laboratories. It is very important for each laboratory to indicate the pathogens sought when the results are reported to the clinician. A report of "No enteric pathogens isolated" is no longer

acceptable, but rather a listing of what was sought and not found must be given (e.g., Negative for *Salmonella, Shigella* and *Campylobacter*). In a survey of almost 3,000 physicians in five states 28% of physicians did not know whether the laboratory tested for *E. coli* O157:H7 and 40% did not know whether cultures had been performed for *Yersinia* spp. or *Vibrio* spp.[119]

TABLE 2-3 The Major Gastroenteritis Syndromes and Most Common Etiologic Agents

Syndrome	Bacteria	Viruses	Parasites	Comments
Inflammatory diarrhea, including dysentery	*Shigella* spp., Enteroinvasive *E. coli*, Enterohemorrhagic *E. coli, Salmonella enterica* various serotypes (e.g., Typhimurium, Enteriditis) , *Campylobacter jejuni* and other *Campylobacter* species, *Vibrio parahaemolyticus, Clostridium difficile*	None	*Entamoeba histolytica*	Involves colon; fecal leukocytes often present
Noninflammatory diarrhea	Enterotoxigenic *E. coli*, Enteroaggregative *E. coli* and *Vibrio cholerae*. Secondary to preformed toxins: *Clostridium perfringens, Bacillus cereus, Staphylococcus aureus*	Norovirus, rotavirus, enteric adenovirus, astrovirus, etc.	*Giardia intestinalis, Cryptosporidium* spp., *Cystoisospora belli, Cyclospora cayetensis,* microsporidia- (fungi)	Involves proximal small bowel; fecal leukocytes usually absent
Diarrhea with systemic disease, including enteric fever	*Salmonella enterica* serotype Typhi, other *Salmonella* spp., *Yersinia enterocolitica, Campylobacter* spp.	None	None	Involves distal small bowel; fecal mononuclear leukocytes may be present

Adapted from reference 108.

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Collection of Fecal Specimens. The collection of diarrheal stools is not difficult. Specimens for detection of all pathogens should be collected in clean (not necessarily sterile), wide-mouthed containers that can be covered with a tight-fitting lid. Screw-top lids are most commonly used, as these are most secure in transit. The containers used for culture should be free of preservatives, detergents, and metal ions. Contamination with urine should also be avoided. If an intestinal parasite such as *E. histolytica*, *G. intestinalis,* or *Cryptosporidium* spp. is suspected, a small portion of stool sample should be placed in preservatives, such as polyvinyl alcohol, or a mercury-free alternative, and 10% formalin. Stool specimens for detection of viruses should follow the manufacturer's recommendations for transport.

In some instances, the collection of a rectal swab rather than feces may be necessary, particularly in newborns or in severely debilitated adults. Rectal swabs may be more effective than feces for recovery of certain strains of *Shigella*, because these organisms are susceptible to cooling and drying. Swabs have also been reported to be more effective than stool specimens[189] for recovery of *Clostridium difficile* in hospitalized patients. The rectal swab should be inserted just beyond the anal

sphincter, avoiding direct contact with fecal material in the rectum. The swabs should be inoculated immediately onto culture media or placed in a suitable transport system to prevent drying. Rectal swabs have also been used for diagnosis of rectal gonococcal infection.

Epidemiologic Considerations in the Evaluation of Patients With Gastroenteritis. A detailed medical history should be taken, including recent travel, exposure to potentially contaminated food or water and the presence of similar disease in friends or family. In particular, travel to countries outside the United States, where certain diarrheal diseases may be endemic, should be solicited. The most common cause of "traveler's diarrhea" (also called "Montezuma's revenge," "Delhi belly," and other colorful names) is enterotoxigenic *E. coli*,[213] but there are a variety of diarrheagenic *E. coli* (e.g., Enteropathogenic, Enteroaggregative, etc.) that may also cause disease in the traveler. Other important pathogens, such as *E. histolytica*, are also more common outside the United States. Poor-quality water and food supplies, marginal cold-storage facilities, and contamination from people preparing foods in countries outside the United States put travelers at risk. In these days of global commerce, however, staying at home is not free of risk either. Epidemics of gastroenteritis have been reported as a result of contaminated food,[49] usually vegetables or fruit that are not cooked before eating, that has been imported from other countries. For instance, a multistate outbreak of *Cyclospora* infection was traced to raspberries imported from Central America only after reports accumulated at national reference centers.[120] It is important to remember that bacteria commonly found on poultry, which will be killed during cooking, may cause disease if uncooked food, such as vegetables, come in contact with the contaminated chopping boards or counters that were used for preparation of the poultry.

Certain locales, foods, and clinical settings are particularly associated with specific pathogens (Table 2-4). A variety of factors may place the individual at risk for infectious enterocolitis, emphasizing the importance of the careful clinical history and reporting of cases to public health authorities.

Upper Intestinal Infections

The upper gastrointestinal tract consists of the esophagus, stomach, and proximal duodenum (i.e., from the oropharynx to the intestine).

Clinical Symptoms. Esophagitis is associated with difficulty swallowing and pain on swallowing (i.e., dysphagia) and by pain that radiates to the back. The gastroesophageal mucosa is at particular risk of ulceration. The most common infectious agents in this site are *C. albicans* and herpes simplex virus, both of which produce erosive disease.

When the stomach and gastroesophageal junction are involved, symptoms include anorexia, a feeling of nausea (occasionally with overt vomiting) and upper abdominal pain. Because of the very low pH of the gastric acid, 99.9% of ingested bacteria are killed within 30 minutes of exposure; therefore, gastritis from direct invasion of the stomach wall is rare. Upper gastrointestinal infection, therefore, is most commonly caused by viruses and preformed bacterial toxins.

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Gastric acid also has an important role to play in protecting the lower gastrointestinal tract from bacterial infection. Antacid therapy, which neutralizes the low gastric pH and gastrectomy, in which most of the acid-forming mucosa is removed, predispose individuals to enteric infections from a variety of bacterial species. Guerrant[107] reports that the dose of *Vibrio cholerae* required to cause infection in normal people (10^8 organisms per milliliter) was reduced to only 10^4/mL in volunteers who were given bicarbonate to neutralize gastric acidity.

TABLE 2-4 Epidemiologic Associations of Gastrointestinal Infectious Agents

Factors	Infectious Agent(s)
Backpacking and drinking from mountain streams	*Giardia intestinalis*
Dairy products	*Salmonella* spp., *Campylobacter* spp., *Yersinia* spp., *Listeria monocytogenes*
East Coast and Gulf Coast	Halophilic vibrios; e.g., *Vibrio parahaemolyticus*, *Vibrio vulnificus*
Egg and potato salads, pastries	*Staphylococcus aureus*
Eggs	*Salmonella* spp.
Fresh fruit	*Cryptosporidium* spp. and *Cyclospora* spp.
Fried rice	*Bacillus cereus*
Hamburger	*Escherichia coli* O157:H7 and other Enterohemorrhagic *E. coli*
	Cryptosporidium spp., *Cystoisospora belli*, *Mycobacterium avium*

patients	complex, *Strongyloides stercoralis* hyperinfection, cytomegalovirus, *Candida* spp.
Shellfish	*Vibrio* spp., noroviruses, hepatitis A virus

Helicobacter pylori is recognized as the most important cause of gastritis and gastric ulcer.[158,180,294] The organism has the unique biochemical property of rapidly and avidly hydrolyzing urea and releasing ammonium ions. The bacterial cells can presumably surround themselves with an alkaline microenvironment, thereby making it possible to survive the highly acid environment of the gastric mucosa. The infectious etiology of peptic ulcer eluded detection for so long because it does not have the classic signs and symptoms of an "acute" infectious disease. The knowledge of this chronic infectious disease has opened new vistas on the spectrum of illness that microbes can produce.

A major factor in upper gastrointestinal infection is ingestion of microbes and/or preformed microbial toxins in food and drink, commonly known as food poisoning. The acute and often fulminant gastritis, accompanied by generalized weakness and vomiting, that may be experienced after ingesting food heavily contaminated with the toxins produced by organisms such as *S. aureus*, *Clostridium perfringens,* and *Bacillus cereus*, does not result from direct bacterial invasion of the stomach wall; rather, it results from the direct emetic action of preformed toxins. These toxins vary in their mechanisms of action. In most instances, although the emesis may be fulminant, it is brief in duration. The diagnosis is solely made on a clinical basis, as toxins are not detected in the clinical laboratory. Public health microbiologists may sample retained food specimens in an effort to detect the pathogen involved and the means of spread, but this is not the task of the clinical microbiologist.

Obtaining Specimens From the Upper Gastrointestinal Tract. Gastric specimens for culture are only rarely obtained and are limited to those few situations for which a diagnosis may not be possible by other means. The bacterial agents of acute toxic food poisoning may be recovered from vomitus material, but this is usually not requested.

Gastric biopsies are performed to detect *H. pylori*.[290,307] Biopsy specimens may be cultured for the recovery of *H. pylori*, a procedure rarely performed in clinical laboratories. Biopsies may be tested for the presence of urease activity, a presumptive test for the presence of *H. pylori*. More commonly biopsies are examined histologically for the presence of gastritis and spiral bacteria in the mucin layer. There is a stool antigen test available for the detection of *H. pylori,* as well as a urea-breath test, both of which have good performance characteristics. Conversely, serologic studies should be discouraged because of suboptimal performance compared with the other diagnostic tests noted.[7,53]

Aspirations of duodenal contents may be helpful in making the diagnosis of giardiasis and strongyloidiasis if repeated stool examinations have failed to detect a pathogen. The use of the commercial "string test" (e.g., Enterotest) is an alternative to endoscopy and biopsy. The Enterotest is a capsule containing a tightly wound string. The string is unraveled for a short distance and the end taped to the patient's cheek; then the capsule is swallowed. In about 30 to 60 minutes, when the capsule has reached the upper duodenum, the string is carefully removed and any mucus adhering to the strand is scraped onto the surface of a glass slide for direct microscopic examination. As noted gastric and/or small intestinal biopsy remains a diagnostic alternative.

Urinary Tract Infections

The urinary tract is divided into the upper urinary tract, composed of the kidneys, renal pelves, and ureters, and the lower urinary tract that consists of the urinary bladder and the urethra. Upper urinary tract infections are most commonly ascending (i.e., infections originate in the urinary bladder and ascend through the ureters to the kidneys). Normally, the vesicourethral valve prevents reflux of urine from the urinary bladder into the ureters. Individuals with urogenital anomalies or with overdistention of the urinary bladder from outflow obstruction, neurogenic malfunctions, or pressure from an enlarged uterus during pregnancy are particularly susceptible to ascending urinary tract infections. Infections of the renal pelvis (pyelitis) and kidney (pyelonephritis) are the most common complications. The infections can be acute or can be recurrent with chronic inflammatory damage.

Upper urinary tract infections less commonly result from hematogenous spread of bacteria into the renal cortex in patients with septicemia. Multifocal abscesses or acute suppurative pyelonephritis are common manifestations.

Urinary tract infections are sometimes divided into uncomplicated and complicated categories.[283,317] Uncomplicated infection is acute cystitis or pyelonephritis in a young woman without underlying urinary tract or systemic disease. If the diagnosis is cystitis, empiric antimicrobial therapy can be instituted without resorting to a culture, because *E. coli* is responsible for the vast majority of these infections. A urinalysis or test for leukocyte esterase (LE) should be performed to demonstrate that the process is inflammatory; if polymorphonuclear neutrophils are absent, a culture should be performed before initiating therapy. A follow-up culture ("test of cure") is not necessary unless symptoms persist.

Cystitis or pyelonephritis in males, children, chronically catheterized patients, and women with recurrent infection, urologic abnormalities or underlying disease is considered a complicated infection. Both urinalysis and urine culture are required for

complicated cystitis and for all patients with pyelonephritis.[283,317]

Clinical Signs and Symptoms

The cardinal clinical manifestations of upper urinary tract infections are flank pain and fever, which is often associated with chills. Frequency, urgency, and dysuria are more suggestive of infections of the urinary bladder and urethra. However, in some patients with pyelonephritis or other upper

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urinary tract infections, particularly those with ascending infections, symptoms consistent with lower tract infections develop first. The differentiation is important because the approach to antimicrobial chemotherapy differs for the two conditions.[283,317]

Lower urinary tract infections typically involve the urinary bladder and/or the urethra. The symptoms are similar for infections in the two sites, so the process is sometimes called the acute urethral syndrome.[285] Frequent and painful urination of small amounts of turbid urine (i.e., symptoms of frequency and dysuria) and suprapubic pain are the usual clinical manifestations. Vaginitis in women and prostatitis in men can produce similar symptoms, but can usually be differentiated clinically. Diagnosis is particularly difficult in elderly persons, in whom fever or leukocytosis may not develop during an infection.

There has been much discussion about the significance of asymptomatic bacteriuria. Although this phenomenon has been documented in many types of patients and clinical scenarios, serious medical problems that necessitate treatment of the bacteriuria occur in only a few situations: pregnant women, women who are undergoing invasive genitourinary procedures, and recipients of renal transplants during the early posttransplantation period.[209]

Host Factors

The prevalence of urinary tract infections varies with the gender and age of the patient. In neonates and infants, urinary tract infections are more common in boys, with an overall prevalence of about 1%. Most of these infections are associated with congenital anomalies. By the time children attend school, there is a higher prevalence in girls compared with boys.[289] This ratio remains consistent into adulthood. High rates of incidence occur in certain conditions, such as diabetes or pregnancy. In the elderly, higher rates of occurrence can be expected for both women (20%) and men (10%) in whom predisposing conditions exist, such as obstructive uropathy from the prostate in men, poor emptying of the bladder from uterine prolapse in women, and

prostate in men, poor emptying of the bladder from uterine prolapse in women, and procedures that require instrumentation in both men and women.

By far the most common population at risk for both symptomatic urinary tract infection and asymptomatic bacteriuria, however, is sexually active women.[288] Although asymptomatic infection in this group does not clearly produce serious medical problems, it may be a predictor of future symptomatic infection.[209] Women are more susceptible to infection than men because of the shorter length of the female urethra. Perineal bacterial microbiota that originate in the gastrointestinal tract are the usual pathogens, especially if the bacteria possess factors that facilitate their binding to the uroepithelium.[246] Sexual intercourse facilitates entry of the bacteria into the female urethra.

A second population that is at increased risk of infection is the chronically catheterized patient.[281] A foreign body, such as an indwelling urinary catheter, guarantees colonization of the catheter within 5 days of placement. The presence of a colonized catheter results in asymptomatic bacteriuria and puts the patient at risk for developing symptomatic infection, including pyelonephritis and urosepsis. When the patient is also afflicted with dementia, as many elderly individuals are, it may be difficult to ascertain whether the infection is symptomatic. Without fever and leukocytosis the only indicators may be subtle changes in personality or mentation. The best person to assess these characteristics may be the primary caregivers, rather than the physician who sees the patient intermittently.

TABLE 2-5 Some Commensal Microbiota and Potential Pathogens in the Urinary Tract

Commensal Microbiota	Potential Pathogens
α/α-Hemolytic streptococci *Bacillus* spp. Coagulase-negative staphylococci Diphtheroids *Lactobacillus* spp.	*Corynebacterium urealyticum*[a] *Enterococcus* spp. *Enterobacteriaceae*[a] *Pseudomonas* spp. *Staphylococcus aureus* *Staphylococcus epidermidis* (elderly men) *Staphylococcus saprophyticus* (young women)

[a]Proteae and *Corynebacterium urealyticum* are urea splitters, alkalinizing the urine and predisposing to stone formation.

Except for the urethral mucosa, which supports the growth of microbiota, the normal urinary tract is usually devoid of bacteria.[141] Urine can easily become contaminated with bacteria from the vaginal canal, the perineum, or indigenous bacterial microbiota in the urethra. Table 2-5 lists selected microorganisms that are considered contaminants and those considered potential pathogens in the urinary tract.

Midstream Urine Specimens. Urine samples are most commonly collected by sampling the midstream flow by the clean-catch technique. Urine collection from women by this technique requires personal education and, in some instances, supervision for best results.[29] The periurethral area and perineum are first cleansed with two or three gauze pads saturated with soapy water, using a forward-to-back motion, followed by a rinse with sterile saline or water.

The labia should be held apart during voiding, and the first few milliliters of urine passed into a bedpan or toilet bowl to remove bacteria from the urethra (Fig. 2-2). The midstream portion of urine is then collected in a sterile, wide-mouthed container that can be covered with a tightly fitted lid. The use of a urine transport tube with a preservative is recommended to prevent overgrowth of contaminating microbiota during transport and processing. The soapy water preparation is usually not required for men; rather, simple cleansing of the urethral meatus immediately before voiding and then collection of the midstream sample is usually sufficient.

Patients seen in a clinician's office or in a clinic are frequently asked to obtain their own urine sample. This practice is acceptable if the patient is given precise instructions for properly collecting the specimen. It is recommended that these instructions be printed on a card that the patient can retain after receiving the verbal description. When the patient does not seem to comprehend or when language is a barrier, the nurse or office assistant should read through the instructions point by point or provide direct assistance in collecting the sample. An example of an instruction card is given in Box 2-2.

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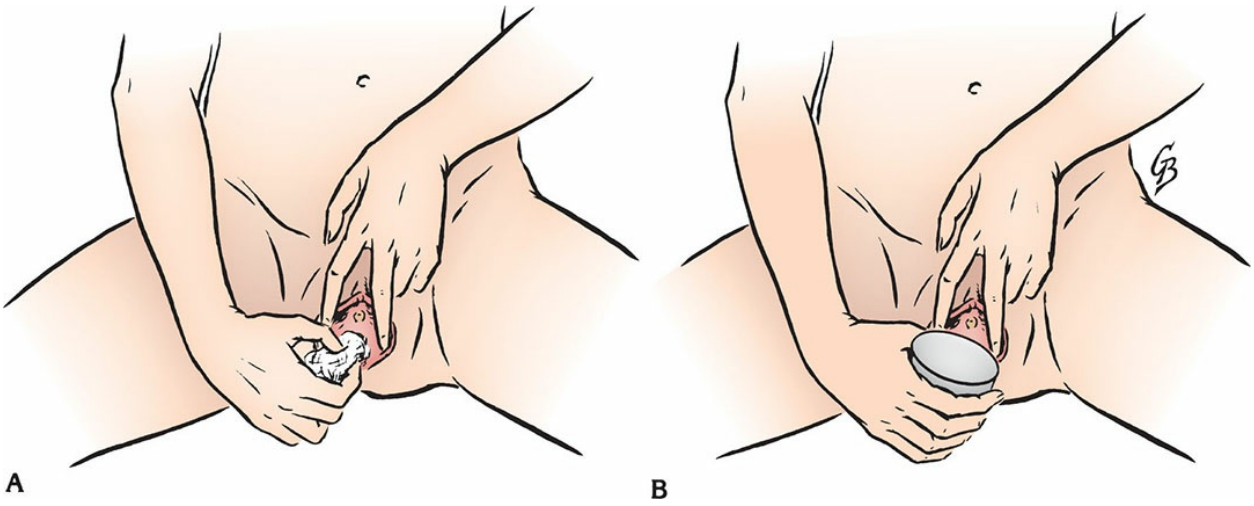

■ **FIGURE 2-2** Midstream clean-catch urine collection. **A.** The labia are separated with the fingers and cleansed with a 4 × 4-inch gauze pad saturated with soap. **B.** The midstream portion of the urine is collected in a sterile container.

The accuracy of the urine collection procedure can be monitored over time by noting the frequency with which urine colony counts range between 10,000 and 100,000 CFU/mL. Most patients will have colony counts outside this range. Those free of infection will have no bacteria or fewer than 10^2 CFU/mL. Infected patients will have 100,000 CFU/mL or more. The frequency of intermediate counts should not exceed 5% to 10% if the urine collection procedures have been performed properly. Specimens should be processed within 2 hours after collection to achieve accurate colony counts.

The BD Urine Collection Kit (Becton-Dickinson, Cockeysville, MD), designed to maintain the bacterial population in urine at room temperature for 24 hours, has been equally as effective as overnight refrigeration of specimens.[126] Although some decrease in colony count may be noted after prolonged storage, the system is recommended for transport of urine samples for which processing will be delayed up to 24 hours. Transport systems using boric acid, which is in included in the BD Urine Collection Kit additives, may offer an alternative method for home or remote collection of urine samples. Jewkes and associates[133] conclude from a study of 84 children that urine collection in boric acid minimizes contamination, although growth of potential bacterial pathogens may also be inhibited in a small number of instances.

Collection of valid clean-catch urine specimens for culture from incontinent elderly men residing in nursing homes also poses a problem. Nicolle and colleagues[210] reported success in diagnosing urinary tract infections in this population of patients by using an external collection device consisting of a sterile condom and leg bag. Before applying the condom, the glans penis was cleaned with soap and water and rinsed with

sterile saline. The leg bag was examined every 10 to 15 minutes until a specimen was obtained. Because contamination with low counts of bacteria occurred in almost 50% of the patients, rapid transport of the specimens to the laboratory and immediate transfer to culture media were required. Bacterial counts greater than 10^5CFU/mL, particularly if obtained on two successive collections, had a high correlation with other indicators of urinary tract infection.

Other Voided Urine Specimens. The purpose of the midstream technique is to collect urine that has been in the bladder after discarding the initial portion that has been in contact with the urethra—and presumably contaminated

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with urethral microbiota. If one is investigating urethritis, however, it is actually the initial portion of voided urine that is of interest. Traditionally, a swab may be inserted into the distal urethra to collect the specimen. The primary application of initial voided urine specimens is the diagnosis of urethritis caused by *N. gonorrhoeae* and *Chlamydia trachomatis*.[135] More commonly, molecular methods are used on urine for the detection of these pathogens.

BOX 2-2

Instructions for Obtaining Clean-Catch Urine Specimens (Females)

1. Remove underclothing completely and sit comfortably on the toilet seat, swinging one knee to the side as far as you can.
2. Spread yourself with one hand, and continue to hold yourself spread while you clean yourself and collect the specimen.
3. Wash. Be sure to wash well and rinse well before you collect the urine sample. Using each of four separate 4× 4-inch sterile sponges soaked in 10% green soap or the equivalent, wipe from the front of your body toward the back. Wash between the folds of the skin as carefully as possible.
4. Rinse. After you have washed with each soap pad, rinse with a moistened pad with the same front-to-back motion. Do not use any pad more than once.
5. Hold yourself apart and allow the first few drops of urine to pass into the toilet bowl. Hold the cup on the outside and pass the remaining urine into the cup.
6. Place the lid on the container or ask the nurse to do so for you.

If one is considering the diagnosis of acute prostatitis, however, prostatic secretions are the specimen of choice. The most common technique is differential culturing of urine before and after prostatic massage. A 10-fold increase in bacterial numbers after massage of the prostate suggests the diagnosis.[72]

Catheter Collections. Catheterization for the express purpose of obtaining a urine specimen should be avoided if possible because of the risk of introducing bacterial pathogens. In a study of 105 women with suspected urinary tract infection,[315] the culture results obtained from midstream, clean-catch urine samples did not differ in sensitivity, specificity, or positive or negative predictive values from parallel straight catheter specimens collected immediately after the midstream samples. Catheterization should be restricted to patients who are unable to produce an adequate midstream sample, and it should be performed with meticulous attention to aseptic technique. The first several milliliters of urine from the catheter should be discarded to wash out any organisms that may have lodged in the catheter tip during transit through the urethra.

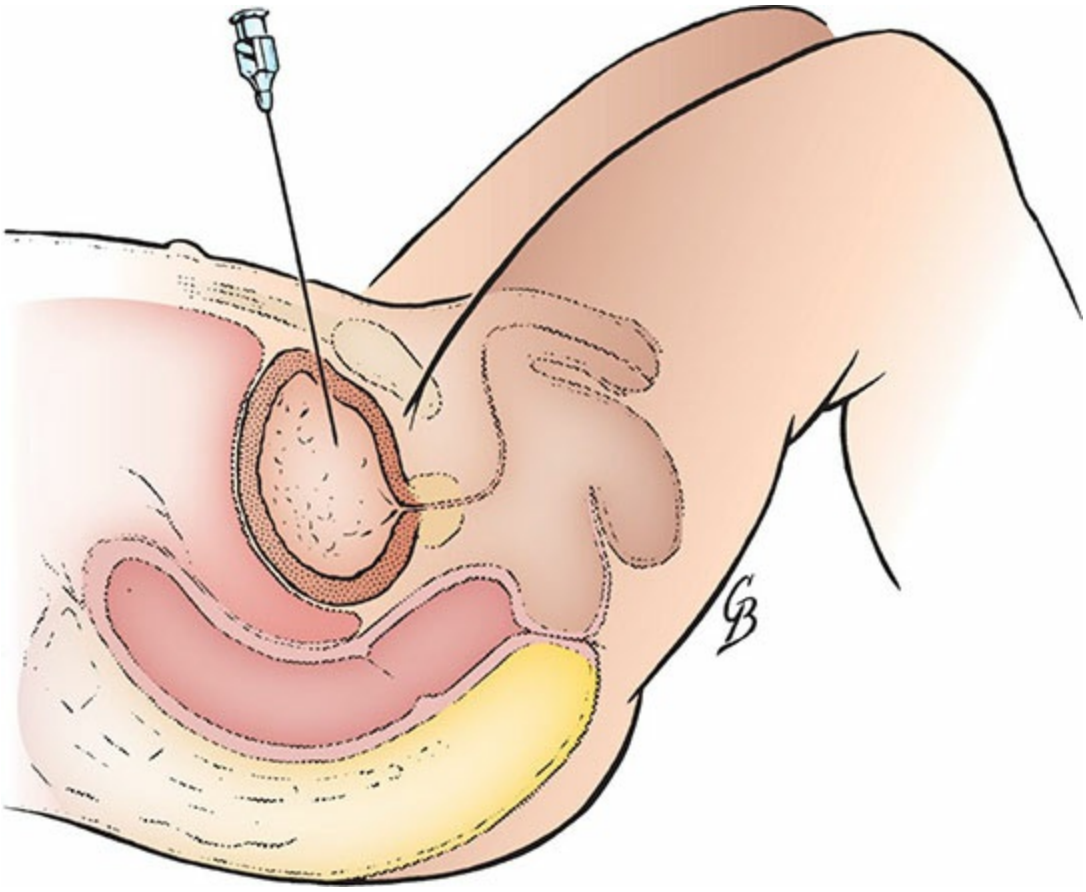
Urine samples from indwelling catheters (i.e., foley catheters) are not recommended, since it is not possible to differentiate the bacteria that have colonized the catheter from potential pathogens. Urine samples should not be obtained from catheter bags. Although it is tempting to use wet diapers or bags as a source of urine in infants, obtaining an interpretable specimen by these means is a major challenge. Foley catheter tips are unsuitable for culture because they are invariably contaminated with urethral or colonizing organisms.[304]

Suprapubic Aspiration. Suprapubic aspirations are reserved almost exclusively for neonates and small children. This technique is illustrated in Figure 2-3. The procedure is best performed when the bladder is full. The suprapubic skin overlying the urinary bladder is disinfected and sterile drapes are put in place. In the immediate site where the aspiration is to be made, an anesthetic solution, such as 1% lidocaine HCl, is injected subcutaneously. An 18-gauge, short-bevel spinal needle is extended into the urinary bladder and 10 mL of urine is aspirated into the syringe. Given the invasive nature of this technique, it is rarely used.

Culture of Urine Specimens

Both selective and nonselective media are required. A combination of 5% sheep blood agar and MacConkey agar is usually sufficient for the recovery of the organisms listed in Table 2-5. Some microbiologists whose laboratories serve primarily ambulatory patients, in whom *E. coli* is the most common pathogen expected, prefer to use eosin methylene-blue (EMB) agar because of the distinctive morphology of *E. coli* on that media. Although some microbiologists incorporate a medium selective for gram-

positive bacteria as well (e.g., colistin–nalidixic acid [CNA] blood agar or phenylethyl alcohol [PEA] agar), the yield from this addition is probably not worth the expense.[44] In many laboratories, inoculation of duplicate plates with both 0.01-mL- and 0.001-mL-calibrated loops is performed for comparison of counts as a quality control check and to facilitate counting colonies.



■ **FIGURE 2-3** Suprapubic urinary bladder aspiration. A needle is directed percutaneously into the urinary bladder just above the symphysis pubis. Urine can be removed with a syringe.

After delivering the inoculum from the calibrated loop, the surface of each agar plate should be completely streaked over all quadrants as demonstrated in Chapter 1 so that semiquantitative colony counts can be performed after incubation. A colony count of 10^5 CFU/mL or greater is the criterion most commonly used to determine whether identification and susceptibility testing of an isolate should be performed. When the colony count is between 10^4 and 10^5 CFU/mL, or when multiple species are recovered, the decision to make identifications and perform susceptibility tests must be

made by each laboratory director, often on a patient-by-patient basis. Some microbiologists use 10^4 CFU/mL as the decision point, because the calibrated loops tend to underestimate the colony count. Similarly, a pure culture is required in some laboratories, while a culture with two pathogens will be evaluated in others. (see Chapter 1 for further discussion of evaluating mixed cultures.)

Cultures of catheterized or suprapubic urine specimens are usually analyzed in detail, even with low colony counts or with the recovery of multiple organism types. Colony counts as low as 10^2 CFU/mL of enteric gram-negative bacilli may be significant in female patients with the acute urethral syndrome[284,285]; however, the physician must alert the laboratory in suspected cases, because semiquantitative urine culture techniques are not designed to detect such low colony counts. The significance of these "low-colony-count" infections has been documented only in women infected with enteric bacilli. Contamination is even more of an issue when evaluating small numbers of organisms. In one study, contaminated specimens were increased by 19% when a 0.01-mL loop was

used.[44] Discussion with the clinician about the significance of such isolates and the need for further work up is appropriate in these situations.

Screening Tests for Urinary Tract Infection

The literature on screening tests for urinary tract infection is voluminous, contradictory and sometimes confusing. It is useful to consider that screening for bacteriuria is a very different task than screening for inflammation.[334] The two will be considered separately.

With the exception of the few situations in which asymptomatic bacteriuria is clinically important, the function of the clinical microbiology laboratory is to test for symptomatic urinary tract infection. Many studies use the presence of 10^5 bacteria as a surrogate end point, but this approach is flawed. It is perhaps useful to think of the problem in the terms used to describe the approach to pneumonia. The diagnosis of urinary tract infection is made by evaluating clinical symptoms and/or testing for the presence of an inflammatory response. Once an infection has been documented, microbiologic culture is useful to determine the etiology of that infection.

Screening Tests for Bacteriuria. The presence of bacteriuria at concentrations that suggest urinary tract infection can be assessed by microscopic examination of the urine, by tests for bacterial products and by cultures. None of the tests detect low

colony counts in urine with adequate sensitivity.

Microscopic Examination. Gram's stain is an inexpensive method to estimate bacteriuria. In one study the presence of at least one organism per oil-immersion field in uncentrifuged urine had a sensitivity of 94% and a specificity of 90% for detecting colony counts of at least 10^5 CFU/mL.[319] This approach has the advantage that the Gram reactivity of the organisms can be characterized. Gram's stain of urine is not used in most clinical laboratories as a screening test, however, because methodically reviewing the smears is too labor-intensive. Examining unstained centrifuged urine as part of the urinalysis has been reported to have a sensitivity similar to Gram's stain on uncentrifuged urine,[44] but this experience has not been universal, and most clinicians do not depend on urinalysis for detection of bacteriuria. Differentiating bacteria from other particles in unstained preparations at moderate magnifications can be a challenge. A useful quality assurance tool is comparison of the results of culture and urinalysis.

Tests for Bacterial Products. One common approach is the use of a reagent-impregnated dipstick, designed to detect the presence of urine nitrite (Griess test) and indirectly to estimate the number of segmented neutrophils by detecting LE activity.[222]The rationale for the nitrite test is that most urinary tract infections are caused by nitrate-reducing members of the family *Enterobacteriaceae*, particularly *E. coli*. This test lacks accuracy when used alone.[136,197] False-positive results may occur if the specimen has been delayed in transit and overgrown with nitrate-reducing bacteria or from drug interference; false-negative results are encountered if the organism causing the infection does not reduce nitrates (e.g., *Enterococcus* spp.) or if the patient is on a vegetable-free diet (loss of an important source of nitrate).

The sensitivity of the combination nitrite–LE strip with bacteriuria at a level of 10^5 CFU/mL is in the range of 79% to 93%, with a specificity of approximately 82% to 98%.[136,222] In a multicenter study of 298 urine specimens with colony counts of fewer than 10^5 CFU/mL,[220] the strip detected 81% of infected patients. In a subset of 204 specimens with pyuria in addition to colony counts fewer than 10^5 CFU/mL (i.e., both inflammation and bacteria), however, the detection rate was increased to 95%.

An alternative approach is detection of catalase, which is produced by most uropathogens, marketed as Uriscreen (Savyon Diagnostics, Ashdod, Israel). It appears to function similarly to nitrite and suffers from the same defects. If the pathogens in a particular population of patients produce catalase, a negative catalase test effectively excludes high colony-count bacteriuria; it offers no advantage over a test for leukocyte esterase.[220,311]

Detection of Bacteriuria by Culture. Conventional culture is a simple and economic approach to screening. Although it does require 18- to 24-hour incubation, the decision to treat is rarely urgent. Some clinicians prefer to inoculate a urine dip slide in the office, rather than send the urine to a laboratory for culture. The dip slide

(SOLAR-CULT; Solar Biologicals, Ogdensburg, NY; Uricult, Orion Diagnostica, Espoo, Finland) is a paddle coated on each side with an agar intended to recover gram-positive and gram-negative bacteria. The paddle is dipped into the urine, drained, replaced in its container, and incubated. The quantity of growth is assessed by comparison to a chart. The dip slides are not waived tests; a clinician who uses them must meet the requirements for nonwaived tests, even if the bacteria are not identified and tested for susceptibility. The advantage of this approach is simplicity and economy for negative specimens. The disadvantage is the difficulty obtaining subcultures for subsequent evaluation of colonies. In the post-Clinical Laboratory Improvement Amendments (CLIA) 1988 era (see Chapter 1), many clinicians prefer the most straightforward approach of sending the original specimen to a diagnostic laboratory.

Screening Tests for Pyuria. LE is produced by polymorphonuclear leukocytes. A reagent strip, impregnated with buffered indoxyl carboxylic acid ester and a diazonium salt, can be used to detect LE activity in the urine. One advantage of this test is that leukocytes need not be viable for LE activity to be detected. When performed alone, this test correlated with 10 or more white blood cells per high-power field (WBC/HPF) in the urine, with a sensitivity in the range of 88% and a specificity of 94%.[155] False-positive LE test findings may result from high urinary levels of ascorbic acid or albumin (>300 mg/dL) or from the effects of preservatives and detergents. Most false-negative results occur when urine WBC counts are in the marginal range of 5 to 10/HPF. Kierkegaard and associates[145] report that 35% of urine samples in their study turned from positive (30 WBC/HPF) to negative (10 WBC/HPF or fewer) by microscopic exam when the urine was delayed in transit for 3 hours. Thus, the LE test may better reflect pyuria than does microscopic enumeration of neutrophils when the interval between collection and processing cannot be controlled.

A useful option to offer clinicians is that of performing a urine culture only if the LE test is positive (i.e., if an

inflammatory process is present). This approach is obviously not appropriate in the few situations in which asymptomatic bacteriuria is of concern or if the patient has neutropenia. The option to order a urine culture regardless of documented pyuria should be offered if the patient has the symptoms of an acute urinary tract infection.

Infections of the Genital Tract

The genital tract consists of external and internal genitalia in both sexes. In males, the

internal genitalia include the testes, epididymis, seminal vesicles and urethra (the prostate was discussed above). In the female, the internal genitalia are ovaries, fallopian tubes, uterus (primarily endometrium), uterine cervix, and the vagina with its accessory glands. The external genitalia consist of the penis and the labia.

Infections, for simplicity, can be divided conceptually into sexually transmitted infections, peripartum infections and vaginitis. The commensal microbiota and common pathogens of the genital tract are summarized in Table 2-6.

Sexually Transmitted Infections[47]

Urethritis and Cervicitis. The one of the most common sexually transmitted infection is caused by *C. trachomatis*, which causes urethritis and cervicitis. In men the symptoms primarily relate to urethritis (i.e., pain on urination and urethral discharge). Asymptomatic infections occur in men, but are less common than in women. In women, a distinctive mucopurulent cervicitis is a common manifestation of infection, in addition to acute urethritis.[36] The most serious complications of these infections is pelvic inflammatory disease, which can produce inflammatory scarring of the fallopian tubes that leads to infertility and ectopic pregnancies. This is especially problematic with *C. trachomatis* infection, which may be asymptomatic; therefore, screening of sexually active women is necessary to prevent these serious complications.[46] *N. gonorrhoeae* produces an infection that is similar to that caused by *C. trachomatis* in both men and women. Therefore many of the molecular diagnostic tests simultaneously detect and differentiate both these pathogens. It is recommended that when one of these infections is considered that tests for both are performed.

Mycoplasma hominis and *Ureaplasma urealyticum* are two causes of nongonococcal urethritis. Other *Mycoplasma* species may also cause disease. These organisms require special culture conditions. Newer diagnostic assays, including nucleic acid amplification assays that target these organisms, are now available through reference laboratories.

Genital Ulcer Disease

Herpes Simplex Virus. Several infectious agents, most sexually transmitted, produce ulcerated lesions in the external and/or internal genitalia.[194] By far the most common is

herpes simplex virus. The initial infection is frequently asymptomatic,[91] and asymptomatic shedding of virus occurs in a small percentage of both women and men.[312] In fact, the rate of viral shedding is very similar, whether a clinically evident

or an asymptomatic infection has occurred.[313] Clinical disease is most frequently accompanied by vesicular lesions with an erythematous base on the glans penis, vulva, perineum, buttocks, or cervix. The vesicles are painful, may ulcerate, and may be accompanied by systemic symptoms—fever, malaise, anorexia, and tender bilateral inguinal adenopathy.

TABLE 2-6 Commensal Microbiota and Selected Etiologic Agents in the Genital Tract

Anatomic Site	Commensal Microbiota	Sexually Transmitted Disease (STD) Etiologies	Non-STD Etiologies
Urethra	*Enterobacteriaceae*, α/γ-streptococci, Enterococcus spp., diphtheroids, coagulase-negative *Staphylococcus*, anaerobes (distal 1–2 cm)	*Chlamydia trachomatis* *Neisseria gonorrhoeae*	
External genitalia and perineal skin	Diphtheroids, coagulase-negative staphylococci, *Micrococcus* and *Kocuria* spp., yeast, *Acinetobacter* spp., *Enterobacteriaceae*	Herpes simplex virus, type 1; human papillomavirus; *Treponema pallidum; Haemophilus ducreyi; Klebsiella granulomatis* (granuloma inguinale); *Chlamydia trachomatis*, serotypes L1–L3 (lymphogranuloma venereum)	*Candida* spp.; *Streptococcus pyogenes*
Vagina	*Lactobacillus* spp., anaerobes, *Enterobacteriaceae*, α/γ-streptococci, *Enterococcus* spp., diphtheroids, coagulase-negative *Staphylococcus* (varies with age)	Human papillomavirus; *Trichomonas vaginalis*	*Candida* spp., bacterial vaginosis; *Staphylococcus aureus* (toxic-shock syndrome)
Endocervix	Normally sterile or minimally contaminated with vaginal flora	Human papillomavirus; herpes simplex virus, type 2; *Neisseria gonorrhoeae, Chlamydia trachomatis*	Cytomegalovirus
Endometrium, fallopian tube, ovaries	Normally sterile	*Neisseria gonorrhoeae, Chlamydia trachomatis*	Mixed aerobic-anaerobic infection (ascending); *Streptococcus pyogenes, Listeria monocytogenes, Streptococcus agalactiae; Actinomyces israelii* (in patients with intrauterine contraceptive devices)
Systemic infections with genital portal of entry		Human immunodeficiency virus (HIV), hepatitis B virus, hepatitis C virus	

Herpes simplex virus has two serotypes, which produce infections that differ both epidemiologically and clinically. Type 1 virus more commonly produces initial infections in infants, children and adolescents. Although traditionally infections caused by this virus are primarily in the upper half of the body, approximately one-third of genital infections are caused by this serotype. Conversely, type 2 virus is more commonly associated with sexual activity and with genital lesion, but infection of the oral and nasal mucosa may occur. The relative prevalence of the two serotypes varies geographically.[273] Infection with type 2 virus is increasing in the United States.[148] The presence or absence of symptoms after infection with type 2 virus is modified by preexisting antibody to type 1 virus[340] and may be influenced by characteristics of the infecting strain.

Herpesvirus establishes latency in the spinal ganglia after a primary infection.

Reactivation of genital herpes infection is more common and illness is more severe if the infecting strain is type 2 than if it is type 1. Recurrences decrease in frequency over time with both types of viruses.[19]

Other Genital Ulcers. In addition to herpes simplex, other genital ulcer syndromes include syphilis, chancroid (caused by *Haemophilus ducreyi*)[240]; lymphogranuloma venereum (caused by L1, L2, and L3 serotypes of *C. trachomatis*)[257]; granuloma inguinale (caused by *Klebsiella granulomatis*) and trauma.[240] The chancres of syphilis differ from those of herpes in that they are painless and have indurated margins with a clean base.[269] Chancroid ulcers, in contrast to syphilitic chancres, are painful and, in contrast to herpetic ulcers, do not have indurated margins. The primary pustule of lymphogranuloma venereum may resemble herpes simplex; however, this condition is usually recognizable by the massive bilateral necrotizing inguinal adenopathy. The primary lesion of granuloma inguinale is a subcutaneous nodule that erodes the surface, from which a beefy red, painless, elevated granulomatous lesion develops.

Although these other agents of genital ulcers are relatively uncommon in most areas of the United States, they are unfortunately increasing in frequency. In particular, syphilis[48] and chancroid[194,195] must be considered in the differential diagnosis in certain geographic areas and in certain patient populations. Chancroid, in particular, may be underreported.[254]

Interactions Between Genital Ulcers and Human Immunodeficiency Virus (HIV) Infection. Patients with genital ulcers of several etiologies are at increased risk for acquiring HIV. In fact, even nonulcerative sexually transmitted diseases, such as trichomoniasis, alter the mucosa and render the infected individual at increased risk for contracting HIV. Additionally, men who are infected with HIV shed type 2 herpes simplex virus more frequently than non–HIV-infected men (even if they have sex with men)[251] and are also quite likely more efficient at shedding HIV in the genital tract if they have ulcerative herpetic lesions.[250]

Venereal Warts. Human papilloma viruses (HPVs) produce exophytic excrescences in squamous epithelium of the skin (i.e., common warts and plantar warts) and mucosal surfaces of the respiratory and genital tracts.[114] Symptomatic anogenital warts (i.e., condyloma acuminata) are usually caused by genotypes 6 and 11. Condyloma acuminata are cauliflower-like excrescences that occur on moist mucosal surfaces, most commonly the vulva, vagina, penis, and anus. Keratotic and smooth pustular lesions occur on dry skin, while asymptomatic "flat warts" may be found in either smooth or dry areas. Untreated infections usually regress spontaneously, but latent or subclinical infection is probably common. The more serious manifestation of HPV infection is the cellular dysplasia and neoplasia that is produced by certain high-risk genotypes, particularly types 16 and 18.[23] Molecular testing for the detection of high-

risk HPV genotypes is now routine, with several excellent FDA-approved methods from which to choose. In addition, two HPV vaccines have been released that target certain high-risk HPV subtypes and are recommended for preteens.

Trichomonas vaginalis. *T. vaginalis* is a sexually transmitted protozoan parasite that infects both men and women. Many men and a substantial number of infected women are asymptomatic. Infection with this parasite classically produces a copious, frothy yellow or yellow-green discharge that collects in the posterior vaginal fornix. Traditionally, the direct microscopic examination to demonstrate the motile organisms has been performed in the physician's office. However, given the need for documentation concerning compliance standards surrounding provider performed microscopy, many physicians choose to submit the specimen to the laboratory. Because motility is lost relatively rapidly, enzyme immunoassays and, more recently, nucleic acid amplification assays have been approved for the detection of this parasite. The latter is particularly important in high-risk individual who are asymptomatic, as it can detect subclinical infections.

Genital Infections Transmitted by Nonsexual Means

Vaginitis and Vaginosis. Vaginitis is caused by a limited number of infectious agents, but noninfectious irritants can also cause inflammation, especially if the vaginal mucosa is atrophic.[276] The disease often involves the vulva (vulvovaginitis). Mixed infections may also occur. Classic presentations for candidiasis and bacterial vaginosis are described below, whereas trichomoniasis was described above. Unfortunately, the symptoms overlap sufficiently that it may not be possible to make a definitive diagnosis clinically.[249] In such instances, it is necessary to document the etiologic agent microbiologically. As many as half of vaginal infections have been reported as not having a demonstrable etiology; this, however, may change as more sensitive methods are employed.[249]

Candidiasis. The discharge in candidiasis is typically more thick and curd-like, and the vaginal mucosa tends to be erythematous. The most common agent is *C. albicans*, but

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other species of *Candida* and even other genera can infect the vagina on occasion.

Bacterial Vaginosis. *Gardnerella vaginalis*, initially thought to be associated with bacterial vaginosis, actually works synergistically with anaerobic bacteria, such as *Mobiluncus* to produce the characteristic malodorous discharge.[280] The recovery of *G. vaginalis* in the absence of mixed anaerobic microbiota and symptoms of bacterial

vaginosis probably constitutes normal vaginal microbiota. It has been reported[78] that bacterial vaginosis (defined by clinical criteria) was not present in 55% of women from whom *G. vaginalis* was isolated. Therefore, culturing for *G. vaginalis* is not recommended because of the poor predictive value of this test. The importance of recognizing bacterial vaginosis clinically and establishing a laboratory diagnosis is emphasized.[122] In a study of 49 women with preterm labor, out of a subset of 12 who had concomitant bacterial vaginosis, 8 (67%) had a 2.1-fold increased risk for preterm birth before 37 weeks of gestation. Bacterial vaginosis was also associated with low birth weight. In the laboratory, bacterial vaginosis is best assessed through the use of the "scored Gram's stain," although newer, molecular methods are now available.

Infections of the Upper Female Genital Tract. These infections result from entry of vaginal microbiota into the upper genital tract.[45,121] The etiologic agents are, therefore, a mixture of aerobic and anaerobic bacteria. *A. israelii,* for example, has been shown to cause endometritis in women using intrauterine contraceptive devices.[75] The etiology is thought to involve the formation of a calcium carbonate nidus on the device, in which the *Actinomyces* species grow. In these infections, if *A. israelii* is present, the infections are more chronic and recalcitrant than if this particular organism is absent.

In the postpartum period, infection is a common cause of fever and even sepsis (i.e., puerperal infections).[84] The most devastating of these infections are caused by *C. perfringens*, typically after an illegal abortion with nonsterile instruments, and *S. pyogenes*, the classic cause of puerperal fever. One of the milestones of epidemiology and infection control was the study of puerperal fever by Semmelweis, who proved that the infection was carried to pregnant patients from the autopsy room by physicians who did not wash their hands.[132] The medical staff responded by ostracizing him; over a hundred years later, adequate handwashing remains a challenge for infection prevention.

Systemic Complications of Genital Infections

Infections in many organ systems result in organisms entering the bloodstream, resulting in disseminated infection and distant metastatic disease. In women, genital infectious agents may also be spread through the peritoneal cavity via the fallopian tubes, and toxins may produce extragenital effects after being absorbed through the mucosa. Dissemination of pathogens through the bloodstream occurs with *N. gonorrhoeae*, *Treponema pallidum*, and any of the agents of upper genital tract infection. *C. trachomatis* and *N. gonorrhoeae* produce a perihepatitis, known as Fitz–Hugh–Curtis syndrome.[146,337] *H. ducreyi*[239] and the lymphogranuloma venereum serotypes of *C. trachomatis*[138] spread to regional lymph nodes, where they produce

swollen, painful suppurative lesions. In addition, the granulomas of lymphogranuloma venereum may also be found in the rectal mucosa in individuals who practice receptive anal intercourse.[227] Granuloma inguinale, also known as donovanosis, does not produce regional lymphadenopathy,[14] but some variants of the infection may produce scarring that can result in lymphatic blockage, lymphedema and even elephantiasis of the external genitals.[259] Rarely, extragenital donovanosis can occur in any part of the body.[229] Finally, herpes simplex virus migrates through the peripheral nerves to the sacral ganglia, as mentioned previously. Reactivation of the virus can result in aseptic meningitis as well as recurrent genital disease.

Genital infections can also affect the fetus and the newborn infant. Some infections, primarily viral, will cross the placenta and infect the developing fetus.[225] These congenital infections include the rubella virus, cytomegalovirus, parvovirus, varicella zoster virus, and HIV, as well as *T. gondii* and *T. pallidum*. Other agents infect the neonate as it traverses the vagina (or in utero if an ascending infection causes chorioamnionitis). These infectious agents, mainly bacterial, include *N. gonorrhoeae* (conjunctivitis); *C. trachomatis* (conjunctivitis and neonatal pneumonia); and agents of neonatal sepsis, prominently herpes simplex virus,[147] *Streptococcus agalactiae*, *E. coli,* and *Listeria monocytogenes*.[123]

Diagnosis of Genital Tract Infections

Diagnosis of Urethritis, Cervicitis, and Vaginitis

Urethritis and Cervicitis. The traditional approach to diagnosis of gonococcal urethritis in men is to examine a Gram-stained preparation of the smear for the presence of intracellular gram-negative, biscuit-shaped diplococci. This finding alone in the discharge from women is insufficient for diagnosis, as nongonococcal *Neisseria* species are part of the normal vaginal microbiota. Although the presenting symptom in females may be a urethral discharge, clinical manifestations are usually more complex, and varying degrees of exudative cervicitis, vaginitis, salpingitis, and pelvic inflammatory disease may be present. Gram's stain is, therefore, recommended only for males with urethral discharge.[135]

Definitive diagnosis requires either the recovery of the etiologic agent in culture or demonstration of specific bacterial nucleic acid. The latter is the current reference standard as the molecular detection of *N. gonorrhoeae* and *C. trachomatis* is more sensitive than culture.[135] If sexual abuse is under consideration or if medicolegal questions are likely to arise, culture (with chain-of-custody documentation of the specimen) may be necessary; consultation with local authorities in such instances is recommended. Detection of antigen has been superseded by molecular approaches and should no longer be used.

Vaginitis. The quickest and least expensive method for diagnosis of vaginal infections is a wet preparation of vaginal secretions. It is possible to detect yeast of *Candida* spp., motile *T. vaginalis* (only if the specimen is examined promptly), and the clue cells of bacterial vaginosis by examining a drop of unstained secretions microscopically.

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Bacterial vaginosis has been diagnosed by several means. Elevated levels of vaginal fluid sialidase activity, probably derived from enzymatic activity of *Bacteroides* species and *Prevotella* species,[30] and an elevation of vaginal fluid pH above 4.5 in conjunction with high levels of *G. vaginalis* (as determined by a specific DNA probe)[57,264] were other markers useful in confirming the diagnosis of bacterial vaginosis. Cook and associates[57] found that a persistent elevated pH and high polyamine and fatty acid levels in vaginal secretions along with clue cells in small numbers were valuable residual abnormalities predicting recurrence of bacterial vaginosis. Clue cells are vaginal epithelial cells that are covered with non-*Lactobacillus* bacteria, causing a change in the refractile index of the cells; they are a result of the altered bacterial microbiota in bacterial vaginosis. False-positive rates as high as 18.5% have been reported because other bacteria can also attach to epithelial cells. A 10% false-negative rate has been reported owing to inhibition of bacterial attachment by IgA. However, when the clue cell test is combined with the amine production or "whiff" test, the predictive negative value of 99% provides a good screen for the absence of bacterial vaginosis. Amine production is detected by mixing equal volumes of genital fluid and 10% KOH; perception of a fishy odor is a positive result.[179]

Microscopic examination for clue cells can be performed with a wet mount or a stained preparation, using the Papanicolaou (Pap) or Gram's stains. With Gram's stain the characteristic shift from predominantly gram-positive bacilli (*Lactobacillus* spp.) to mixed microbiota is also useful for making the diagnosis.[187] The Nugent scoring method is the most widely adopted means of scoring the Gram's stain for bacterial vaginosis.[212]As discussed previously, culture is not useful for diagnosis of vaginosis. There are newer molecular methods for bacterial vaginosis that have been or are soon to be released that warrant review.

If it is not possible to promptly examine a specimen from a symptomatic patient for the presence of *T. vaginalis*, there is a convenient and sensitive method for the transport of *T. vaginalis*. The InPouch TV Test (Biomed Diagnostics, White City, OR) consists of nutrient medium in a plastic pouch. After introduction of vaginal secretions, the pouch is resealed. It can then be examined immediately as a wet

preparation and/or incubated for subsequent periodic observation of motile trophozoites. Although less sensitive than culture or a wet preparation (in which the motility of the parasites facilitates detection), trichomonads can be visualized with Gram's stain or the Pap stain.

T. vaginalis may be submitted in Diamond's medium for culture, but this is not commonly performed and usually reserved for patients with refractory disease wherein an isolate will be needed to send for susceptibility testing. More recently, an capillary flow enzyme immunoassay (i.e., OSOM, Sekisui Diagnostics, Framingham, MA) became available, which is FDA cleared and CLIA waived for use in physician's offices; it reportedly has 82% to 95% sensitivity and a 97% to 100% specificity.[42,128]Additionally, molecular methods are now currently available and FDA cleared for use in the laboratory. Of these, the APTIMA *T. vaginalis* assay (Hologic, San Diego, CA) has the highest sensitivity and specificity, which are both between 95% and 100%. Laboratory-developed assays, with similarly high sensitivities and specificities are available through commercial reference laboratories. The high sensitivities of these nucleic acid amplification assays afford the detection of subclinical disease, which is important for early treatment and the prevention of disease transmission in high-risk individuals.

The direct examination (i.e., KOH/calcofluor white or Gram's stain) is usually sufficient to demonstrate the overabundance of yeast in women with *Candida* vulvovaginitis. Culture for yeasts has a limited role in diagnosis. In patients with recalcitrant or recurrent infections, fungal culture may be used to identify the type of yeast present and perform susceptibility testing.

Diagnosis of Genital Ulcer Disease and Venereal Warts. Diagnosis of genital ulcer disease may be by direct microscopy, immunofluorescence microscopy, molecular methods or culture, depending on the organism and the clinical situation. Herpetic ulcers may be sampled for viral culture or for demonstration of characteristic viral cytopathology. Herpes simplex virus produces multinucleated giant cells with intranuclear inclusions. They can be visualized after staining with Wright's stain (or Wright–Giemsa stain), hematoxylin–eosin stain, or the Pap stain. Alternatively, immunofluorescence or immunoperoxidase reagents can be used to stain viral antigen in epithelial cells or demonstrate the antigen in an enzyme immunoassay.[287] More recently has been the release of several FDA-approved nucleic acid amplification assays, most of which both detect and differentiate herpes simplex virus 1 and 2 from lesions.

The Donovan bodies of granuloma inguinale can be visualized (with difficulty) by staining the intracellular bacteria in mononuclear cells, using Wright's stain (or an equivalent stain), hematoxylin–eosin (used for primary histologic study of biopsies), or the Pap stain.[65,241] The classic culture technique for the diagnosis of chancroid used

rabbit blood agar, which is not available in most microbiology laboratories.[113] Fortunately, *H. ducreyi* can be cultivated on a variant of the enriched chocolate agar used for *N. gonorrhoeae*.[63,113] If the modified media are not available, enriched chocolate agar may be substituted. The presence of hemin (i.e., X factor) is needed for the growth of this organism. *H. ducreyi* is labile and does not survive well in transport. If standard transport media, such as Amies media, are used and the specimen is refrigerated, the bacteria can be recovered with acceptable accuracy.[64] Lymphogranuloma venereum is usually diagnosed serologically, but the agent can be cultivated if material from a suppurative regional lymph node is cultured for *C. trachomatis*.[257] None of the FDA-approved assays are cleared for this specimen type, so validation studies would be necessary to offer this type of testing, which is difficult given the low prevalence of infection in most settings.

The diagnosis of primary syphilis was classically made by darkfield microscopy of scrapings from the ulcer base.[157] The scrapings must be examined immediately, because visualization depends on having live, motile treponemes. This, however, is not commonly performed. Immunofluorescence reagents that react with *T. pallidum* have been available from the Centers for Disease Control and Prevention, but their continued availability is unsure. With these reagents a dried smear can be stained after transport to the laboratory.[130] Serologic diagnosis is the mainstay of diagnosis, the algorithm

of which has undergone significant changes. In brief, these changes allow for initial testing to be performed using treponemal assay (i.e., screening for *T. pallidum* IgG) followed by the use of the nontreponemal test (i.e., rapid plasma reagin [RPR]) on positive specimens. This change in the order of testing (i.e., traditionally nontreponemal tests have been performed first) affords higher throughput testing on automated instrument, which allows for the "screening out" of many specimens in a highly efficient manner.

Diagnosis of venereal warts must be made by molecular means because human papillomaviruses do not grow in cell culture and serologic responses are not dependable. The test for "low-risk" subtypes, which was performed in the past, is no longer recommended.[114] There are now three FDA-approved platforms for the detection of high-risk subtypes. One utilizes signal amplification technology, whereas the other two employ nucleic acid amplification. One of the latter has been cleared by the FDA as a "primary screen," which may represent a substantial change in the use of this test in women's health. Although it has been cleared for this purpose, at the time of this writing the major medical societies have not yet endorsed this approach.

Collection of Urethral Specimens From Males

When urethral discharge is scant and intracellular diplococci are not seen in a random sample, collecting an early-morning specimen before urination may be helpful. Exudate may be expressed from the urethral orifice by gently applying circumferential pressure to the penis; if material is not readily obtained, the tip of a narrow-diameter cotton, rayon, or Dacron swab on a plastic or aluminum shaft may be inserted 3 to 4 cm into the anterior urethra. The swab should be left in place for a few seconds to allow the fibers to become saturated with the exudate. If a culture for *C. trachomatis* is being obtained, the swab should be rotated 360 degrees to dislodge some of the epithelial cells. Urine specimens for nucleic acid amplification tests, which offer superior sensitivity, are now most commonly performed. Occasionally, however, culture may be needed, so the specimen collection portion above has been retained. Collection kits designed for each commercial assay are supplied by the manufacturer. The first portion of the urine, which contains urethral cells and bacteria that have been washed out, is collected, in contrast to the midstream urine that is obtained for the diagnosis of cystitis. The manufacturers of kits vary in the specifics of their recommendations for culture.

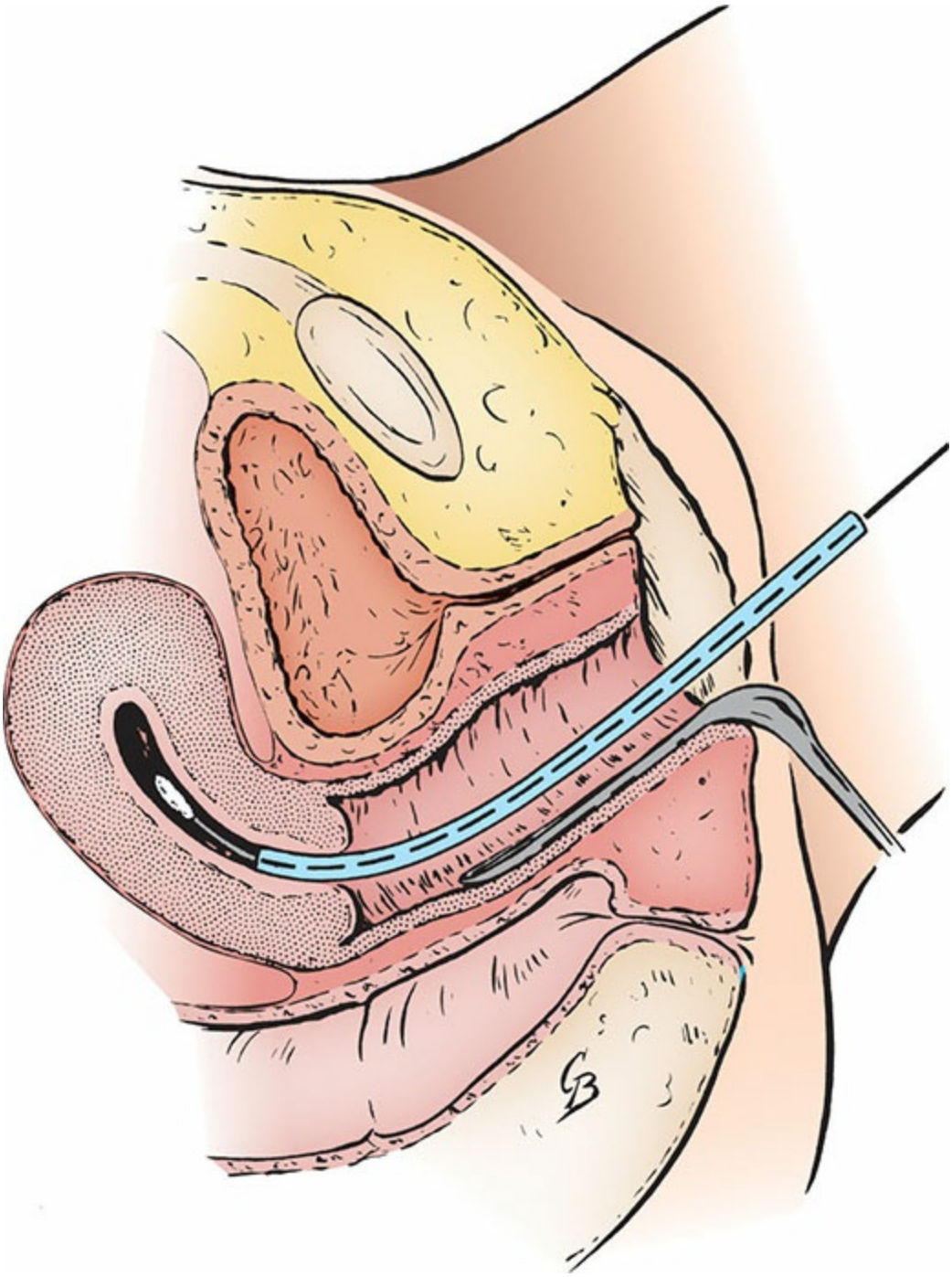
Collection of Genital Specimens From Females

In females with signs and symptoms of acute genital infection, samples are most commonly obtained from the uterine cervix. Cervical specimens are collected with the aid of a speculum after removal of the cervical mucus with a large swab. A smaller swab with a plastic shaft and a Dacron or polyester tip is recommended for obtaining the specimen.[174] The tip of the swab is inserted a few millimeters past the cervical os, rotated firmly to obtain both exudate and cervical cells, and removed, taking care not to touch the lateral walls of the vaginal canal. Urethral samples are more commonly obtained when screening asymptomatic women, and are obtained as a urine specimen. As it is for males, urine is a satisfactory specimen for diagnosis of infection in women. Although slightly less sensitive than cervical specimens, it is less invasive and may obviate the need for a speculum exam. Specific recommendations vary by manufacturer.

Vaginal secretions can be aspirated or collected with a swab. Specimens from the upper genital tract are difficult to obtain from below without contaminating the specimen with vaginal microbiota. Endometrial specimens are best obtained by inserting the swab through a narrow-bore catheter that has been introduced into the cervical canal, as illustrated in Figure 2-4. By using this technique, there is less chance of contaminating the specimen from secretions of the cervical os or the vaginal canal.[181] For specimens from the fallopian tubes and ovaries, laparoscopy or surgical approaches are necessary.

Collection of Specimens From Genital Ulcers

Ulcers should be cleaned to remove surface debris and contaminating bacteria. For darkfield examination or preparation of smears, a scalpel blade should be used to abrade the base of the ulcer and transfer the material onto a glass slide. For culture, either a washing of the base or a cotton swab may be used.[113] If intact vesicles are present, a swab may be used to collect fluid for Tzanck preparations or herpes culture. Swabs for bacterial culture may be placed in Amies' or Stuart's transport media.



■ **FIGURE 2-4** Endometrial culture technique. Through a speculum, a catheter is introduced into the cervical os and a swab extended through the catheter into the endometrial cavity. This helps prevent contamination of the swab by contact with the vaginal wall or the cervical os.

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Infections of the Bones and Joints

Clinical Presentation and Diagnosis

Infections of the bones and joints may develop after bacteremic spread of the pathogen from a distant site, by direct extension from an adjacent infection or by introduction from the environment through trauma. The most common pathogenic organism in bacteremic spread is *S. aureus*, but other microbes may also reach the skeletal system by this route, including *Borrelia burgdorferi* (Lyme disease) and agents of systemic mycosis, such as *B. dermatitidis* and *Coccidioides* spp. Infections that originate in adjacent tissue are often polymicrobial and commonly include anaerobic bacteria. Traumatic lesions reflect the environmental microbiota introduced at the time of injury.

The symptoms of septic arthritis are fever, pain, and swelling of the affected joint(s).[266] Osteomyelitis may be associated with local pain and tenderness and with restriction of movement.[163] Systemic symptoms may be present in both types of disease. If the affected bone is in the extremity, the problem is usually evident. In acute vertebral osteomyelitis, there is often point tenderness over the affected vertebra. If however, the infection is subacute, there may be nonlocalized back pain or no local symptoms at all, especially in the elderly.[61] Osteomyelitis may become chronic, whereas septic arthritis is less likely to enter a chronic phase.

The diagnosis of osteomyelitis must be accomplished by surgical biopsy of the affected bone. Multiple specimens are often submitted from these surgeries. In addition to culture, it is important to have correlative histopathology. The fluid in a septic joint may be aspirated for microscopic examination and culture. Joint fluid clots readily, so special approaches are necessary. The fluid may be inoculated directly into blood culture bottles[127,153] or into the Isolator system,[342] an effective method for recovering single pathogens. Alternatively, the fluid may be inoculated into a sterile tube that contains sodium polyanethol sulfonate (SPS), the anticoagulant commonly added to blood culture bottles; after transport to the laboratory, the unclotted fluid can be processed as usual for any other normally sterile body fluid. If clotted joint fluid is received in the laboratory, cell counts cannot be performed, but the clot can be homogenized for Gram's stain and culture. It is essential to include chocolate agar when joint fluid is cultured, because fastidious bacteria are prominent pathogens. For infections that are thought to have originated with a bacteremia, blood cultures may yield the pathogen.[61] On occasion, recovery of *S. aureus* from blood in the absence of a clear source may be the first clue to vertebral osteomyelitis.

Infections of the Central Nervous System

The most common infections of the central nervous system (CNS) are encephalitis (brain), myelitis (spinal cord), meningitis (membranous covering of the brain and spinal cord) and meningoencephalitis. When meningitis and encephalitis (meningoencephalitis) occur together, they are most commonly part of the same infectious process. Infections of the soft tissue (e.g., epidural abscess) and bone (osteomyelitis) may impinge on the nervous system. Infections of the peripheral nervous system, which include neuroborreliosis (a variant of Lyme disease), leprosy and tuberculosis, are infrequent.

Meningitis

Patients with early acute meningitis may experience an influenza-like syndrome—sore and stiff neck, headache, low-grade fever, and lethargy. In elderly, debilitated, or immunosuppressed patients, an unexpected alteration in mental status may be the only clue to infection. Various degrees of confusion, agitation, disorientation, or coma may be observed. A positive Brudzinski's sign (i.e., resistance to passive neck flexion) and Kernig's sign (i.e., inability to extend the leg when the thigh is flexed at a 90-degree angle with the trunk) are signs of meningeal irritation. Subacute or chronic meningitis caused by tuberculosis or fungal infections may present with signs of increased intracranial pressure (e.g., papilledema, nausea, and vomiting) and mental changes, such as disorientation, confusion, personality change, and stupor.[190]

The cerebrospinal fluid (CSF) is normally water-clear, has no more than five lymphocytes per milliliter, has a glucose concentration between 45 and 100 mg/dL depending on the level of blood glucose, a protein concentration between 14 and 45 mg/dL and is sterile. The classic alterations attributable to meningitis are summarized in Table 2-7. Bacterial infections, which typically are accompanied by large numbers of polymorphonuclear leukocytes, are often called septic or purulent meningitis, whereas infections without a pronounced neutrophilic response (mycobacterial, viral, and most fungal infections) are called aseptic meningitis.[56] The common lay term "spinal meningitis" is not inaccurate, but it is not informative because it is rare for only one segment of the meninges to be infected; the term is often used for meningococcal meningitis, but obviously any agent can affect the spinal membranes.

Infectious agents can get to the CNS through the bloodstream or by direct extension from adjacent structures.

TABLE 2-7 Cerebrospinal Fluid Abnormalities in Meningitis

Parameter	Bacteria	Mycobacteria	Fungi	Viruses	Exceptions
Polymorphonuclear neutrophils	High to very high	High	High	Absent to elevated early; usually absent late	May be absent in *Cryptococcus neoformans*, in the profoundly immunocompromised host
Monocytes	Variable	Variable	Variable	Usually present	
Glucose	Low	Low	Low	Normal	May be low in herpes simplex infections
Protein	High	High	High	Elevated	

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Herpesviruses and rabies virus reach the CNS by retrograde extension up peripheral nerves from spinal ganglia, but this route is unusual for other agents.

Meningitis is most prevalent in certain age groups or in patients with various underlying diseases. Meningitis in the neonate most commonly results from infection that is acquired from the mother in utero or during vaginal delivery.[6,344] *S. agalactiae* and *E. coli* are the organisms most often recovered; *L. monocytogenes*, various members of the *Enterobacteriaceae*, *Pseudomonas* spp., *Elizabethkingia meningosepticum*, *S. aureus*, and miscellaneous anaerobic bacteria are recovered less frequently. For reasons that are unknown, *Citrobacter koseri* (*diversus*) can cause a devastating meningoencephalitis in the neonate.[104] Herpes simplex virus type 2 produces meningitis in neonates after acquisition during vaginal delivery, which may progress to fully disseminated disease.[296]

N. meningitidis causes infections in all age groups. *H. influenzae* type B was the most common cause of acute bacterial meningitis in the 6-month to 5-year age group, but this infection has been virtually eradicated by an effective vaccine. Similarly, *S. pneumoniae* meningitis, which may result from bacteremia or extension of infection from adjacent sinuses or the middle ear, has been substantially reduced because of vaccination.

In adults, *S. pneumoniae* remains an important cause of bacterial meningitis. In young adults, *N. meningitidis* is also common pathogen for which a vaccine exists; this vaccine is highly recommended, but not mandated. *Listeria* is an important food-borne cause of meningitis, which preferential infection of individuals with underlying disease, such as malignancy. In the elderly, meningitis follows Shakespeare's ages of man, with the pathogens of the neonatal period making a reappearance. *E. coli* and other gram-negative bacilli are high in prevalence[190] and *S. agalactiae* is also a factor.[76]

Viral meningitis in all age groups is caused primarily by enteroviruses.[231] A large number of viruses that are transmitted by ticks and mosquitoes (arthropod-borne viruses or arboviruses) cause CNS disease uncommonly.[41] The West Nile virus has appeared in the United States since the last edition of this text and has subsequently

become endemic.[207]

C. neoformans, *M. tuberculosis* and the dimorphic fungi *H. capsulatum* and *Coccidioides* species cause indolent or chronic forms of meningitis in both intact hosts and in those with altered defenses. Recurrent aseptic meningitis (Mollaret's meningitis) is typically caused by herpes simplex virus,[295] but the diagnosis must be made by molecular means. *E. coli*, *K. pneumoniae*, and staphylococci are most commonly recovered from patients with posttraumatic or postsurgical meningitis. Approximately 50% of acute meningitis associated with ventricular shunts is caused by coagulase-negative staphylococci; other skin microbiota, such as *Corynebacterium* spp. and *Propionibacterium acnes* are also important causes.[190]

Naegleria fowleri, a free-living amoeba, produces a necrotizing meningoencephalitis termed primary amoebic meningoencephalitis, particularly in patients who swim in warm, brackish water.[172] *Acanthamoeba* spp. and *Balamuthia mandrillaris* are causes of chronic granulomatous meningoencephalitis. These parasites are addressed more fully in Appendix II.

Encephalitis and Brain Abscess

Brain abscess and encephalitis are the most serious infections of the CNS.[182,232,331] Encephalitis is most commonly caused by viruses, although it can also be produced by other agents, such as *M. pneumoniae*. The etiologic agent of most encephalitis syndromes is unknown.[100] Herpes simplex virus type 1 is the most frequently documented cause of sporadic encephalitis. The reactivated virus travels up the olfactory nerve to the temporal lobe of the brain, where it produces a necrotizing encephalitis that is distinctive because of the anatomic location. The diagnosis is unlikely in the absence of evidence of temporal lobe disease or a pleocytosis (inflammatory response) in the CSF, although exceptions have been described, particularly in children.[151] In the past, temporal lobe biopsy was required to achieve a definitive diagnosis. This practice is now very infrequent, with the diagnosis largely be achieved by herpes simplex virus PCR of the CSF.

Varicella-zoster virus also produces a localized encephalitis, but less commonly. This usually occurs in the setting of a patient with ophthalmic zoster (a recrudescent infection, usually years after chickenpox). The virus travels up the fifth cranial nerve and produces a necrotizing infection, often with vasculitis and presumably small vascular infarcts.[149] The clinical presentation can even mimic that of noninfectious stroke. This, too, is largely diagnosed by varicella zoster virus PCR of the CSF.

Other types of sporadic or epidemic encephalitis affect the brain diffusely; clinical and radiographic data are not diagnostic of a particular etiology, which must be made with serologic, microbiologic or molecular techniques. Rabies, fortunately extremely rare, is uniformly fatal if untreated; if associated with an animal bite, the diagnosis is

obvious, but contact with an animal is not always obvious and the diagnosis may not be made until autopsy.[211] Unfortunately, the rabies virus is now listed amongst the agents that can be transmitted by organ transplantation.

Anaerobic bacteria are frequently recovered from brain abscesses; anaerobic streptococci, *Prevotella melaninogenica*, *Bacteroides* spp., *Fusobacterium nucleatum*, *Eubacterium* spp., and *P. acnes* are the most commonly encountered species. The aerobic bacteria most commonly recovered include α and β-hemolytic streptococci, *S. aureus*, *S. pneumoniae* and gram-negative bacilli, both *Enterobacteriaceae* and nonfermentative organisms. *Nocardia* spp. and the certain dematiaceous fungus such as *Cladophialophora bantianum* (formerly *Xylohypha bantiana*) are particularly neurotrophic and may also produce brain abscesses.[258] A necrotizing encephalitis can result from extension of a zygomycetes fungus from a sinus into the eye and brain (i.e., rhinocerebral zygomycosis), particularly in patients with neoplastic or immunosuppressive disease and in diabetic patients who are experiencing ketoacidosis.[178]

Brain abscess may result from bacteremic spread, particularly in patients with bacterial endocarditis, wherein a single organism is usually recovered.[205] Conversely, there may be direct extension from an infection in an adjacent sinus or in the middle ear; in these instances the infection is often polymicrobial.[242] Most viridans streptococci are recovered as part of a polymicrobial infection, but one particular *Streptococcus*, known colloquially as "*Streptococcus* Milleri group," causes abscesses in several organs, including the brain, often as a sole pathogen.[54]

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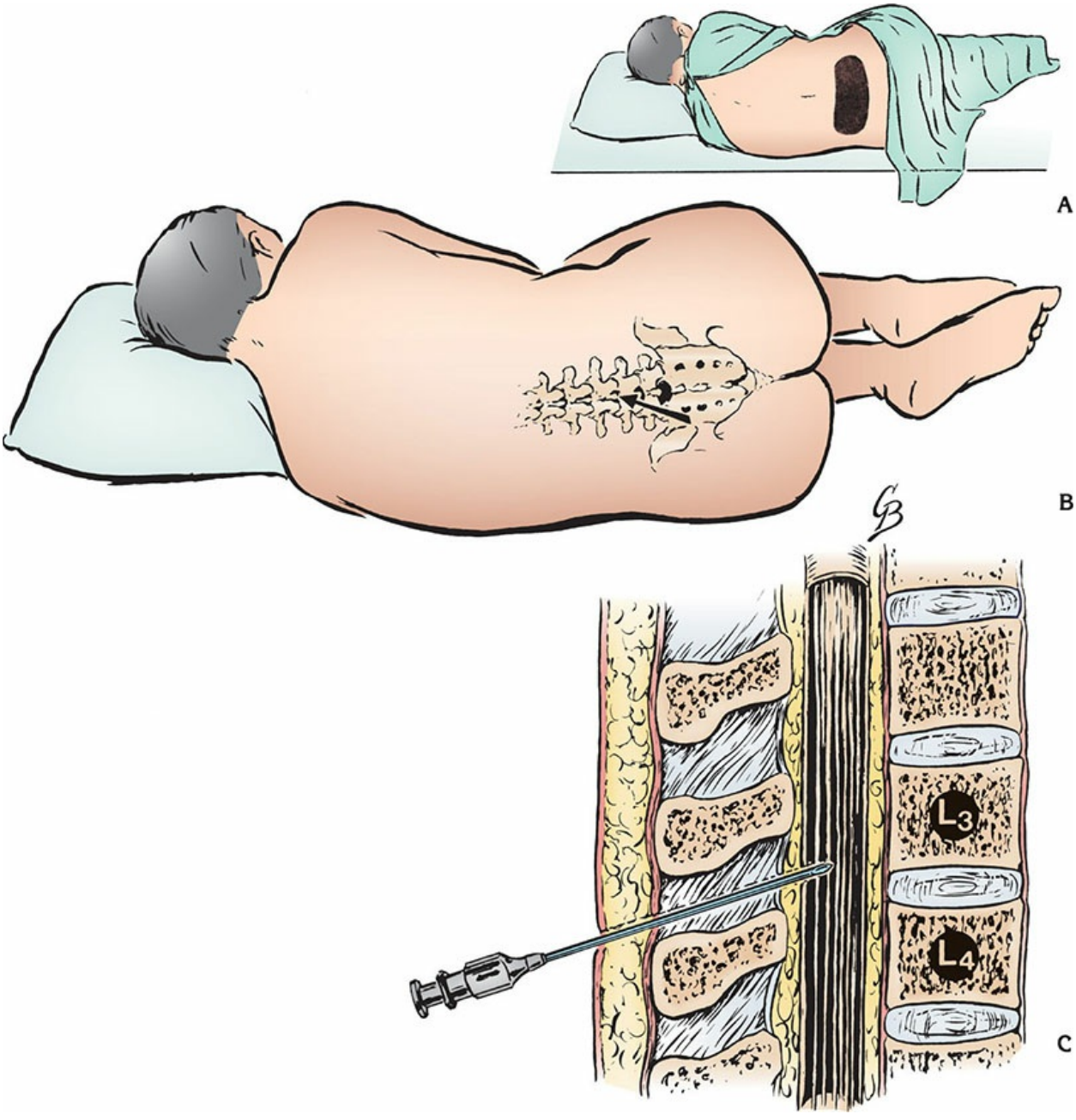
Several parasitic infections can cause necrotizing infection or a mass lesion in the brain. These include *Acanthamoeba* spp. and related free-living amoebae,[172] *Taenia solium* causing cysticerci[262] and *T. gondii*.[87] Cerebral malaria, caused by *Plasmodium falciparum*, is an often fatal complication in which the cerebral capillaries are occluded and the brain becomes hypoxemic.[116,275]

Diagnosis of Central Nervous System Infections

Collection of Specimens. CNS specimens for culture include CSF (obtained either by subdural or ventricular aspiration, or lumbar puncture), brain abscess (obtained by aspirate) and brain tissue (obtained by surgical biopsy). The spinal tap technique is illustrated in Figure 2-5.

CSF obtained from lumbar spinal puncture is the most common CNS specimen received in the laboratory. Optimally, three separate tubes of CSF fluid are submitted:

Tube 1 for cell counts and differential stains
Tube 2 for Gram's stain and culture
Tube 3 for protein and glucose, or for special studies, such as Venereal Disease Research Laboratory (VDRL; a serologic test for syphilis), cryptococcal antigen testing, or cytology, depending on the clinical situation.



■ **FIGURE 2-5** Spinal tap technique: The patient lies on his or her side, with knees flexed and back arched to separate the lumbar vertebrae. **A.** The patient is surgically draped, and an area overlying the lumbar spine is disinfected. **B.** The space between lumbar vertebrae L3

and L4 is palpated with the sterilely gloved forefinger. **C.** The spinal needle is carefully directed between the spinous processes, through the intraspinous ligaments into the spinal canal.

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Assessing the Inflammatory Response and Microscopic Techniques. An orderly approach to the processing and culture of CSF specimens should be implemented. The time from when a specimen is obtained to when it is received in the laboratory should be monitored, as should the time from receipt to result. Prolonged transport time and process-to-result time may have deleterious effects on patient care. Examining stained smears of CSF sediment is helpful both in establishing a presumptive diagnosis and in providing guidelines for the selection of culture media. Microorganisms can often be detected in Gram-stained or methylene-blue-stained CSF smears if they are present in concentrations of at least 10^4 to 10^5/mL. Some suggest that gram-negative organisms are often better observed in the methylene-blue-stained smear because the deep blue-staining cells are easier to differentiate from the background debris.[62] Safranin stains the background pink-to-red in Gram's stain, tending to obscure any pink-staining bacteria. The addition of 0.05% basic fuchsin to the safranin counterstain improves the staining of gram-negative organisms. Smalley and Bradley[271] suggest that the LE test may substitute for the performance of cell counts on body fluids suspected of harboring bacteria, but this has not become a common practice. In a study of 63 culture-positive peritoneal fluids, 85.7% also had positive LE reactions. Six of the nine culture-positive and LE-negative fluids grew only a few colonies of coagulase-negative staphylococci, isolates of questionable clinical significance. DeLozier and Auerbach[68]reported an overall sensitivity of 84.4% and a specificity of 98.1% using the dipstick LE test on spinal fluids collected from 800 patients with suspected meningitis. The sensitivity of LE in culture-proven cases of bacterial meningitis was only 73%. They concluded that the LE is an adjunct to, but not a substitute for, CSF cell count and chemistry determination in the initial laboratory assessment of bacterial meningitis. The use of antigen testing of CSF specimens for bacterial pathogens has largely been abandoned and is often discouraged. Soon to be released multiplex molecular diagnostic assays hold great promise in the more rapid detection of the agents of meningitis.

Direct Detection of Antigen and Nucleic Acid. The initial enthusiasm for the rapid diagnosis of bacterial meningitis by antigen detection has been tempered by more recent experiences.[237] When latex particle agglutination tests for *H. influenzae* type B, *S. agalactiae*, *N. meningitidis,* and *S. pneumoniae* were performed on 1,540 CSF

specimens obtained from patients with suspected acute bacterial meningitis, antigen was detected in only 27 samples.[230] The positive antigen results were helpful only in the treatment of neonates with group B streptococcal infections. These authors considered the latex test not to be cost-effective. In addition, occasional false-positive agglutination results in an unacceptably poor predictive value of a positive result. Use of these tests has declined dramatically and cannot be recommended.

In contrast, direct detection of cryptococcal antigen in the spinal fluid is almost as sensitive as culture and has often replaced the less sensitive India ink test in most laboratories. Latex agglutination and enzyme immunoassay kits have been developed. In a study of 218 CSF specimens, which included 16 retrospective and 6 prospective patients with proven cryptococcosis, 2 latex antigen kits performed with 100% sensitivity compared to culture.[339] False-positive tests occur occasionally, caused at least in part by the presence of rheumatoid-like factors in specimens or inappropriate specimen transport.[333] Although infections with *Trichosporon* are very infrequent, specimens from such patients have produced false-positive cryptococcal antigen-detection results. Treatment of the specimens with pronase decreases spurious reactions, but does not completely eliminate them.[245] The sensitivity of latex tests on serum, but not on CSF, is increased by treating the specimen with pronase.[111]

Molecular techniques have been developed for diagnosis of selected infections, primarily those caused by herpes simplex virus and enteroviruses.[248,306] These application by far exceed the sensitivity of culture-based methods for the detection of these viruses from these specimens and have become the standard of care for the detection of these pathogens from CSF. Although not yet commercially available at the time of this writing, there are multiplex PCR assays in development that target the causative agents of meningitis and meningoencephalitis. These will use platforms that are currently FDA-approved and in use for the detection of respiratory viruses and gastrointestinal pathogens.

Serologic Diagnosis. Serology is a useful diagnostic tool for confirmation of infections by many of the arboviruses, such as the West Nile virus. These are important as the period of viremia is transient and serologic results may exceed reverse transcription-polymerase chain reaction (RT-PCR) of CSF specimens in sensitivity. Detection of IgM in CSF is currently the method of choice in selected laboratories where the performance of the test is carefully controlled and monitored.[176,224] The ratio of CSF immunoglobulin to measles virus compared to serum antibody levels has been used for the diagnosis of subacute sclerosis pan encephalitis (SSPE).

Diagnosis by Culture. Culture is the diagnostic standard for bacteria and fungi. The CSF specimens from patients with chronic, often basilar, meningitis caused by *M. tuberculosis* and the dimorphic fungi, *H. capsulatum* and *Coccidioides* are often

negative. The diagnosis in these instances often relies upon the assessment of a meningeal biopsy by both culture and histopathology. Culture of viruses from these specimens is largely not performed, given the superiority of molecular methods. The most important question in meningitis is whether a bacterial agent that could be treated with antibiotics is present, so Gram's stain and bacterial culture are essential.

In preparing CSF specimens for culture, the fluid should be centrifuged to concentrate any bacteria that may be present. Centrifugation is also recommended for the recovery of *M. tuberculosis* in patients suspected of having tuberculous meningitis. A total volume of at least 6 mL, which need not be collected all at once, should be processed to increase the likelihood of recovering mycobacteria.[298] As an alternative to centrifugation, the CSF can be passed through a 0.45-γm filter (Millipore, Bedford, MA) to concentrate the microbial cells. The filters should be placed face down on the surface of the agar and moved to a new location after 24 hours of incubation. An excellent review of the approach to the laboratory diagnosis of meningitis has been published by Gray and Fedorko.[105]

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Wounds, Abscesses, and Cellulitis

Clinical Presentation

The accumulation of pus, either within an abscess, exuding from a sinus tract or from a mucocutaneous surface, is one of the cardinal indicators of a suppurative infection. Varying degrees of redness, pain, and swelling may also be present. Exogenous wound infections include those associated with traumatic injury or decubitus pressure ulcers (bedsores),[166] animal or human bites,[90,293] burns,[67,185] or foreign bodies in the skin or mucous membranes.

Endogenous wounds and abscesses may be associated with appendicitis, cholecystitis, cellulitis, dental infections, osteomyelitis, empyema, septic arthritis, sinusitis, or other internal infections. Many of these processes are nosocomial (i.e., acquired in healthcare institutions), contracted after invasive procedures, surgical manipulations or the placement of prostheses. Conversely, these may be derived from hematogenous spread of microorganisms from a primary site of infection to distant (i.e., metastatic) sites. Finally, there may be direct extension of bacteria from an adjacent site of infection or from ruptured viscera, particularly the large intestine. Anaerobic bacteria are commonly present when the infectious site is adjacent to the intestine or when the wound is contaminated with fecal microbiota.[208]

Certain bacteria are associated with particular clinical situations. For example,

Pasteurella multocida is commonly found in wounds that result from animal bites.[90,293] *P. aeruginosa* is a common pathogen when a penetrating injury of the foot occurs in patients who are wearing sneakers.[131] *P. aeruginosa*, *Candida* spp., and a variety of filamentous fungi are common causes of burn wound infections,[170] but herpes simplex virus has also been noted in this population patients.[93] Aerobic gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, strict anaerobic bacteria, and aerobic gram-negative bacilli frequently cause infections in patients with diabetes.[139]

Many wound infections and abscesses are polymicrobial, particularly those that result from fecal spillage, bedsores, and infections in diabetic patients. There is considerable dispute about the value of identifying and testing the antimicrobial susceptibility of multiple isolates, even if the specimen is a biopsy of tissue. Problems of sampling make it difficult to ensure that all pathogenic species have been recovered. A particularly instructive example of this phenomenon is a study of abscesses, in which multiple cultures of each specimen were performed over a 24-hour period; 11 of 37 total anaerobic strains were not recovered in the initial plating.[12] A case can be made, therefore, for empiric therapy based on the likely pathogens expected in a particular clinical situation, particularly in instances of polymicrobial infection.[28]

Cellulitis is a soft tissue infection that spreads through the planes of the superficial connective tissue rather than producing a mass lesion, which is what occurs when an abscess is produced. It is often possible to see the redness and feel the heat and swelling of the process by palpation of the overlying skin.[291] Cellulitis is most commonly caused by staphylococci and streptococci, but in certain situations, gram-negative bacilli and anaerobic bacteria are implicated. A deeper infection that spreads through the connective tissue (i.e., the fascia) is termed necrotizing fasciitis. This is characteristically caused by organisms such as *S. pyogenes* and *C. perfringens,* but may be caused by other bacteria. It is a rapidly progressive disease that must be treated as a medical and surgical emergency.

Diagnosis of Wound Infections, Abscesses, and Cellulitis

Collection of Specimens. Surface wounds are rapidly colonized with environmental bacteria. Therefore, if there is an underlying wound infection or associated cellulitis, a superficial specimen collected on a swab is more likely to reflect this colonizing microbiota than the true cause of the infectious process. Even if the true pathogen is recovered among the denizens of colonizing microbes, it is not possible to determine which of the many organisms recovered is the cause of disease. Therefore, a deep biopsy of tissue following curettage of the superficial aspects of the wound, or aspiration of loculated fluid/pus from the depths of wound or underlying abscesses using an approach through nearby decontaminated skin, are the most desirable

methods for collecting material to examine. The skin through which the aspiration will occur should first be decontaminated with surgical soap and 70% ethyl or isopropyl alcohol, chlorohexidine, or a similar disinfectant. In the past, the aspirating syringe was often used as the transport container, provided the needle was capped. This procedure is no longer acceptable because of the risk that a blood-borne virus will be transmitted by a needlestick. The aspirated material should be discharged into an anaerobic transport vial and promptly sent to the laboratory for processing. The syringe with the needle should not be recapped, as this also represents a needlestick hazard; rather, following the discharge of the specimen into a transport container, the needle and syringe should be discarded into an approved "sharps" container.

Aspiration of material from an area of cellulitis, with or without first injecting sterile normal saline may be attempted, but the results are usually unsatisfactory. Punch biopsies may provide more satisfactory results.[291] Regardless of whether aspiration or biopsy is attempted, these should be performed at the leading edge of the cellulitis. Blood cultures are usually not useful in this clinical situation.[219] Commonly, the therapeutic approach is defined by empiric knowledge of the pathogens expected in a given clinical situation.

Microscopic Examination of Specimens. Gram's stain should be performed on all specimens (see Chapter 1). There are several scoring systems for wound specimens that all work toward the same end (i.e., identifying those specimens wherein the culture workup will most likely demonstrate the agent of a wound infection rather than colonizing microbiota).[263] Morphologic clues to the etiology may suggest additional diagnostic procedures beyond those requested by the clinician; (e.g., anaerobic or fungal cultures). If a biopsy of tissue is received, histologic examination of hematoxylin-eosin–stained sections will disclose the type of inflammatory response, which should direct the ordering of subsequent histochemical stains for bacteria and fungi,

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and possibly immunohistochemical stains for the assessment of potential viral inclusions.

Culture. Nonselective, selective/differential and enriched nonselective media should be used to recover both eugonic and fastidious bacterial species. Anaerobic culture is appropriate if tissue or aspirated material has been obtained, but is inappropriate for surface cultures and swabs.

Quantitative microbial cultures have been advocated for culture of wounds, by some, to determine the significance of isolates, predict the likelihood of burn wound

sepsis, and determine the probability that wound healing will proceed smoothly. Quantitative culture of biopsied tissue is complex, expensive, and time-consuming; tissue must be weighed on an analytic balance, homogenized in a measured amount of broth, diluted serially, and inoculated onto multiple agar plates. These maneuvers are difficult for most laboratories to accomplish. It is clear that infections by *S. pyogenes* are clinically important, no matter what the quantity of bacteria present.[238] Currently this approach is not endorsed, with the exception of perhaps uncommon circumstances, such as burn wound infections. The problem is complicated still further by the evidence that a single biopsy of a wound will not give an accurate picture of the entire microbiota of chronic wounds.[253]

There are data that support the use of surface cultures rather than biopsies[28]; although sometimes these are unavoidable, the difficulty in the interpretation of the findings from these versus a deeper, surgically obtained biopsy remains a fact. Similarly, semiquantitative cultures (i.e., performed routinely by serial streaking of quadrants on agar plates) have provided information that is equivalent to the more difficult quantitative approaches.[40] Obviously, if no microbes are recovered, a qualitative culture provides the same information as a quantitative one.

Eye Infections

Clinical Presentation

Infectious agents may become introduced to any part of the eye, either externally or from an endogenous source. External infections usually involve the superficial structures—the conjunctiva and the cornea—unless there has been a penetrating injury that introduces microbes into the globe. Infections from endogenous sources often include seeding of the eye from microorganisms present in the blood (e.g., endocarditis) or reactivation of latent viruses or parasites (e.g., cytomegalovirus or toxoplasmosis).

Conjunctivitis. The conjunctiva is a thin membrane that covers the eyelid (palpebral conjunctiva) and reflects onto the outer surface of the eyeball, the sclera (bulbar conjunctiva). The central cornea is not covered. The conjunctiva may be infected by a variety of many microbes, most of which inhabit the upper respiratory tract. Inflammation (conjunctivitis) produces redness (pink eye), itching and a discharge, which may be mucous or purulent.[161,292] The exudates in bacterial infection are particularly thick, sticky and encrusted, so the eyelids may stick together. Acute noninfectious inflammation may also develop in patients with seasonal allergies. Conjunctivitis is highly contagious; the infection can be transferred easily to the other

eye or to other individuals by contact (e.g., rubbing the infected eye and then the normal eye).

The most common bacterial pathogens are *Staphylococcus aureus*, *H. influenzae*, *Streptococcus pneumoniae* and *P. aeruginosa*. Acute conjunctivitis can result from two sexually transmitted pathogens, *C. trachomatis* and *N. gonorrhoeae*, which can affect sexually active adults or neonates who acquire the infection during vaginal delivery. The most common viral etiology is adenovirus, which can cause epidemic disease, with or without associated pharyngitis (i.e., epidemic pharyngoconjunctival fever).

Keratitis. Keratitis, inflammation of the cornea, is a much more serious infection than conjunctivitis. Rather than the temporary discomfort of conjunctivitis, keratitis can result in scarring and blindness. It is caused by infectious agents in every class. The most common bacterial agent is *S. aureus*.[164] Filamentous molds, especially *Fusarium* spp. and *Aspergillus* spp., and yeast, most commonly *Candida* spp. produce a lesion that may resemble bacterial infection, delaying diagnosis and appropriate treatment.[150] Herpes simplex virus can cause an ulcerative lesion called dendritic keratitis because of the branching pattern of the lesions.[173] Reactivated varicella-zoster virus (shingles) can produce a similar keratitis, if it affects the ocular branch of the trigeminal (i.e., fifth cranial) nerve.[287] In people who wear contact lenses, *Acanthamoeba* spp. (a free-living amoeba) can produce ulcerative lesions that are frequently very painful (Appendix II).[172]

Interstitial keratitis, produced when blood vessels grow into the cornea from the conjunctiva, is the most common cause of blindness in the world. Trachoma is a form of chronic keratoconjunctivitis that is produced by *C. trachomatis*.[138] The scarring that can result from recurrent disease has six million people worldwide. The World Trachoma Initiative aims to eliminate this treatable infection by 2020. In parts of Africa, a filarial parasite, *Onchocerca volvulus*, which is transmitted by black flies (*Simulium* spp.), produces an intense inflammatory response when migrating worms die, resulting in river blindness.[110] Other causes of interstitial keratitis include *T. pallidum* (syphilis),[159] *Mycobacterium leprae* (leprosy), and *M. tuberculosis* (tuberculosis).

Keratitis (and blindness) can also be produced by noninfectious injury, such as trauma, ultraviolet radiation (i.e., the reason for not looking directly at the sun, particularly during an eclipse) and conditions that diminish the tears that lubricate the cornea.

Uveitis and Endophthalmitis. The most serious infections are those that affect the interior of the eye. Endogenous infections reach the eye through the bloodstream. A

variety of bacteria, fungi, and viruses may be responsible. Likely the most common cause is endocarditis, wherein the eyes, as well as various other organs, are seeded by the continuous bacteremia. Other endogenous infections include reactivation of latent viruses and parasites, such as cytomegalovirus and *T. gondii,* respectively. Exogenous infections are usually associated with penetrating trauma to the eye. A special category is postsurgical infection. An exogenous parasitic source is the infection with animal helminthic parasites, such as *Toxocara canis, T. cati,*

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or *Baylisascaris.* The infection of humans by these parasites results in visceral larva migrans, wherein the immature larvae wander since they are in the wrong host; these infections commonly involve the eyes and the CNS.

Diagnosis of Eye Infections

Collection of Specimens. Conjunctivitis is usually diagnosed with a swab of the affected conjunctiva, which can be placed in an appropriate transport medium. All other infections are appropriately collected by an ophthalmologist. Keratitis is addressed by scrapings of the affected lesion; if a bacterial or fungal etiology is suspected, material is often inoculated directly onto appropriate media by the clinician. Specimens for the diagnosis of endophthalmitis must be collected surgically.

Microscopic Examination. Depending on the pathogen suspected and quantity of specimen, Gram's stain, calcofluor white or Wright's stain (or equivalent) should be performed. If tissue is obtained surgically, appropriate stains for infectious agents should be done.

Culture. The appropriate media to inoculate depends on the clinical assessment of the most likely etiologies. Most of the commonly encountered bacterial and fungal pathogens can be cultivated on routine media. For certain pathogens, such as the agents of syphilis, leprosy, and parasitic infection, the diagnosis must be made microscopically or serologically.

Infections of the Blood

Clinical Presentation and Pathogenesis

Bacteremia and Septicemia. The suffix "-emia" refers to the circulatory system. Bacteremia, fungemia and viremia are states in which bacteria, fungi, and viruses, respectively, circulate through the vascular system. Signs and symptoms may be

present, but are not invariable. If the patient is not aware of the illness (i.e., the circulating microbes), then the condition is termed "silent" or "subclinical." In contrast, septicemia (sepsis) is a clinical syndrome characterized by fever, chills, malaise, tachycardia, hyperventilation and toxicity or prostration. Septicemia results when circulating bacteria multiply at a rate that exceeds their removal by phagocytes. The symptoms are produced by microbial toxins and/or cytokines produced by inflammatory cells.[217] It now appears that the immunostimulation of cytokines is followed by a series of important immunosuppressive events.[125] Failure of multiple organs is an important component of fatal sepsis, but the pathogenetic mechanisms that lead to death remain unclear.

Sepsis is traditionally associated with gram-negative bacteria, which contain endotoxin.[184] It is now well appreciated, however, that gram-positive bacteria and fungi can also cause the sepsis syndrome.[5,82]

Types of Bacteremia. Bacteremia may be transient, intermittent or continuous, reflecting several mechanisms by which bacteria enter the bloodstream. Transient bacteremia occurs when organisms, often members of the normal microbiota, are introduced into the blood by minimal trauma to membranes (e.g., brushing of teeth, straining during bowel movements or medical procedures[160]). Intermittent bacteremia occurs when bacteria from an infected site are periodically released into the blood from extravascular abscesses, spreading cellulitis or infections of body cavities, such as empyema, peritonitis or septic arthritis. Continuous bacteremia usually occurs when the infection is intravascular, such as infected endothelium (bacterial endocarditis or aneurysms) or infected hardware (arteriovenous fistulas, intraarterial catheters, or indwelling cannulas). The source of organisms may not be determined, however, in up to one-third of bacteremias.

Bacteremia may result from an infection in an organ or tissue (secondary bacteremia). Often, however, the primary site is not evident (primary bacteremia). In this situation it is possible that a transient bacteremia was not efficiently cleared by host defense mechanisms. At least in the case of *S. aureus,* it has been shown that colonizing bacteria in the nose may be the source of the systemic infection.[309] The factors that trigger dissemination from the anterior nares remain unclear, but simple mechanical spread to the skin and subsequent infection of wounds or intravascular devices is one possibility.

When an infection in an organ spills over into the blood (e.g., bacteremic pneumococcal pneumonia), the severity of the infection is often increased and the prognosis for the patient worsened.[204] Conversely, bacteremia can result in disseminated infection in distant organisms, a phenomenon that is termed "metastatic infection."

Bacteremia can also be classified as community-acquired or nosocomial. It can occur in immunocompetent or immunosuppressed hosts. The types of organisms and the prognosis of the resulting infection vary greatly depending on these factors, as well as on the age of the patient.[88,168,191,268]

A variety of bacteria have been recovered from the bloodstream, both gram-positive[221] and gram-negative.[71] Over the past several decades, there has been a clear shift in the nature of the infecting microbiota. The number of anaerobic isolates has decreased over that time, whereas the number of isolates of yeast and clinically significant coagulase-negative staphylococci has increased.[329] For reasons that are unclear, bacteremias with nonfermenting gram-negative bacilli are more often polyclonal (more than one molecular type) than are bacteremias with other gram-negative bacilli.[330] Changes in presurgical prophylaxis protocols and the use of intravascular access lines have likely had an impact in this area.

Some specific microbes have distinct clinical significance. *Clostridium septicum* is frequently associated with neoplastic disease, particularly carcinoma of the colon,[152] and may result in distant metastatic abscesses. Similarly, bacteremic *Streptococcus bovis* is commonly associated with endocarditis and with colonic disease, including carcinoma of the colon.[17] Rarely, episodes of *C. perfringens* bacteremia result in sudden, dramatic hemolysis, which can be rapidly fatal[302]; the hemolysis is caused by clostridial toxins, but it is unclear why the fatal hemolysis occurs only in a small proportion of clostridial bacteremias.

Risk Factors and Prognosis. Several mechanisms play a role in the removal of microorganisms from the bloodstream.

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In healthy and immunocompetent hosts, a sudden influx of bacteria is usually cleared from the blood within 30 to 45 minutes. The liver and spleen play the primary role in clearing bacteria; intravascular neutrophils play only a minor role. Encapsulated bacteria are more difficult to eliminate, but specific antibodies (opsonins) enhance clearance.[34] Not surprisingly patients that have been splenectomized are at increased risk for infections by these encapsulated bacteria. Patients who are debilitated, immunodeficient or immunocompromised are at increased risk because circulating bacteria may not be cleared from the blood for hours.

Other risk factors have been investigated by Weinstein and associates.[326] In their study of 500 episodes of bacteremia and fungemia, the overall mortality rate was 42%, with approximately half of these deaths attributed directly to septicemia. Risk factors and the relative mortality rates for each are shown in Table 2-8. Bryan[37] also emphasizes how positive blood cultures identify a population of patients at high risk of

death; patients with positive blood cultures were 12 times more likely to die during hospitalization than those with negative blood cultures. From these experiences, it is imperative that the laboratory perform blood cultures correctly and report accurate results as soon as possible.

The timely detection of bacteremia and fungemia, followed by expeditious identification of pathogens and determination of susceptibility to antimicrobial agents can have great diagnostic and prognostic importance. The mortality rate from septicemia may be 40% or higher in certain populations of hospitalized patients.[326] Although underlying diseases are important determinants of fatal outcomes, approximately half of the deaths can be attributed directly to the infection.[37] Prompt initiation of appropriate antimicrobial therapy is demonstrably important for preventing morbidity and mortality.[167] Initial therapy must be empirically based on likely pathogens and typical patterns of antimicrobial susceptibility. The microbiology laboratory plays its most important role when the actual pathogen and/or the antimicrobial susceptibility deviate from that predicted by the clinician (i.e., the organism is not covered by empiric therapy). The fatality rate for patients who are treated appropriately is significantly less than that for patients who receive ineffective antibiotics.[329] Prompt correction of the empirically chosen course of action is dependent on speedy provision of data by the laboratory. This is more appropriate than ever with the introduction of FDA-cleared molecular diagnostic products that can rapidly and accurately achieve both a genus- and species-level identification, as well as detect key genetic determinants of resistance. Additionally, the application of MALDI-TOF affords the rapid and accurate identification of most bacterial pathogens. In addition to all the important patient care reasons for implementing these technologies, a financial case can also be made for expeditious provision of results.[18] Any activity that reduces complications and shortens hospital stay will result in major financial savings for the institution as well as improved care for the patient.

Intravascular Infection. The most common intravascular infection is endocarditis, infection of the endothelial lining of the heart.[16,247,320] The earlier division of illness into acute and subacute forms is no longer considered particularly useful. Although almost any organism can produce endocarditis on occasion, the majority of infecting organisms are gram positive. Most important among these are the viridans streptococci of the oral cavity and *S. aureus*. Patients are most at risk if they have endocardial abnormalities or previous valve surgery.[77] Endocardial damage was formerly most commonly the result of rheumatic fever. With the virtual disappearance of that disease, congenital or developmental abnormalities, such as bicuspid aortic valves and prolapsed mitral valves, have assumed greater importance. Fibrin-platelet thrombi on the surface of abraded endocardium serve as site for bacterial

adherence for those organisms that transiently circulate in all of us (e.g., after brushing our teeth). It has been shown that certain microbes, particularly streptococci and enterococci, have an enhanced ability to adhere to these thrombi.[60] The thrombi and associated bacteria form excrescences (i.e., vegetations), which can be visualized by radiographic or echographic techniques. Uninfected and infected thrombi cause marantic and infective endocarditis, respectively.

TABLE 2-8 Mortality Rates and Risk Factors Associated With Bacteremia[326]

Condition	Mortality (%)	Relative Risk of Death
Age of Patient		
20	13.8	1.00
21–40	32.8	2.33
41–50	42.9	3.06
>50	49.8	3.55
Type of Organism		
Nonfermenters (*Pseudomonas aeruginosa*)	27.7	6.84
Enterobacteriaceae		
Escherichia coli	35.5	3.3
Klebsiella pneumoniae	48.0	4.52
Gram-positive cocci		
Staphylococcus aureus	32.7	3.08
Streptococcus pneumoniae	22.0	2.08
Enterococci	45.5	4.28
Unimicrobial bacteremia	37.7	
Polymicrobial bacteremia	63.0	5.96

Fungi	67.7	
Source of Infection		
Intravenous catheter	1.1	1.00
Genitourinary	14.9	1.35
Foley catheter	37.8	3.38
Surgical wound (and burns)	42.9	3.88
Abscess	51.2	4.65
Respiratory infection	52.3	4.73
Predisposing Conditions		
Surgery	16.3	0.78
Trauma	27.3	1.30
Diabetes mellitus	30.0	1.43
Corticosteroids	33.3	1.59
Renal failure	37.5	1.79
Neoplasm	42.1	2.01
Cirrhosis	71.5	3.40

Although gram-positive bacteria are the most common etiologic agents of endocarditis, some fastidious gram-negative bacilli[33,81] and some fungi[4,192] can produce the infection. Somewhat more fastidious gram-negative bacteria, such as *Aggregatibacter aphrophilus*, *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Cardiobacterium* spp. and *Kingella kingae*, are known to cause endocarditis. In the past, special culture techniques were needed for these, but the advanced blood culture systems currently in use are adequate for recovery of these bacteria. The detection of endocarditis caused by *P. acnes* may require extended incubation. Fungi and some of these gram-negative bacilli are notable for their propensity to form large vegetations that can break off and travel through the bloodstream to distant sites (septic emboli). Most instances of endocarditis involve the left side of the heart, which is the high-pressure side of the system. If, however, bacteria are directly injected into the venous system, as occurs with illicit intravenous

injection of drugs, right-sided endocarditis, often caused by a *Bacillus* spp. *Candida* spp. or *P. aeruginosa*, may result.[332]

In a minority of patients with signs and symptoms of endocarditis, it is difficult or impossible to isolate an etiologic agent (i.e., culture-negative endocarditis). Diseases that cause marantic endocarditis may be responsible, but certain microbes are not readily recovered by conventional blood culture techniques. Prominent among these agents are *C. pneumoniae*, *Coxiella burnetii* (Q fever), and *Bartonella* spp.[32,96,183,303] The identity of the etiologic agent is usually achieved using serologic or molecular methods.

Patients who have recently implanted intravascular hardware are usually infected with bacteria that are indigenous to the skin, most commonly coagulase-negative staphylococci and diphtheroids, or, less commonly, microorganisms that cause wound infection, such as gram-negative bacilli, *S. aureus* or fungi.[223] Prosthetic-valve endocarditis occurs in 3% to 6% of patients who receive either mechanical or bioprosthetic (i.e., valves made from animal tissues) grafts[310]; the infections can be divided conceptually into early (<60 days after surgery) and late stages. In the early stage, the skin and wound microbes predominate. In late stages, the organisms that infect native valves are found.

The most serious complications of endocarditis are rupture of a cardiac valve with resultant heart failure and metastatic disease caused by embolization of pieces of the infected vegetation. Sudden cardiac decompensation is a particular problem in endocarditis caused by *S. aureus*, which may represent a surgical emergency. Renal failure and strokes can result from septic emboli to the kidney and brain, respectively.

Catheter-Associated Bacteremia and Sepsis. A special kind of intravascular infection has been the result of technologic advances that allow the life-saving use of indwelling vascular catheters. Catheters may simply be the portal of entry for bacteria that colonize the skin adjacent to the entry point or they may serve as a foreign body that harbors microbial microcolonies. Sepsis and serious metastatic disease may result from catheter-related infections.[228] In patients with neoplastic disease, intravascular catheters may be in place for prolonged periods; this group of patients, who may have profound neutropenia, are at great risk for serious and life-threatening infection.[80] In addition to the effects on quality of care and quality of life, catheter-related infections put a major financial burden on institutions.[3] Although central venous catheters are most commonly used and are most commonly the route of infection, intraarterial catheters may also present a risk.[282]

The most common organism found in catheter-related infections is coagulase-negative *Staphylococcus*, which is a common indigenous bacterium on the skin and possesses characteristics, such as the presence of a biofilm, that facilitates colonization

of catheters.[129] The most serious infections, however, are caused by *S. aureus* and gram-negative bacilli. There are now standardized methods that should be employed under observation to insert and change central venous catheters help to diminish, albeit not eliminate, the central venous catheter infections. Catheter-related bloodstream infections are quality metrics that are monitored by hospital leadership.

Collection of Blood Cultures

The critical factors that must be decided by laboratory directors include the type of collection, number and timing of blood cultures, the volume of blood to be cultured, the amount and composition of the culture medium, when and how frequently to subculture and the interpretation of results.[234,236,329]

Contamination With Skin Microbiota. Every precaution should be taken to minimize the percentage of contaminated blood cultures.[323] Isolates of *Corynebacterium* spp., *P. acnes,* and *Bacillus* spp. other than *B. anthracis* are usually contaminants, albeit line-related infections may also be caused by these bacteria. Most isolates of gram-negative bacilli, yeast, β-hemolytic streptococci, *Streptococcus pneumoniae*, *Enterococcus* spp., and *Staphylococcus aureus* are clinically significant. The most problematic isolates are coagulase-negative staphylococci, because although these species are increasingly causes of true bacteremia, they are also indigenous microbiota of the skin.

A quality assurance monitor tracing the incidence of contaminated samples should be performed regularly in all microbiology laboratories. Less than 3% of blood cultures should be contaminated. Bates and associates[15] estimate that a contaminant blood culture can lead to an increase of as much as 20% to 39% in a patient's hospital bill from an extended stay for intravenous antibiotic therapy and additional tests. They also emphasized the need for paired blood culture sets to indicate likely contamination if only one turns positive. If collection technique is good and contamination minimized, the cost of contaminants becomes acceptable.[316] Inappropriate antibiotic use can be discouraged by a laboratory policy wherein single isolates of coagulase-negative staphylococci not be tested for antimicrobial susceptibility

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routinely. Several approaches to this determination are provided by Weinstein.[323]

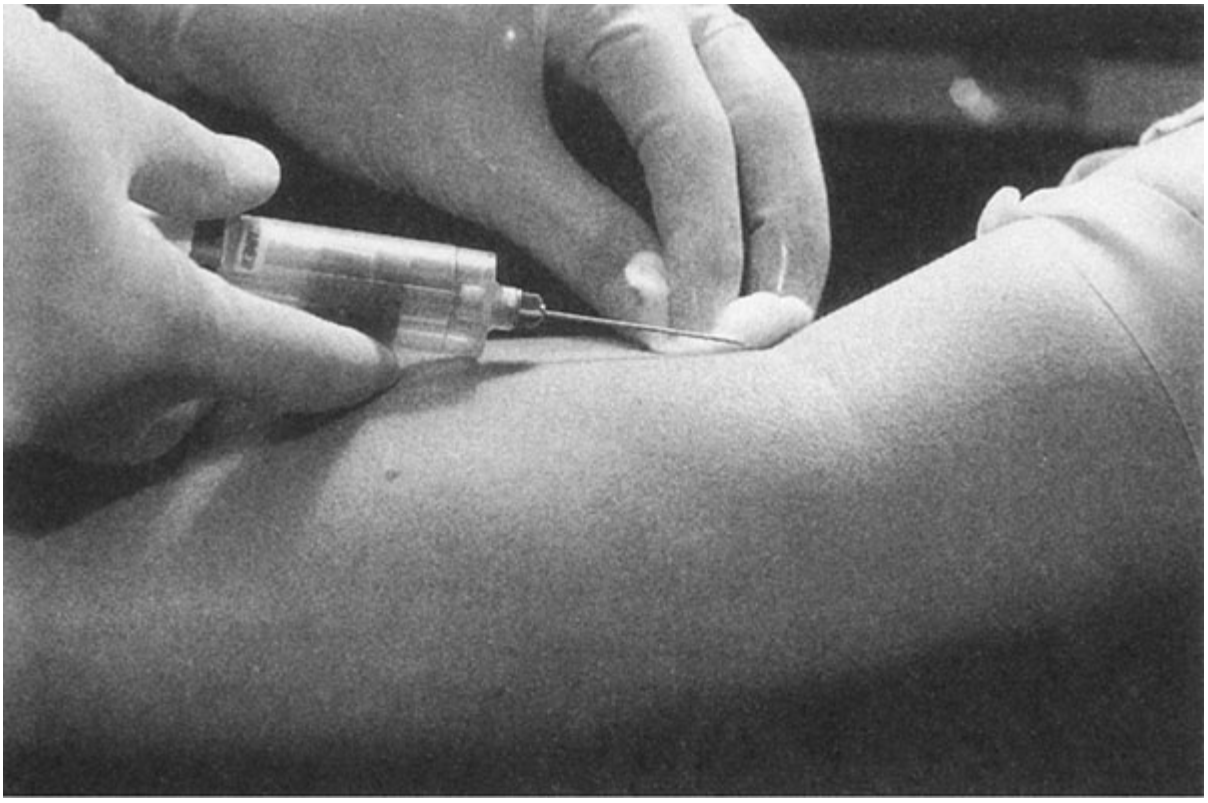
A variety of techniques have been proposed for determining whether an isolate of coagulase-negative *Staphylococcus* represents contamination. Unfortunately, none works well. Neither the number of positive bottles,[196] identification of the isolate to the level of species,[325] time to initial positivity[143] or the number of other negative

sets[143] distinguished clinically significant from insignificant isolates. Species of coagulase staphylococci other than *S. epidermidis*, *S. capitis*, and *S. haemolyticus* were almost always clinically insignificant, but these three species accounted for 98% of the significant isolates and 89% of the insignificant isolates.[325] The only way to determine with certainty whether two or more isolates represent the same strain is to perform molecular typing, a technique that is not available in most laboratories. Use of clinical data in combination with the antibiogram of the strains[143] or a combination of antibiogram and biochemical pattern (as opposed to identification)[323] have been proposed as means for determining whether multiple isolates represent multiple contaminants or a single infecting strain. The expanded use of MALDI-TOF now affords the ability to identify *Staphylococcus* to the species level in a rapid and inexpensive manner. This, at least, allows the laboratory the opportunity to assess if two coagulase-negative *Staphylococcus* species are members of the same species or not. If not, both may be contaminants and it is not possible to determine which, if any, is associated with a bloodstream infection. If, however, both are of the same species, then the likelihood of that organism representing a true infection is increased.

To reduce the chance of introducing contaminating organisms from the skin, the venipuncture site should ideally be prepared as follows: (1) wash with soap, (2) rinse with sterile water, (3) apply 1% to 2% tincture of iodine or povidone–iodine and allow to dry for 1 to 2 minutes (povidone–iodine) or 30 seconds (tincture of iodine), and (4) remove the tincture of iodine with a 70% alcohol wash. In practice, the soap wash is usually omitted; however, the combined use of iodine compound and alcohol to disinfect the venipuncture site is essential. If the site must again be palpated after the iodine–alcohol preparation, the finger of the glove must be disinfected or a sterile glove worn. If povidone–iodine is used, step 4 must be omitted. Be sure, however, that the povidone–alcohol solution is dry before making the venipuncture. A packaged skin preparation kit performed similarly to individual iodophor and alcohol pledgets.[335] There are increasing demands on the time of healthcare personnel, so there is a strong temptation not to allow the prolonged contact required for povidone–iodine solutions. Tincture of iodine, which is effective after contact with the skin for 30 seconds, has obvious advantages and has been reported to be more effective than povidone–iodine,[165] but is less acceptable to personnel. Other disinfectants have been suggested for preparation of venipuncture sites,[323] with variable acceptance. There are other acceptable alternatives, such as decontamination with chlorohexidine that may be explored.

Blood cultures may be obtained either by using a needle and syringe (Fig. 2-6) or by a closed system, consisting of a vacuum bottle and double-needle collection tube. Obtaining blood for culture from indwelling intravenous or intraarterial catheters should be discouraged. Although some authors have found a good correlation between

cultures drawn through a catheter or by venipuncture, most investigators have found a significantly increased risk of recovering skin organisms if an intravascular catheter is used.[39,86] The hope is that a patient will be saved the discomfort of a venipuncture, but recovery of a coagulase-negative *Staphylococcus* may actually result in more discomfort from additional venipunctures undertaken to address the questionable isolate.[323] If an intravascular catheter is used, a second culture should be drawn by venipuncture for comparison.



■ **FIGURE 2-6** Venipuncture technique for blood culture using a sterile needle and syringe. A tourniquet is applied to the upper arm above the venipuncture site to distend the antecubital veins. The site has previously been prepared with tincture of iodine and alcohol. The blood is removed with the syringe and needle and injected into an appropriate blood culture bottle. Rubber gloves must be worn during this procedure.

The practice of changing needles after the venipuncture and before injecting the blood into culture bottles has been replaced by direct injection with the original phlebotomy needle, because hepatitis or HIV infections can be acquired from accidental needlesticks. The majority of investigators who have studied this issue have concluded that there is no significant difference in blood culture contamination rates between patients for whom needles were switched between phlebotomy and injection

of the bottles and those for whom needles were not switched.[51,154,272] A meta-analysis of the issue did reveal more frequent contamination when needles were not changed, but the risk of acquiring a blood-borne viral infection greatly outweighs the benefits gained from changing needles.

One important factor in the quality of blood culture collection that is often overlooked is the training of the collectors. Multiple investigators have demonstrated that a trained phlebotomy team can obtain blood cultures with fewer instances of contamination than random individuals, no matter what their educational level.[322,323] It is recommended that phlebotomists are thoroughly trained and that the contamination rates for each phlebotomist is maintained and monitored by the phlebotomy supervisor. In this manner, lapses in technique can be readily uncovered and retraining instituted.

Number and Timing of Cultures. The number of specimens collected is less important than the total volume of blood cultured. Multiple studies have documented the

importance of volume; most are consonant with the finding that the yield increases to approximately 30 mL of blood in adults.[328] If a set of two bottles, each containing 10 mL of blood is submitted, two sets will satisfy the volume criterion. If the bottles only hold 5 mL each, or if a full complement of blood is not inoculated by the clinician, a correspondingly greater number of sets must be collected to satisfy the volume requirement. A lower total volume of blood is usually needed to document endocarditis, because the bacteremia is continuous. Some have suggested that a single aerobic culture may be adequate for patients with community-acquired pneumonia.[215] Organizing a blood culture order so that it consists of the phlebotomist filling of two sets of blood culture bottles approximately 15 minutes apart, with each set (i.e., a set contains one aerobic and one anaerobic bottle) representing a 20 mL assessment of blood (i.e., 10 mL per bottle) is a practical approach that covers all scenarios.

In a study of hospital blood culture practices, Schifman and associates[252] discovered that the incidence of solitary blood cultures ranged from 1% to 99%, with a median of 26%. They estimated that as many as 18,000 episodes of bacteremia might be missed annually because an inadequate volume of blood was collected. Between 20% and 30% of the solitary blood cultures they reviewed were not clinically indicated; most of the others were ordered by physicians who were unaware that one culture was not sufficient. Focused intervention and global education reduced solitary blood cultures from 40% to 24% in one hospital, and unnecessary collections were reduced from 38% to 12.5% in another institution. Unfortunately, the improvement of

performance after education has a short half-life; constant monitoring (e.g., as part of an ongoing quality assurance program), and/or electronic interventions are required. Solitary blood cultures present the additional problem that single isolates of coagulase-negative staphylococcus are difficult to interpret, as described above.

Blood cultures should be drawn before the use of systemic antimicrobials if possible. If therapy has been started, cultures should still be obtained, but negative results must be interpreted with circumspection.

Traditional guidelines were that multiple cultures should be taken at different times if intravascular infection and continuous bacteremia was suspected. Most authorities now believe that there is little to be gained by spacing cultures over time. Fever is often a somewhat delayed response to the entry of bacteria into the blood system, so collection of the specimens as soon as possible after a fever spike is recommended. As noted above, at least two venipunctures should be performed, but all necessary specimens (usually two to three sets, each containing two bottles) can be collected either at one time or approximately 15 minutes apart.[156]

After collecting an adequate volume of blood and initiating antimicrobial therapy, there is nothing to be gained by collecting additional specimens until the results of the initial sets are known.[103] The practice of a standing order for daily cultures should not be condoned. Most bacterial isolates are recovered by continuous-monitoring systems within 72 hours, so it is reasonable to wait at least 3 days before collecting additional specimens. A good rule is that it is preferable to consider the possibility of another cause rather than blindly repeating a nonproductive diagnostic approach. If clinical conditions change, however, additional specimens are indicated. "Breakthrough bacteremia" (positive cultures and new or persistent symptoms in the face of antimicrobial therapy) suggest the possibility of antimicrobial resistance and are a poor prognostic sign.[327] It should be remembered, however, that newer blood culture media that contain resins for removal of antimicrobial agents may result in the apparent persistence of pathogens; an assessment of the symptomatic status of the patient is of critical importance.

Culture Media. The media used in blood culture bottles are multipurpose and nutritionally enriched: tryptic or trypticase soy, supplemented peptone, brain–heart infusion, Columbia CNA agar, and brucella broths are commonly used. All are commercially available; however, variations in the composition of the same type of medium by different manufacturers makes comparisons and conclusions about the comparative yields of bacteria from each difficult to assess.

Most commercially available blood culture media contain the anticoagulant SPS in concentrations varying from 0.025% to 0.05%. In addition to anticoagulant properties (anticoagulation is a desired effect because certain bacteria do not survive well within the clot, where phagocytosis by neutrophils and macrophages remains active), SPS

also inactivates neutrophils and certain antibiotics, including streptomycin, kanamycin, gentamicin, and polymyxin, and precipitates fibrinogen, β-lipoproteins, β_{1C}-globulin, and other components of serum complement. SPS may also inhibit the growth of certain bacteria—*Peptostreptococcus anaerobius*, *N. gonorrhoeae,* and *N. meningitidis*. The inhibitory effect of SPS can be neutralized by adding gelatin to the medium, to a final concentration of 1%.

Many current blood culture bottles incorporate synthetic antibiotic-removing resins. Recovery of pathogens, and, unfortunately also skin contaminants, is clearly improved by these additions.[323] In a study of 6,839 paired blood cultures,[142] the use of resin media significantly improved the recovery of members of the family *Enterobacteriaceae*, *Enterococcus* spp., *S. pneumoniae* and viridans streptococci.

Traditionally, blood culture sets consisted of a bottle designed to recover aerobic bacteria and one engineered for anaerobic bacteria. In actuality, the majority of all types of bacteria are recovered in both bottles. The change in the pattern of bacteremias over time described above has led some investigators to suggest that an "anaerobic" bottle be limited to situations in which anaerobes might be expected (e.g., in patients with abdominal disease processes).[199] Recovery rates from three different paired bottle systems were compared: (1) one aerobic and one anaerobic bottle (5 mL of blood each); (2) two aerobic bottles (5 mL each); and (3) two aerobic bottles plus an extra anaerobic bottle when anaerobic infection was clinically suspected. The third approach had the largest yield of isolates. From their data, these investigators concluded that the use of two aerobic bottles with selective culturing for anaerobes will potentially increase the number of clinically important isolates by at least 6%.[199] The policy of substituting a second "aerobic" bottle for the "anaerobic" bottle is not a common practice, probably in part because of the difficulty of deciding which patients would benefit from the practice and the impossibility of leaving the decision to the clinical staff. Another reason is likely that others have demonstrated that anaerobic bacteremias are not predictable, so an anaerobic bottle should be included in every blood culture set.[59] It has been shown that many facultatively anaerobic bacteria (the majority of human pathogens) grow well in the "anaerobic" bottle.[55] It should be noted that the policy at one institution, where a decreasing incidence of anaerobic bacteremias had also been documented,[74] included two aerobic components in addition to the nonvented "anaerobic" bottle.

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BOX 2-3

Guidelines for Obtaining Blood Cultures[a]

Ideally, a trained phlebotomy team should collect all blood cultures according to established protocols. The frequency of likely contaminants in cultures collected by each member of the team should be monitored so that team members can improve their performance.

Blood cultures are indicated when a serious febrile illness that will require antimicrobial therapy is present or when an infection in an organ system might be elucidated by isolating a pathogen from the blood (e.g., meningitis or pneumonia).

Compare the name and medical record number on the requisition with that of the stated identity of the patient. Two patient identifiers should be used. Establish the site(s) for venipuncture and clean the site with an iodine compound followed by alcohol (preceded by surgical soap and water if possible).

Two blood culture sets, each with an aerobic and an anaerobic bottle within the set, is recommended. The second set should be drawn in the manner described above from a second phlebotomy site. There should be at least two venipuncture sites. All venipunctures may be collected at one session, but some recommend temporally separating the phlebotomies by about 15 minutes. For adults, each blood culture bottle should be filled with 15 mL of blood. Therefore, upon completion there should be two aerobic and two anaerobic blood culture bottles, each filled with 10 mL of blood, for a total blood culture assessment of 40 mL.

Variations on this theme may occur from institution to institution, and there are different guidelines for pediatric patients which often employ body mass to guide the amount of blood drawn.

Use all available safety devices, such as needles with safety features, a safety transfer device for inoculating blood into bottles, and a tube holder adapter, when a winged collection device is used. Dispose of all sharps in a protected container.

Do not force blood into the bottles.

Transmit the inoculated bottles to the laboratory as quickly as possible.

[a]Adapted from reference 83.

Blood cultures for mycobacteria and fungi should be considered in appropriate clinical situations. Dimorphic fungi, particularly *H. capsulatum,* and filamentous molds require special attention.[171] *Candida* spp. are effectively recovered by the current, automated blood culture systems.[188]

A summary of recommendations for collecting blood cultures is presented in Box 2-3.

Systems for Processing Blood Cultures[214]

Manual Blood Culture Systems. Two of the manual commercial systems are variations on the classic agar–broth combination bottles, known as Castañeda bottles. For laboratories that do not have the resources for a more automated system that monitors bacterial growth continuously, they represent a reasonable choice.

The Oxoid Signal Blood Culture System. The *Oxoid Signal Blood Culture System* (Thermofisher) is a single-bottle blood culture system that uses the production of CO_2 to detect early bacterial growth. The main blood culture bottle is similar to those used in other broth systems; however, the system uses a second plastic chamber, known as the signal chamber, which is fitted at the bottom with a long needle. After the blood sample to be cultured has been inoculated into the main bottle, the signal chamber is connected by inserting the needle through the rubber stopper and positioning it below the surface of the culture medium. Growing and metabolizing bacteria produce CO_2. The resulting in increased pressure, which forces liquid into the signal chamber; this can be directly visualized and used to prepare Gram's stain and subculture. This system has been evaluated favorably.[203,255] A higher than normal number of false-positive results and a lower than normal yield of anaerobes remain problems. Weinstein and coworkers[324] found an improved yield of organisms with improved bottles that have an increased head–gas space and are subjected to agitation.

BBL Septi-Chek Blood Culture System. The Septi-Chek biphasic agar-slide system (BD Diagnostic Systems, Sparks, MD) is also widely used. The system uses a standard blood culture bottle that contains either brain–heart infusion broth or trypticase soy broth. The bottle is designed for connection to a second plastic chamber that contains a paddle with agar surfaces. After the primary bottle is inoculated with the blood specimen to be cultured, the plastic-contained "slide" is screwed on. This slide contains a trisurface paddle faced with chocolate, MacConkey, and malt agar strips. The first "subculture" is made after 4 to 6 hours of incubation at 35°C by inverting the bottle and allowing broth to enter the slide's chamber, thereby flooding the agar surfaces. The bottle is then again placed upright for continued incubation. The bottle can be inverted again at regular intervals to reinoculate the agar media on the paddle.

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Lysis-Centrifugation Blood Culture System

Wampole Isostat/Isolator Microbial System. The Isolator system (Alere, Waltham, MA) is widely accepted as an alternative blood culture method that is particularly useful for recovery of fastidious or slow-growing organisms. It is the method of choice for dimorphic fungi, *Malassezia furfur* and *Legionella* spp.[24] There

was a reduction of mean recovery time for yeasts from 4.9 days using a conventional biphasic broth–agar system to 2.12 days with the Isolator. The mean recovery time of 8.0 days for *H. capsulatum* compared with 24.14 days for the biphasic system. The recovery of *H. capsulatum* using the Isolator system has not been surpassed by the automated systems. An overall 36.6% increase in the recovery rate of fungi from blood cultures was documented when the Isolator system was used. This method should be considered when other fastidious organisms, such as *Bartonella henselae*,[169,270] are suspected, if attempts at culture are being considered. The Wampole Isolator Microbial System (Alere) is a special tube that contains saponin, a chemical that lyses both the red and the white blood cells. Approximately 7.5 to 10 mL of blood are added to the tube, which is then thoroughly mixed by inverting the tube several times, so that the lysis reaction can go to completion. The tube is then placed into an angle centrifuge and spun at 3,000 rpm for 15 minutes to concentrate any microorganisms that may be present. After centrifugation, the sediment is aspirated and subcultured to appropriate media. A smaller version that does not require centrifugation is available for use in infants and young children; the use of the pediatric version for adults should be discouraged or prohibited because an insufficient volume of blood is cultured.

The Isolator system is also the method of choice when quantitative cultures of blood are desired. The CFU/mL can be calculated from the volume of blood processed and the number of colonies present on the agar surfaces.[341]

A twofold-to-eightfold increase in contamination rates over conventional systems is the major problem with the use of the Isolator. It has been suggested that contamination can be reduced by using dry agar plates, disinfecting the work area and processing samples in a vertical laminar air hood.[144] Another drawback is the labor needed to process each individual blood culture, each of which requires centrifugation and agar plate inoculation; this is substantially more labor intense than simply determining the quantity of blood present in a bottle of an automated system and placing it onto the instrument.

Examination of Manual Systems. Blood culture bottles should be incubated at 35°C and examined visually for evidence of growth (i.e., hemolysis, gas production, or turbidity) during the first 6 to 18 hours after collection. For those using conventional broth media, bottles should be examined against bright fluorescent bulbs or with incandescent transmitted light. The surface of the sedimented blood layer should be examined because discrete colonies may be detected. Blind subcultures to chocolate agar plates should be made from all nonagar broth and continuous blood culture bottles within 12 to 24 hours after collection, after which the plates are incubated aerobically in 5% to 10% CO_2 at 35°C. Blind anaerobic subcultures are usually not done in most laboratories. It is generally agreed, however, that both aerobic and anaerobic subcultures of all visually positive blood culture bottles should be set up. In

a study of 20,155 blood culture bottles (trypticase soy broth and thiol broth),[202] only 32 trypticase soy bottles and 10 thiol bottles turned positive after 7 days of incubation. Fifteen of the 32 trypticase isolates and all of the thiol isolates were either recovered in other cultures or were not considered clinically significant, indicating that holding manual blood cultures beyond 7 days is unnecessary. Most institutions have gone to holding blood cultures for only 5 days.

The routine microscopic examination of macroscopically negative blood culture bottles after 24 hours of incubation is not indicated because the number of organisms that can be detected by Gram's stain (about 10^5 CFU) is not appreciably less than the 10^6 to 10^7 CFUs required to produce visible turbidity of the broth.[236] Acridine orange stains are more sensitive, detecting 10^3 to 10^4 CFU/mL. Tierney and associates reported a 16.8% increase in the early detection of septicemia by examining macroscopically negative blood culture broths with the acridine orange stain.[299]

Automated and Computerized Blood Culture Systems. The introduction of continuous-reading, automated, and computerized blood culture systems represented an important advance in clinical microbiology practice. The use of manual blood culture systems has decreased with the introduction of these systems. Three such systems, the BacT/ALERT 3D (bioMérieux, Durham, NC), the BD BACTEC (BD Diagnostic Systems), and the VersaTREK (ThermoScientific, Cleveland, OH) are commonly used in the United States. Each of these systems alerts the microbiologist that a culture is positive, after which the relevant bottles can be removed for Gram's stain and subculture. More recently, FDA-approved multiplex molecular diagnostic assays are available for use on blood cultures that signal positive, which affords a more rapid species-level identification and, in some instances, the detection of genetic determinants of resistance. The media selected for subculture, as well as some of the molecular assays, can be chosen based on the Gram reaction and morphology of the microbes. If organisms are not visualized, a blind subculture should be performed and the bottle returned to the instrument for continued incubation. Multiple studies have demonstrated that bottles need be incubated only for 5 days when the continuous monitoring systems are used. In the future it may be possible to reduce the incubation time even further.[58]

The BacT/ALERT 3D Blood Culture System. As the first continuous-monitoring blood culture system developed and marketed in the United States, the BacT/ALERT3D (formerly BacT/Alert) system has been implemented in many clinical laboratories. Each blood culture bottle has the capacity to receive 10 mL of blood. As microorganisms grow in the blood–broth mixture, CO_2 is liberated. A CO_2-sensitive chemical sensor that is separated from the blood–broth mixture by a unidirectional CO_2-permeable membrane is bonded to the bottom of each bottle. In

the presence of CO_2, the color of the sensor turns from green to yellow, although the light-sensitive detector built into the instrument reacts before a color change is apparent.

Each bottle is placed bottom-side into a receiving well in the data unit, directed by a barcode on the bottle label, which is integrated in the computer to match the patient identification data for each. Each data unit is a cabinet about the size

of a small refrigerator which serves as a self-contained incubator, shaker and detection device, with a capacity to hold either 240 or 120 bottles, depending on the model. Multiple modules are through the same computer controls, yielding a total of 1,440 bottles that can be monitored. The wells are arranged in two rows within a horizontal rack that gently rocks back and forth when the door to the data unit is closed. At 10-minute intervals, a light beam from emitting diodes (one for each well) is projected through an excitation filter to reflect off the CO_2-sensitive sensor in the bottom of each bottle. The reflecting light is directed through an emission filter to a photosensitive detector that, in turn, is connected to a computer compiler. As soon as the accumulation of CO_2 is sufficient in the bottle to alter the sensor, an audible or visible "alert" is generated, and the position of the positive bottle is immediately flagged by the computer. Positive bottles can be immediately removed and further processed.

*The **BD BACTEC** Blood Culture Systems.* The BACTEC system consists of a self-contained incubator, agitator and detection device, similar in appearance to the BacT/ALERT 3D system. There are three sizes: model 9240 holds 240 bottles, model 9120 holds 120 bottles and model 9050 accommodates 50 bottles. Multiple modules can be linked to the same computer control unit. Similar to the BacT/Alert, each bottle has a sensor disk bonded to the inner surface of the bottom. The one operational difference between the BacT/Alert and the BACTEC systems is that the latter uses fluorescent, rather than spectral light to detect changes in the concentration of CO_2 in the broth–blood mixture. As CO_2 is produced in each bottle, its sensor emits a fluorescent light that passes an emission filter on the pathway to a light-sensitive diode. Bottles are placed bottom down into receiving wells that are monitored once every 10 minutes. The voltage of the current reading of the diode is compared with the previous reading. If the voltage change exceeds a preset delta value, the microcomputer flags the bottle as positive. The position of the positive bottle is indicated on the computer screen so that bottle can be pulled for further processing. A graph illustrating the progress of CO_2 production can be brought up on the computer screen at any time.

The VersaTREKBlood Culture System. The VersaTREK blood culture system (ThermoScientific (previously TREK Diagnostic Systems), Cleveland, OH) differs from the BacT/ALERT3D and the BD BACTEC systems in the following ways: (1)

the production of CO_2 is monitored manometrically, (2) both gas consumption and production are monitored, and (3) changes in the concentrations of H_2 and O_2 in addition to CO_2 are detected.

The data unit is also a cabinet that serves as a self-contained incubator, agitator and detector. Units with a capacity of 96-to-240 or up to 528 bottles are currently available. After inoculation of up to 10 mL of venous blood, each bottle is fitted with a disposable connector, which includes a recessed needle that penetrates the septum of the blood culture bottle. Each bottle is then placed in a defined position on a carrying rack that is aligned such that the connector attaches directly to a sensing probe located at the top of each position. Once the bottle is properly aligned, the pressure of the head–gas is continuously monitored. A reading is taken every 12 minutes. When the change in reading exceeds a delta value, lights are illuminated that indicate the position of any positive bottle.

A reading may occur during a phase of consumption of H_2 and O_2. Oxygen consumption is accelerated at the time replicating organisms enter the log phase of growth. A reading may be possible, therefore, early in the incubation period before a detectable amount of CO_2 is produced. Testing multiple gases is a theoretical advantage for the electrostatic precipitator (ESP) system, especially for the detection of asaccharolytic microorganisms that may not produce sufficient CO_2 for detection by the indicator.

Comparative Studies

The comparative performance of these blood culture systems has been extensively studied. Depending on the design of the study, the spectrum of microorganisms being recovered from clinical specimens, the volume of blood being cultured and the exact types of bottles and media formulations being compared, one system may emerge as slightly superior or inferior to another. Improvements in formulations of media, sensitivity of detectors, and design of instruments continue to be made; the results of a study performed some months ago may not necessarily reflect the current technology. Therefore, each laboratory director and supervisor must weigh published and verbal comments from individuals who have used the system and the needs of the local laboratory when determining whether a new system should be implemented. These are all FDA-approved, excellent instruments; the decision to be made is which is the best fit for the individual laboratory.

The advantages of continuous-monitoring blood culture systems include a decrease in laboratory workload, a decrease in the number of false-positive results and pseudobacteremia (because of decreased handling and sampling of the bottles), and a significant increase in the speed of detection and in the rate of microbial recovery. Disadvantages include a limited selection of media, the large size of the instruments

(this is less of a problem currently, as smaller platforms exist), and expense. The decrease in laboratory workload is primarily because a technologist's time can be dedicated to processing only the positive cultures instead of loading and unloading instruments or subculturing and observing predominantly negative specimens. An adjustment in staffing during off hours must be made because cultures may become positive at any time of day.

Special Considerations

Fastidious Organisms and Endocarditis. Although most pathogens are detected in a few days, some fastidious microbes grow slowly. Some of these organisms produce endocarditis. It is, therefore, appropriate to prolong incubation beyond the routine cutoff period when requested by the clinician. A discussion with the clinician is warranted to determine if they have a clinical suspicious for a particular fastidious microorganism. This is useful in several ways. In some instances, the clinician may be unaware of advances in blood culture instrumentation and may be interested in extended incubation for an organism that is readily recovered by the current systems (e.g., *Cardiobacterium* spp.). Alternatively, they may be interested in extended incubation for bacteria that will not grow in culture (e.g., *Coxiella burnetti*) or are highly unlikely to grow in culture (e.g., *Bartonella* spp.). In such instances, directing them to serologic and/or molecular solutions would

be appropriate. Finally, they may be interested in organisms, such as *P. acnes,* that may require extended incubation, in which case it should be performed. A problem with some rare, fastidious pathogens is that they do not generate sufficient CO_2 to trigger the detector. A useful maneuver, if this is suspected, is blind staining of bottles with acridine orange after incubation for 7 days and/or the total period of incubation. Acridine orange is preferred to Gram's stain because it is more sensitive and the smear can be examined more quickly.

Intravascular Catheter Cultures. Two basic approaches have been taken to the diagnosis of catheter-associated bacteremia. One requires removal of the catheter; the other does not.[193]

The initial suggestion for diagnosis—and probably the most common approach even today—was a semiquantitative procedure.[175] The tip of the catheter is rolled across the surface of an agar plate and the resulting colonies are counted after overnight incubation. A statistical association of >15 CFU with catheter-associated sepsis was established and subsequently confirmed by other investigators. Variations on this procedure have included culturing multiple segments, sonication of the catheter tip,

and washing the interior of the catheter,[265] but the simplicity of the original procedure has appealed to most microbiologists.

The second approach is performance of quantitative blood cultures, simultaneously collected through the catheter and by venipuncture.[92,226] The theory is that if the catheter is secondarily infected by a bacteremia from another source, fewer CFUs will be detected in the blood drawn through the catheter than in that collected by venipuncture. If the catheter was the source of the bacteremia, the reverse will obtain. The simplest method for performing the quantitative cultures is to use the Isolator blood collection system.[92] Similarly, some have examined time-to-detect in catheter-drawn and peripherally-drawn blood cultures as surrogates for quantitative blood cultures. The use of quantitative cultures and time to detection has not been widely deployed in clinical laboratories.

The advantage of the quantitative culture technique is that the catheter can be left in place if it is not implicated in the bacteremia. It should be noted, however, that it is possible to change a catheter over a wire, so that it is not necessary to perform a new procedure to maintain vascular access; in that case, a decision about removing the new catheter can be made after the results of culturing the first catheter are known.[193] In either case, an intravascular catheter is left in place if the catheter is determined not to be the cause of the sepsis. The usefulness of catheter-associated cultures is of great debate, because of the increased possibility of contamination and limited-to-no additional benefit compared with peripheral blood cultures. The approach to catheter-associated cultures should be made after thorough literature review and consultation with the ordering physicians regarding usage.

Tissues and Biopsies. Tissue samples for culture should be delivered promptly to the laboratory in a suitably capped, sterile container. If the sample is very small, it may be placed on a piece of sterile, moistened filter paper. Gauze is sometimes used, but it may be very difficult to recover small specimens from the interstices of the gauze. Specimens that have been placed in formalin are not suitable for culture.

Bone marrow cultures may be helpful in establishing the diagnosis of granulomatous diseases such as brucellosis, histoplasmosis, and tuberculosis. Using the Isolator system or other lytic systems to process the bone marrow samples may improve the recovery of bacteria, particularly if the infections are caused by intracellular organisms.

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CHAPTER 3

Laboratory Diagnosis by Immunologic Methods

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Antigens and Antibodies: Basic Definitions

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Immunofluorescence Techniques

Immunofluorescence Techniques for Antigen Detection
Immunofluorescence Techniques for Antibody Detection

In clinical microbiology laboratories, culture of microorganisms from patient specimens remains the principal method for the diagnosis of infectious diseases. During the 1940s and 1950s, serologic techniques, such as Oudin and Ouchterlony immunodiffusion, that were developed in research settings were introduced into clinical laboratories. Subsequently, other methods that exploited basic immunologic concepts, such as complement fixation (CF), were introduced as methods for retrospectively documenting the host immune response to infection. The development of enzyme immunoassay (EIA) and hybridoma technology has completely altered the role of serology in the diagnosis of infectious diseases. Methods that were originally developed for antibody detection were subsequently reconfigured for direct detection of microbial antigens in patient specimens. Newer immunoserologic techniques have expanded the role of the laboratory in patient care and have gained widespread acceptance as helpful diagnostic tools. All of these techniques have their foundations in basic immunologic concepts. After a brief review of these concepts, the newer applications of immunoserology to patient care will be discussed.

Antigens and Antibodies: Basic Definitions

An **antigen** is a substance that evokes the formation of antibodies in an animal that is immunized with that particular antigen. An antigen is generally immunogenic, that is, it has the capability to stimulate antibody formation and to specifically combine with the antibodies that are formed against it. Not all molecular structures composing an antigen are equally immunogenic; those molecular structures that are immunogenic and that are recognized by antibodies are called **immunodominant antigenic determinants** or **epitopes**. The unique characteristics of each antigenic epitope depend on the types and sequences of amino acids in proteins, the chemical and structural compositions of polysaccharides, glycoproteins, and nucleic acids, and their secondary, tertiary, and quaternary structures. Different molecules may share antigenic determinants and will be recognized by antibodies directed against these determinants. For example, the C1 portions of the light chains of the various immunoglobulin classes (discussed below) contain common antigenic determinants that allow them to be recognized by the same antibodies. These antigen/antibody combinations are termed **cross-reactive**. Cross-reactions of antibodies with common or closely related antigens may be clinically important in some disease states. For example, studies with monoclonal antibodies have shown that certain streptococcal M protein antigens and the group A streptococcal carbohydrate antigen *N*-acetyl-β-D-glucosamine cross-react with cardiac

myosin and several other proteins (e.g., tropomyosin, vimentin) found in heart muscle and heart valve tissue.[46,62,100] In this model, antibodies developed during acute streptococcal pharyngitis subsequently bind to these cross-reactive epitopes in heart tissue, thereby activating the complement cascade and resulting in immunologically mediated damage to cardiac muscle and adjacent tissues and the development of acute rheumatic fever (ARF).[104] A similar mechanism is believed to operate in the pathogenesis of poststreptococcal glomerulonephritis. Antibodies elicited against various non-M protein antigens of "nephritogenic" group A streptococci (e.g., cytoplasmic endostreptosin, extracellular "nephritis strain–associated protein [NSAP]," streptococcal pyrogenic

exotoxin B [speB]) may react with renal tissues to produce glomerular injury.[44,154]

Antibodies belong to a group of structurally related glycoprotein molecules found in the blood and extracellular fluids and are known collectively as **immunoglobulins (Igs)**. These molecules are produced by B lymphocytes that express surface-bound Igs of a single specificity. When the antigen receptors on these cells encounter the appropriate binding ligand, the B cells proliferate and secrete soluble antibodies against the target antigen. Individual plasma cells produce quantities of single antibody molecules that have the same antigen-binding specificities. In addition, some B cells function as antigen-processing cells that subsequently present the antigen to T lymphocytes, stimulating additional antibody production and the induction of cellular immune responses.[27] The genesis of cellular and humoral immune responses and the cellular interactions that result in production of specific antibodies is beyond the scope of this text and may be found in other resources devoted to immunology and immunogenetics.[27]

Immunoglobulins can be divided into five classes based on their structure: IgG, IgM, IgA, IgD, and IgE (Fig. 3-1). IgG molecules have a molecular weight of about 150 kDa, are composed of two light chains and two heavy chains, and have two sites for binding to specific antigen (Fab sites). The non–antigen-binding site, composed of portions of the two heavy chains, is called the Fc region. Molecules of the IgG class are actively transported across the placenta and provide passive immunity to the newborn infant at a time when the immune mechanisms of the infant are in development. IgGs are divided into four subclasses, termed *IgG_1, IgG_2, IgG_3,* and *IgG_4*. IgG_1 is the major Ig in serum and is able to fix and activate complement. IgG_2 and IgG_4 are the major Igs produced in response to polysaccharide antigens and, therefore, to encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b. IgG_3 is produced in greater quantities during the

secondary immune response and is particularly important for viral neutralization. IgM molecules have a molecular weight of about 950 kDa and are composed of five monomers, each of which resembles a single IgG molecule. The five monomeric structures are joined to each other by disulfide bonds on the Fc region of each monomer and by a 15-kDa molecule called the J chain, which is necessary for aggregation of the pentameric structure. IgM is the first Ig class produced by the fetus and is the first Ig to appear in the circulation following immunization or infection. IgM is also helpful in complement fixation. The appearance of IgM in the serum is transient, and its presence usually indicates recent infection. However, IgM responses may be noted during reactivation of latent viral infections and during reinfection with the same or closely related agents. In addition, IgM antibodies may persist for weeks to months, depending on the agent and the host's immune competence. Unlike serum IgG, IgM does not cross the placenta; so demonstration of IgM against an infectious agent (e.g., rubella) in fetal or cord blood indicates a congenitally or perinatally acquired infection. Serum IgG appears 4 to 6 weeks after infection and usually persists for life. IgA antibodies occur either as monomers (160 kDa) or dimers (400 kDa) and account for about 15% of the total serum Ig. IgA is the principal antibody class found on mucosal surfaces and in extracellular secretions (e.g., colostrum, saliva, tears, mucin, intestinal, respiratory, and genital tract secretions). IgD is similar in structure to IgG, has a molecular weight of 175 kDa, and comprises only about 0.2% of the total serum Ig. This antibody class is found primarily on the surfaces of immature B lymphocytes and

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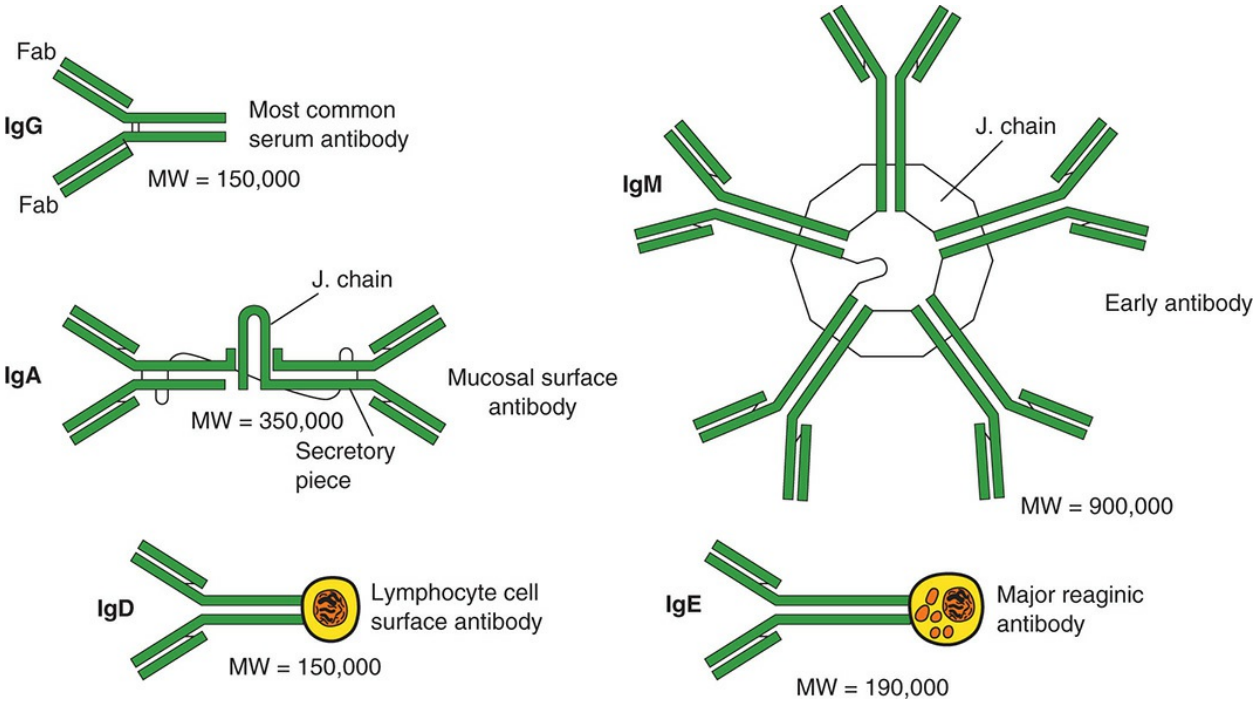
acts as a cellular antigen receptor. IgE is a 190-kDa Ig that is present only in trace amounts in serum. This Ig is noncovalently bound by the Fc region to mast cells and basophils.[50] Binding of antigen to IgE activates the degranulation of these cells, leading to the synthesis of peptide mediators of hypersensitivity. Therefore, IgE antibodies play major roles in allergic reactions (e.g., anaphylaxis). These antibodies may also be transiently or persistently elevated in serum during intestinal helminth infections.[63,134]



■ **FIGURE 3-1** Classes of human Igs. Antibodies belong to five structural and functional classes, designated IgG, IgM, IgA, IgD, and IgE. The basic structural unit of members in each class consists of two pairs of polypeptides (two heavy chains and two light chains) joined by disulfide bonds, and each unit has two antigen-combining sites. Some Ig types have other structural components (J chain in IgM, secretory piece in IgA).

Serologic diagnosis of infections with bacterial, viral, fungal, or parasitic agents in the clinical laboratory is accomplished by detection of specific antibodies in patient serum specimens. As mentioned, IgM is the first antibody to appear after infection; so a serologic diagnosis of recent infection may be obtained by performing an IgM-specific test on a single serum specimen collected early in the clinical course. Diagnosis of recent infection can also be made by the detection of IgG antibodies in paired specimens. The first specimen is collected within 5 to 7 days after the onset of symptoms; the second is collected during convalescence 2 to 4 weeks later. A fourfold or greater rise in the antibody titer between the acute and the convalescent specimen suggests a recent or intercurrent infection. The antibody titer is defined as the reciprocal of the dilution of serum that still gives a positive test for the presence of antibodies. For example, if an acute-phase serum specimen is reactive at the highest dilution of 1:8 (titer of 8), and the convalescent specimen is reactive up to a dilution of 1:64 (titer of 64), the titer of the second specimen is greater than fourfold the titer of the first specimen ($8 \times 4 = 32$ and $64 > 32$). This fourfold rise in titer suggests infection with the agent within the recent past. Use of a fourfold titer increase to delineate recent infection is based on normal test variation and is predicated on testing

of both acute and convalescent specimens in the same procedure at the same time. Single serum specimens may be tested to determine the immune status of individuals to certain agents (e.g., rubella, varicella zoster, etc.); in these cases, test performance must include not only positive and negative controls, but also additional sera of known reactivity to serve as calibrators for the interpretation of test results.

Serologic diagnosis of infectious diseases is retrospective, as some time must elapse between infection with an agent and the detection of an immune response. However, serologic procedures have broadened in scope to include not only the detection of antibodies in serum, but also the detection of antigens in a variety of specimen types. For the rest of this chapter, methods for serologic detection of both antibodies and antigens will be addressed in general terms, with specific applications of these methods being addressed in subsequent chapters.

Monoclonal Antibodies

Antigens, by their nature as macromolecules having primary, secondary, tertiary, and quaternary structures, constitute a "mosaic" of antigenic determinants. An outgrowth of basic serologic principles and techniques has been the attempt to "purify" antigens to reduce the heterogeneity of antibodies developed against them. Antigen molecules with only a single epitope are rarely encountered; rather, hundreds or even thousands of potential antigenic determinants may exist on a cell surface or within the mix of other substances. When these mixed antigens are injected into an animal, an equal number of lymphocyte clones are stimulated. Even though each clone produces a specific antibody, the final result is a highly heterogeneous mixture of antibody molecules, the specificity and affinity of which are often unknown and difficult to control from batch to batch. When these **polyclonal antisera** are used in immunologic test systems involving infectious agents, cross-reactivity may be noted either because antigenic determinants are shared by different species or because mutations may have led to the evolution of epitopes sufficiently close in specificity to produce detectable cross-reactions. Attempts to produce pure antibodies through absorption with cross-reacting antigens or to prepare "clonal" from "polyclonal" antisera via techniques such as affinity column chromatography have only been partially successful.

As the science of serologic testing evolved, the view was held that the availability of an antibody having a high degree of molecular homogeneity and with specificity for a single, antigenic epitope without cross-reactivity would solve many of the problems encountered in the use of polyclonal antibodies. Highly specific **monoclonal antibodies**, the product of a single clone of lymphoid cells, gradually emerged as a by-product of the investigations in cell fusion and hybridoma technology conducted by Kohler and Milstein.[85,175] Because of their discovery, it is now possible to isolate

cloned lines of individual lymphocytes that produce unique, monospecific antibody molecules. Monoclonal antibodies refer to a uniform, homogeneous, molecular species of Ig, rather than to a heterogeneous array of Igs as is produced during the usual immune response. The principal feature of this technology was not that a single line of monoclonal antibody (MAB)-producing cells could be isolated, but rather that these mouse lymphocytes could be "fused" with mouse myeloma cells to produce hybrid cells having two inherent properties: (1) the capability of producing monospecific antibodies (acquired from the parent lymphocytes) and (2) the ability to grow permanently in culture (the characteristic "immortality" acquired from the transformed myeloma cells). Thus, individual monoclonal antibodies can be produced in a continuous and almost endless supply (Box 3-1).

Monoclonal antibodies have been developed against clinically relevant antigens of many bacterial, viral, fungal, and parasitic agents, and reagents prepared with these antibodies are used in many commercially available EIA and immunofluorescence test kits. In addition to the direct detection of microbial structural antigens (e.g., capsular polysaccharides, outer membrane protein antigens), monoclonal antibodies have also been developed for the detection of microbial virulence factors, such as toxins produced by enterohemorrhagic *E. coli* (e.g., Shiga and Shiga-like toxins).[80,102] This approach introduces a new way of evaluating the relationship of microorganisms and infectious diseases. Instead of the conventional focus on detection and identification of the organisms themselves, these reagents allow specific detection of microbial virulence factors that may be shared by several bacterial species associated with a given symptom complex. For example, it may be more important to know that an enteric toxin is the cause of hyperosmotic diarrhea, rather than to receive the information that the patient is infected with *Shigella* species or an enterotoxigenic *E. coli* strain.

BOX 3-1

Procedure for Production of Monoclonal Antibodies[85,175]

1. **Selection of Antigen**
   Monoclonal antibodies can be produced against any substance recognized as an antigen by the immune system of the animal being injected. Using a pure antigen is ideal. In fact, certain antigens, such as chemically purified drugs used for assays (e.g., digoxin), may be homogeneous. Even so, one can never guarantee that an antigenic determinant will consist of only one epitope. The fact that impure antigens can be used in MAB production is a chief advantage over

conventional methods used to produce polyclonal antibodies.

2. Animal Immunization

The chief objectives in the immunization procedure are to prime the immune system of the animal to avidly recognize all antigens injected, to maximally stimulate B-lymphocyte clones, and to have the spleen cells divide at a high rate. In the production of monoclonal antibodies, the BALB/c mouse strain is most commonly used. The antigen is injected subcutaneously or intraperitoneally, with the simultaneous injection of Freund's adjuvant. Injections are repeated at weekly intervals, and a final "booster" injection is given intravenously approximately 3 days prior to harvesting the spleen cells. At the end of the injection schedule, the animal is killed and the spleen is aseptically removed.

3. Fusion of Splenic Lymphocytes and Myeloma Cells

The animal spleen is placed in sterile culture medium containing antibiotics. The splenic tissue is teased to release cells and to form a slurry. This material is passed through a mesh to obtain single cells. Ficoll is added, and the slurry is centrifuged to remove RBCs. Polyethylene glycol (PEG) is added to the slurry to reduce cell-to-cell surface tension; this brings the cells into close proximity to one another, allowing their membranes to fuse. Dimethylsulfoxide (DMSO) is added to the fusion mixture to maximize cell contact even more. Finally, the cells are packed into a pellet by gently centrifuging the mixture for 5 minutes. Thus, at the end of these steps, the preparation consists of unfused myeloma cells, unfused lymphocytes, and a few fused hybrid lymphocyte-myeloma cells (It should be recognized that splenic lymphocytes and myeloma cells fuse with a frequency of only about 1 per 10^5 or 10^6 cells.).

4. Selection of Hybrid Lymphocyte-Myeloma Cells

Unfused myeloma cells rapidly outgrow the hybrids and must be removed in some manner. The myeloma cells used for fusion are grown in the presence of 8-azaguanine, a drug that causes the cells to permanently switch off the production of hypoxanthine phosphoribosyl transferase (HPRT), an enzyme that is needed to continue growth. If these HPRT-negative cells are suspended in a medium containing hypoxanthine, aminopterin, and thymidine (HAT medium), only the hybridoma cells will grow successfully. The hybridoma cells inherit HRPT from the splenic lymphocytes with which they have fused and will survive. The unfused myeloma cells, unable to synthesize DNA because of inability to produce HPRT, will be killed by the aminopterin in the selective HAT medium. It should also be remembered that unfused splenic lymphocytes do not survive beyond a few days in culture medium; therefore, the fused hybrid lymphocyte-myeloma cells alone survive in the HAT medium.

5. Cloning the Hybridoma Cells

The single hybrid cells producing the desired antibody must be isolated and grown as a clone. Two techniques can be used: (1) limiting dilution and (2) growth in an agar gel medium. In the limiting or doubling dilution technique, the suspension of hybrids (after maximum growth) is diluted and distributed into a series of sterile

wells in a microtiter plate. The dilutions are so calculated that each well contains an average of only one cell that can then be replaced as a single antibody-producing clone. In the alternative method, using agarose gel supplemented with serum, amino acids, and antibiotics, the dividing hybrid cells form tiny, sphere-like clusters. These spheres can be selected with a Pasteur pipette and transferred to microtube wells for further culture and ultimately for assay to determine whether the desired antibody is being produced.

6. Screening for Desired Antibodies
In the fusion step of the procedure of producing monoclonal antibodies, many lymphocytes other than those producing the desired monoclonal antibodies may have fused. In fact, less than 5% of the hybrid cells out of those selected actually produce the desired specific antibodies. Thus, assays of the selected cell lines are required to determine if the desired antibody is being produced. Radioimmunoassays, enzyme-linked immunosorbent assay (ELISA), precipitin techniques, and blotting techniques can be used for this phase of the procedure.

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7. Mass Production of Monoclonal Antibodies
Once the desired clone of hybrid cells has been selected, the next step is the production of large quantities of monoclonal antibodies. The peritoneal cavity of mice, preferably the same strain that was used for the initial immunization step, can be used to grow the selected hybrid cell clone. First, the peritoneal cavity is injected with an organic irritant, such as pristane, to produce a chemical peritonitis. Next, the selected hybrid cell line is injected into the peritoneal cavity. Within days, a tumor known as a hybridoma develops. This tumor produces large quantities of monoclonal antibodies that can be harvested by aspirating the ascitic fluid from the mouse's peritoneal cavity. A tumor-bearing mouse will survive for 4 to 6 weeks, during which time large quantities of antibody can be harvested. Hybridomas can also be grown in tissue cultures in which highly purified antibodies are produced without the potential for contamination from serum, nonspecific interference from ascites proteins, or cross-reactivity of histoincompatibility antibodies derived from the mouse tissues.

Types of Antigen: Antibody Reactions Used in Diagnostic Serology

Precipitin Reactions

The basic type of antigen:antibody reaction is the precipitin reaction. This reaction is found in test systems that allow the free diffusion of antigen and antibody fronts toward one another. At a critical point of interface, where the concentrations are optimal, a visible precipitate composed of combined antigens and antibodies forms. In a **single-diffusion** system, antibody is incorporated into an agar gel into which antigen is allowed to diffuse. In the tube (Oudin) immunodiffusion method, antigen is overlaid onto agar-containing antisera, and one or more precipitin lines form at zones of equivalence. In **radial immunodiffusion**, antibody is incorporated into agar that is coated onto a glass slide. Material containing antigen is then placed into a circular well cut into the agar. During incubation, the antigen diffuses into the agar and a ring of precipitate forms. The square of the radius of the ring is directly proportional to the amount of antigen present in the material. Using a constant dilution of antiserum, semiquantitative antigen concentrations may be determined by comparing the diameters of the precipitin reaction produced by solutions of known antigen concentrations with that of the ring produced by an unknown solution. The most commonly used conventional immunodiffusion procedure is **double diffusion**. In this technique, both antigen and antibody are placed in wells adjacent to one another, and the materials diffuse out and toward one another. A line of precipitate then forms between the lines when concentrations of equivalence are reached. Double diffusion may require incubation for up to 48 hours before an interpretable result is obtained. **Countercurrent immunoelectrophoresis (CIE)** employs double diffusion technology, but uses an electric current running through the agarose support matrix to speed up the migration of the antigen and antibody toward one another. These methods may be used for the detection of either antibody or antigen in body fluids.

One precaution in performing precipitin procedures is to recognize the possibility of false-negative reactions due to prozone or postzone phenomena. If antibody is in excess (i.e., in concentrations far in excess of available antigen), a false-negative (prozone) reaction occurs, because molecular lattices, which make up the visible precipitate, do not form. In contrast, postzone reactions occur when antigen is in excess and binding sites on the antibody become saturated with antigen such that lattice formation characteristic of precipitin reactions does not occur. In cases wherein high concentrations of antigen or antibody are anticipated, false-negative prozone or postzone phenomena, respectively, can be avoided by repeating the tests using serial dilutions of the specimen. The zone of equivalence is defined as the range of reactant ratios that results in maximum precipitation of both antigens and antibodies.

Double diffusion methods are still used as an aid in the diagnosis of systemic fungal infections, including blastomycosis, histoplasmosis, coccidioidomycosis, paracoccidioidomycosis, and penicilliosis due to *Penicillium marneffei* (i.e., fungal immunodiffusion testing).[64,95,192] In fungal immunodiffusion, purified antigens from

these systemic fungal pathogens are reacted with patient and control sera containing antibodies in double diffusion tests. The development of precipitin lines of identity with the positive control sera and with the serum of the patient indicates the presence of antifungal antibodies. The presence or absence of certain precipitin bands (e.g., the H and M bands seen in *Histoplasma capsulatum* infection, anti-BAD1 [formerly WI-1] antigen bands in *Blastomyces dermatitidis* infection) may have diagnostic and prognostic significance for patient management.[83] Although specific, these tests suffer from inadequate sensitivity, and their use has been eclipsed by newer serologic (e.g., antigen detection, EIAs) and molecular diagnostic methods (e.g., gene amplification).[192]

Immunodiffusion is also the basis of the exoantigen test for identification of systemic fungal pathogens. In this method, an agar slant growing a mycelial culture of the organism to be identified is extracted with water:merthiolate overnight. This aqueous extract is concentrated and reacted in a double diffusion test with antisera against *H. capsulatum*, *B. dermatitidis*, *C. immitis*, and *Aspergillus* species along with appropriate antigen controls. After 24 hours incubation, the immunodiffusion plate is examined for lines of identity between the mycelial extract, the fungal antisera, and control antigens for each organism.[78] Although this method is still used for

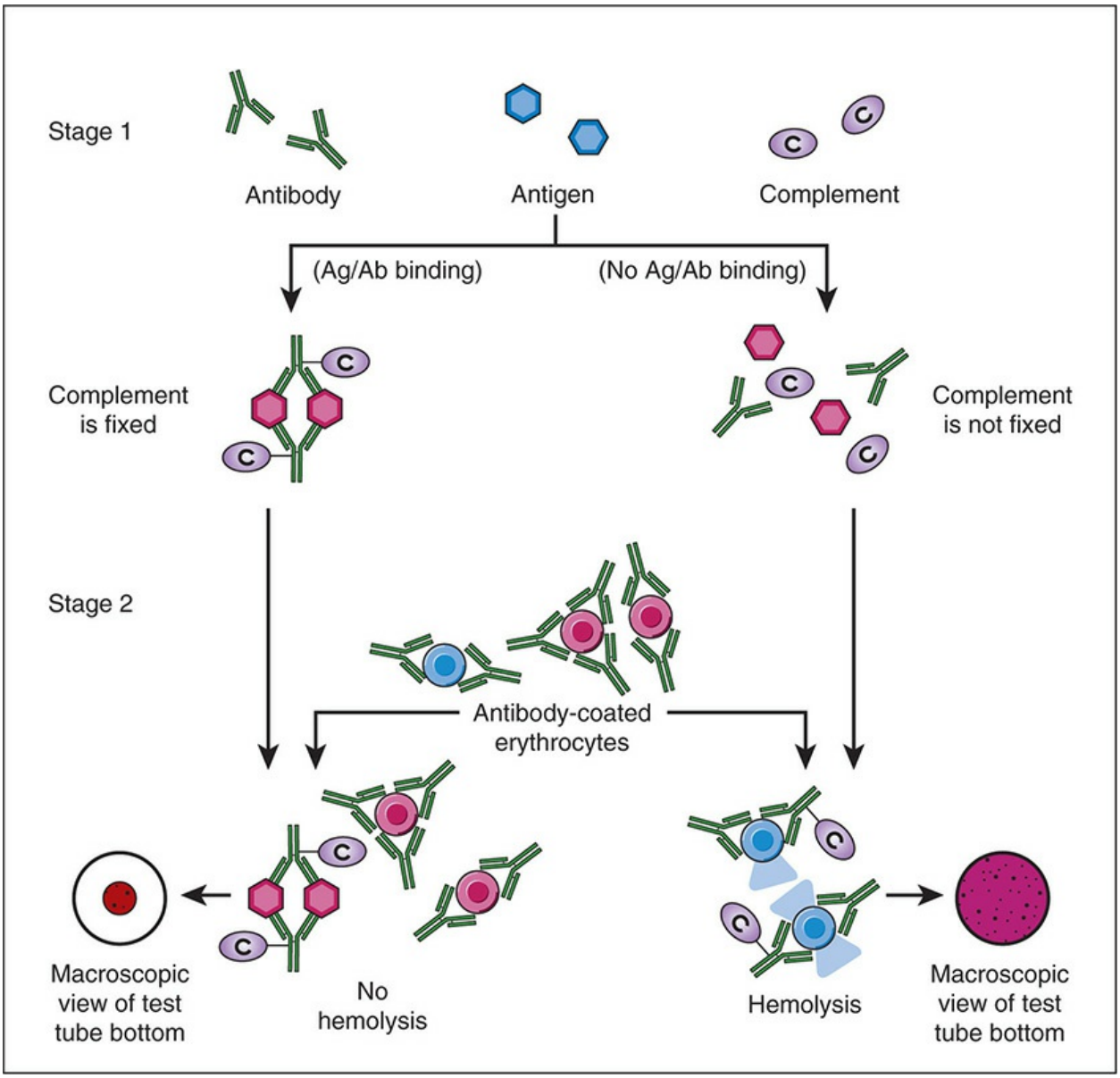
identification of systemic fungal pathogens, the availability of acridinium ester–labeled chemiluminescent nucleic acid probes for these agents offers certain advantages, including the use of younger, less mature colonies, clear-cut results, rapid turnaround time, and specific identification.[168,192]

Complement Fixation and Hemagglutination Inhibition

Over the last three decades, serologic methods for the diagnosis of infectious diseases have changed significantly. Older methods include **complement fixation** (**CF**) and **hemagglutination inhibition (HAI**). CF and HAI can be used either to identify viral antigens (i.e., usually for identification of viruses in tissue cultures) or to detect antibodies in patient serum and certain other specimen types (e.g., cerebrospinal fluid [CSF]). These tests have been widely used to retrospectively document infections, particularly those caused by microbes that are not easily detected by culture methods. In clinical laboratories, these methods have been largely superseded by other serologic methods (e.g., EIAs) and by nucleic acid–based methods of pathogen detection (e.g., polymerase chain reaction [PCR]).

In order to detect antibodies in patient serum, the serum specimen is first heated at 56°C for 30 minutes to inactivate endogenous complement, and an aliquot is placed in

the well of a microtiter plate. A titered amount of complement and antigen (specific for the antibody to be detected) are added to the microwell, mixed, and incubated overnight. If antibody in the patient serum complexes with (i.e., recognizes) the antigen, the two molecules combine and complement becomes "fixed' to this antigen–antibody complex. If antibody against that antigen is not present, no immune complexes form and complement remains free or "unfixed." In the next step, red blood cells (RBCs, generally from sheep) that are "sensitized" with anti-RBC antibodies are added to the microtiter well. If the complement was bound to immune complexes during the first step, it is not available to bind to the antibody-coated erythrocytes; so the sensitized RBCs are not lysed and settle to the bottom of the well as a "button." Conversely, if immune complexes were not formed during the first step, complement is free to bind to the antibody-coated RBCs, resulting in lysis of the erythrocytes. Lysis of the cells produces a diffuse reddish discoloration of the reactants within the microtiter well. The CF procedure is outlined in Figure 3-2 and presented in Chart 3-1.



■ **FIGURE 3-2** CF testing. Antigen, antibody, and complement are mixed in stage 1. If the antigen and antibody bind, complement is fixed and will be unable to act on antibody-coated erythrocytes added in stage 2. The final reaction appears as an absence of hemolysis. If the antigen and antibody do not bind in stage 1, complement is not fixed and remains free to act on the antibody-coated erythrocytes added in stage 2. The final result appears as hemolysis of the erythrocytes. Unhemolyzed cells settle into a button in the center of the bottom of the test tube, whereas hemolyzed cells produce a reddish discoloration of fluid, with very few, if any, cells settled in the center of the bottom of the tube. Ag/Ab, antigen-antibody. (Redrawn from Leland DS. Clinical Virology. Philadelphia, PA: Saunders, 1996.)

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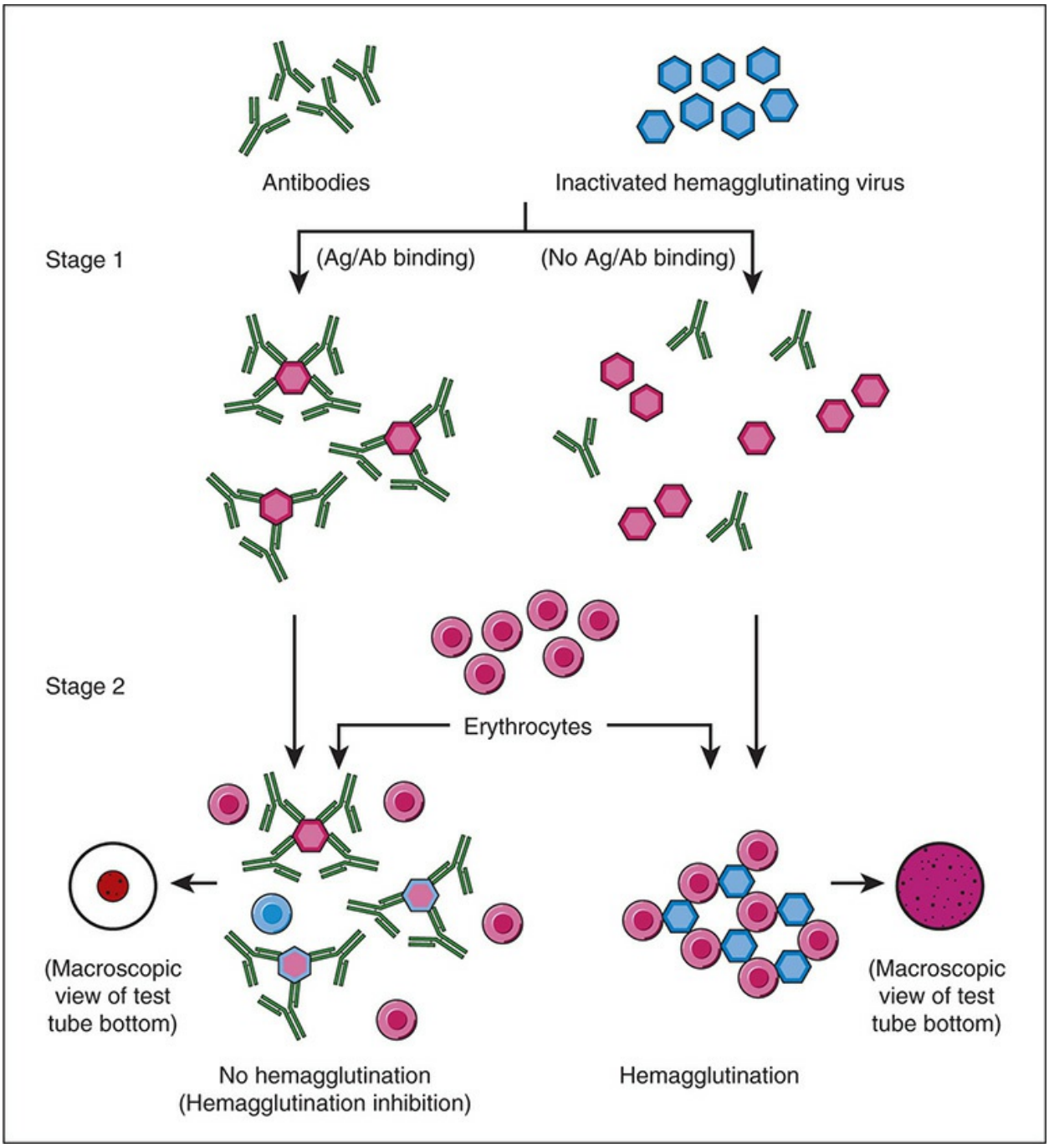
The CF test continues to be useful for the diagnosis of certain infections, particularly histoplasmosis and coccidioidomycosis. For histoplasmosis, complement-fixing antibodies appear 4 to 8 weeks after infection and persist for years.[192] A fourfold rise in the CF titers or a single titer of 1:32 or greater suggests active infection. The CF test is less sensitive for diagnosis of histoplasmosis in immunocompromised hosts who may not mount an adequate immune response. In one study, the antibody response in patients with AIDS and disseminated histoplasmosis ranged from 67% to 80% compared with 86% in patients without underlying immunosuppression.[195] In coccidioidomycosis, elevated levels of antibodies can be detected by CF in over 90% of cases; CF titers decline with effective therapy and rise in cases of relapsing disease or treatment failure.[6,192] As with histoplasmosis, the sensitivity of the CF test is lower in immunocompromised patients (e.g., AIDS, transplant recipients).[5,19,166] The CF test is also useful for the diagnosis of coccidioidal meningitis. Complement-fixing antibodies can be detected in the CSF in more than 80% of cases.[73] CF antibody titers in the CSF decline with adequate therapy and increase with central nervous system disease progression.

Certain viruses (e.g., rubella, influenza/parainfluenza viruses, respiratory syncytial virus [RSV], measles, mumps) have surface antigens that are hemagglutinins; that is, they are able to bind to the *N*-acetylneuraminic acid on RBCs and form cross-linkages that result in macroscopic agglutination. Antibodies directed against a specific virus bind to these hemagglutinins, thereby inhibiting macroscopic agglutination of the RBCs. This reaction is the basis of the HAI assay for detection of viral antibodies (Fig. 3-3).[42,130] Patients with infections caused by hemagglutinating viruses can be diagnosed retrospectively by detecting a fourfold or greater rise in the titer of antibodies that inhibit the ability of the virus to agglutinate erythrocytes. Although antibody detection for rubella and other viral agents is now done by EIA or indirect



■ **FIGURE 3-3** HAI testing is performed in two stages. In stage 1, hemagglutinating viruses and antibodies are mixed. In stage 2, erythrocytes are added. If the antibodies bind to the virus in stage 1, the virus is inhibited and fails to hemagglutinate the erythrocytes. If the antibodies do not bind to the virus in stage 1, the virus remains active and hemagglutinates the erythrocytes in stage 2. Hemagglutination appears as a layer or "shield" of tiny aggregates in the bottom of the tube. Unagglutinated cells settle into a button in the center of the bottom of the tube. Ag/Ab, antigen-antibody. (Redrawn from Leland DS. Clinical Virology. Philadelphia, PA: Saunders, 1996.)

immunofluorescence in most clinical laboratories, HAI is still performed for standardization and validation of new assays (e.g., EIAs for viral serology) and in public health and reference laboratories for surveillance and diagnosis (e.g., serology for *Flaviviridae*, *Bunyaviridae*, *Togaviridae*).[42,68,72,122,130] The HAI procedure is presented in Chart 3-2.

Agglutination Reactions

Agglutination reactions can be defined as the specific immunochemical aggregation of particles (erythrocytes, latex particles, staphylococcal cells) coated with antigen or antibody that can be used to detect either soluble antibodies or antigens, respectively. Antigens or antibodies are attached to these particles either by intramolecular electrical forces or by covalent bonds. Clumping of the carrier particles occurs as an indicator of antigen–antibody interactions occurring on the surface of the carriers. Initially, erythrocytes were used as carriers. These erythrocytes had to be treated with tannic acid or other agents to stabilize the cell membrane, and then formalinized to increase the nonspecific absorption of peptides, proteins, and polysaccharides. For some proteins, chemical coupling agents were used to covalently link the proteins to the red cells; these agents worked for some proteins, but not for others. In 1955, uniformly sized (i.e., 0.81 nm diameter) polystyrene latex beads were demonstrated to be the ideal carrier for antibody molecules, since the hydrophobic surfaces of the beads specifically and irreversibly bind to the Fc region of IgG molecules. Antigens such as complex polysaccharides and proteins could also be linked to latex beads via sulfate groups that are present on the surface of the beads as a result of the manufacturing process. At present, latex beads are the most commonly used carriers in agglutination assays. Latex agglutination tests are usually performed on slides or plastic-coated cardboard cards. Latex beads coated with specific antibodies are the basis for cryptococcal antigen (capsular galactoxylomannan) latex agglutination tests (e.g., Cryptococcal Antigen Latex Agglutination System [CALAS], Meridian Bioscience, Inc., Cincinnati, OH; Crypto LA, International Biological Laboratories, Inc., Cranbury, NJ), latex particle agglutination assays for direct detection of group A streptococci in throat swab specimens, and commercial products for serologic grouping of β-hemolytic streptococci (e.g., Slidex Strepto-Kit, bioMérieux, Inc., Marcy l'Étoile, France; Streptex Streptococcal Grouping kit, Remel, Lenexa, KS).[9,33,93,163,174] Latex agglutination tests were the first-generation rapid tests for direct detection of group A streptococcal antigen in throat swabs specimens. The sensitivities of these antigen-detection latex agglutination tests ranged from 62% to 96%; specificities in most cases exceeded 97%.[57,93,164] These tests have largely been replaced by lateral flow

immunoassays or other nonculture molecular assays. Latex agglutination is still used for streptococcal grouping from positive cultures and for cryptococcal antigen detection. The cryptococcal antigen test is performed on CSF and serum specimens, but it has also been used for antigen detection in bronchoalveolar lavage (BAL) and other specimen types.[14,143,199] The sensitivities of the commercial cryptococcal latex tests vary from 83% to 97%, with specificities of 93% to 100%, depending on the manufacturer.[174] These tests generally provide a rapid diagnosis of cryptococcal meningitis in over 90% of cases.[192] Latex particles can also be conjugated to antigens, thereby allowing latex agglutination to be used for the qualitative and semiquantitative detection of antibodies (e.g., rubella). These tests were convenient, rapid, easy to perform, detected both IgG and IgM antibodies, and were fairly specific. However, latex agglutination tests were less sensitive for detecting low titers of antibody or seroconversions.[87,92,159] Latex agglutination tests for determining immune status to rubella, cytomegalovirus (CMV), and varicella zoster virus (VZV) are no longer commercially available.

Staphylococci may also be used as carrier agents. Strains of *Staphylococcus aureus* (e.g., ATCC 12498) have a high content of protein A in their cell wall. This protein is able to specifically bind the Fc region of IgG, leaving the Fab regions available for binding of antigen.[48] Because of the nature of protein A and its specific interaction with IgG molecules as a functional carrier, staphylococcal coagglutination is used primarily for antigen detection and is the method used in some commercial kits for serologic grouping of β-hemolytic streptococci (e.g., Phadebact Streptococcus Test, Bactus AB, Huddinge, Sweden). Following extraction of antigen from the organisms by chemical or physical means, the antibody-coated staphylococci are mixed with the extract. Visible agglutination of the staphylococcal cells is a positive test. Commercial coagglutination tests are also available for culture confirmation of *Neisseria gonorrhoeae* and *S. aureus* (i.e., Phadebact Monoclonal GC test, Phadebact Staph Aureus test, Bactus AB, Huddinge, Sweden).

Another type of agglutination reaction is the basis of a technique called **immune electron microscopy** (**IEM**). This technique is used for visual detection of viral agents that are noncultivable, such as hepatitis A, some of the newer agents of hepatitis, and noncultivable gastroenteritis viruses (e.g., Norwalk and Norwalk-like viruses, noroviruses) in stool filtrates.[94,137,148] Specific antiviral antibodies are used to cause aggregation of the viral particles; these aggregates are detected by the electron microscope more easily than individual, dispersed virions. The immunospecific aggregation of viral particles increases electron microscopic detection of viruses by 100- to 1,000-fold, thereby enabling detection of as few as 10^5 to 10^6 virus particles/mL of stool filtrate.

Solid-Phase Immunoassay Methods

EIAs for Antibody Detection

Solid-phase immunoassay refers to the binding of either antigen or antibody to a variety of solid materials, such as polystyrene microtube wells or plastic/latex beads. Solid-phase immunoassays designed for the detection of antibody in an unknown sample have antigen bound to the solid phase. The initial reaction occurs when the specimen to be tested is incubated for a prescribed time in contact with the solid phase. Specific antibody binds to the immobilized antigen. After the reaction mixture is washed to remove extraneous material, an antiglobulin is conjugated with a "tag" and incubated in the reaction vessel. In commercially available EIA systems for detecting human antibodies, the conjugate is frequently

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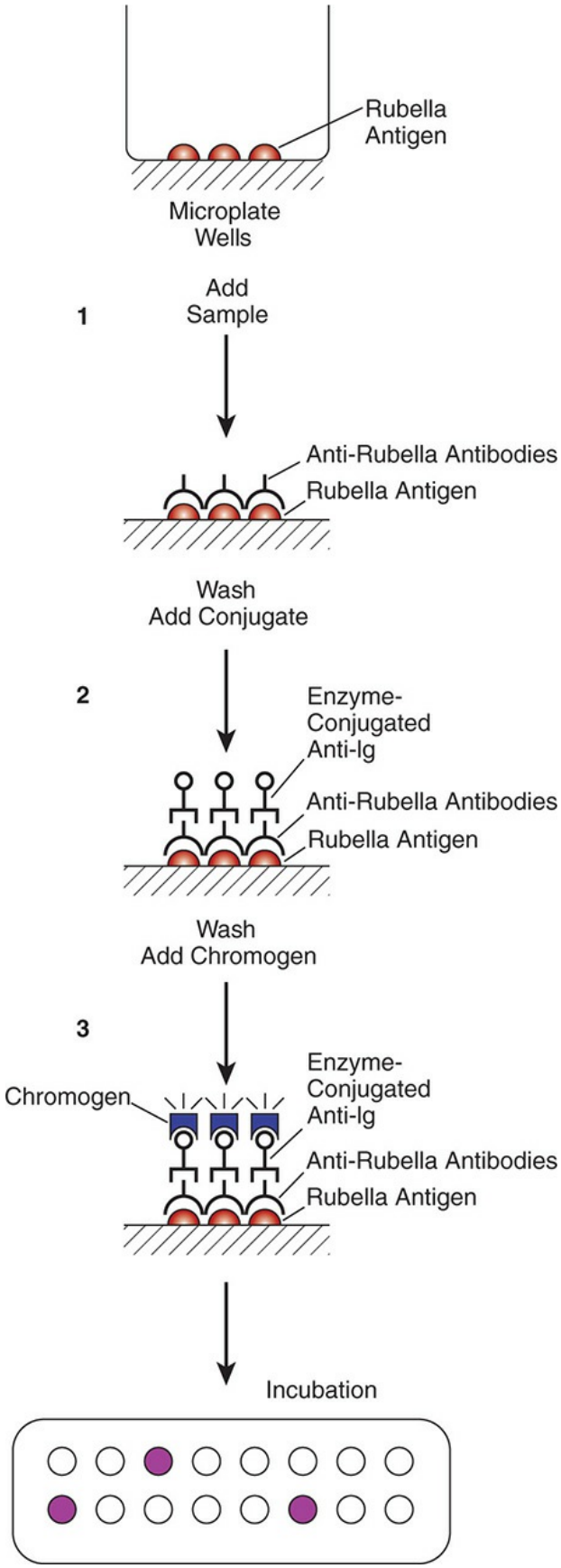
alkaline phosphatase–or horseradish peroxidase–labeled antihuman Ig raised in goats or manufactured as monoclonal antibodies. If the initial antigen–antibody reaction has occurred, the antiglobulin (with its enzyme tag) binds to the antibody. The final step in these assays is the addition of the enzyme substrate, yielding a colored end product that is detected visually or spectrophotometrically. A positive reaction indicates that antibody was present in the original sample, and the intensity of the reaction is proportional to the concentration of antibody in the specimen. A representative EIA procedure for detection of rubella antibodies is shown in Figure 3-4. Commercially available EIA kits for detection of various antibodies in sera are widely available and have supplanted, in many cases, time-consuming and labor-intensive procedures such as CF and HAI for viral serology.

The chemistry of antibody conjugates used in EIA techniques is not limited to antigen/antibody interactions, as exemplified by avidin, streptavidin, and biotin. Avidin and streptavidin are glycoproteins that are purified from egg white and from *Streptomyces avidinii*, respectively. Biotin is a component of the B_2 vitamin complex and is a cofactor involved in CO_2 transfer in a number of carboxylase reactions. Avidin molecules are able to stoichiometrically bind biotin residues in much the same way as an antibody/antigen interaction.[194] In EIA formats, goat antihuman globulin attached to biotin molecules can be used as the conjugate to detect human antibodies that have bound to an antigen fixed to the solid phase. After a wash step, enzyme-labeled avidin (or streptavidin) is added. After incubation and washing, addition of the enzyme substrate yields a colored end product. Alternatively, biotinylated antiglobulin may be detected by adding unlabeled avidin, and, after a wash step, biotinylated enzyme is added. The biotinylated enzyme binds to the avidin, and, after washing,

addition of the enzyme substrate yields a colored end product.

Serologic procedures for antibody detection that are based on EIA technology were designed to increase their utility as specific diagnostic or confirmatory test methods. The Western immunoblot procedure grew out of a need to determine antigenic specificities of antisera and involved the electrophoretic separation of antigens on polyacrylamide gels, followed by electrophoretic transference and replication of the gel pattern on nitrocellulose paper.[103] Probing the transblotted antigens with antisera was then used to detect and characterize the specificities of polyclonal and MAB preparations. The Western immunoblot procedure for HIV-1 antibodies is the best known application of this technique (Fig. 3-5). In the original HIV-1 Western immunoblot procedure, HIV-1 grown in tissue culture is partially purified away from the cell culture and solubilized by detergent treatment (sodium dodecyl sulfate) and a reducing agent (2-mercaptoethanol). Using polyacrylamide gel electrophoresis, the HIV-1 proteins are fractionated based on molecular weight, with low-molecular-weight proteins migrating further than high-molecular-weight proteins and glycoproteins. A sheet of nitrocellulose paper is placed on the gel, and the proteins are electrophoretically "transblotted" onto the nitrocellulose sheet. The sheet is cut into strips for use as "solid phase" in the assay. Serum that is repeatedly reactive in the HIV-1 EIA is diluted and incubated with the nitrocellulose strip. If present, HIV-1 antibodies bind to specific viral antigens on the strip. After washing, the strips are incubated with goat antihuman antibodies conjugated with horseradish peroxidase or alkaline phosphatase. After another wash step, enzyme substrate is added to the strip. Colored bands


Rubella
Antigen
Microplate
Wells
1
Add
Sample
Anti-Rubella Antibodies
Rubella Antigen
Wash
Add Conjugate
2
Enzyme-
Conjugated
Anti-Ig
Anti-Rubella Antibodies
Rubella Antigen
Wash
Add Chromogen
3
Chromogen
Enzyme-
Conjugated
Anti-Ig
Anti-Rubella Antibodies
Rubella Antigen
Incubation

■ **FIGURE 3-4** Principles of EIA. This figure shows the EIA procedure for the detection of antibodies against rubella virus. Purified rubella antigens are absorbed onto microplate wells. In step 1, serum is added and incubated. Antirubella antibodies, if present, bind to the antigen. Following a wash step, antihuman Ig that is conjugated with an enzyme is added (step 2). After a second wash step, a chromogenic enzyme substrate is added. Absorbances for individual wells are read spectrophotometrically, and test results are interpreted by comparison, with positive and negative controls performed in the same test.

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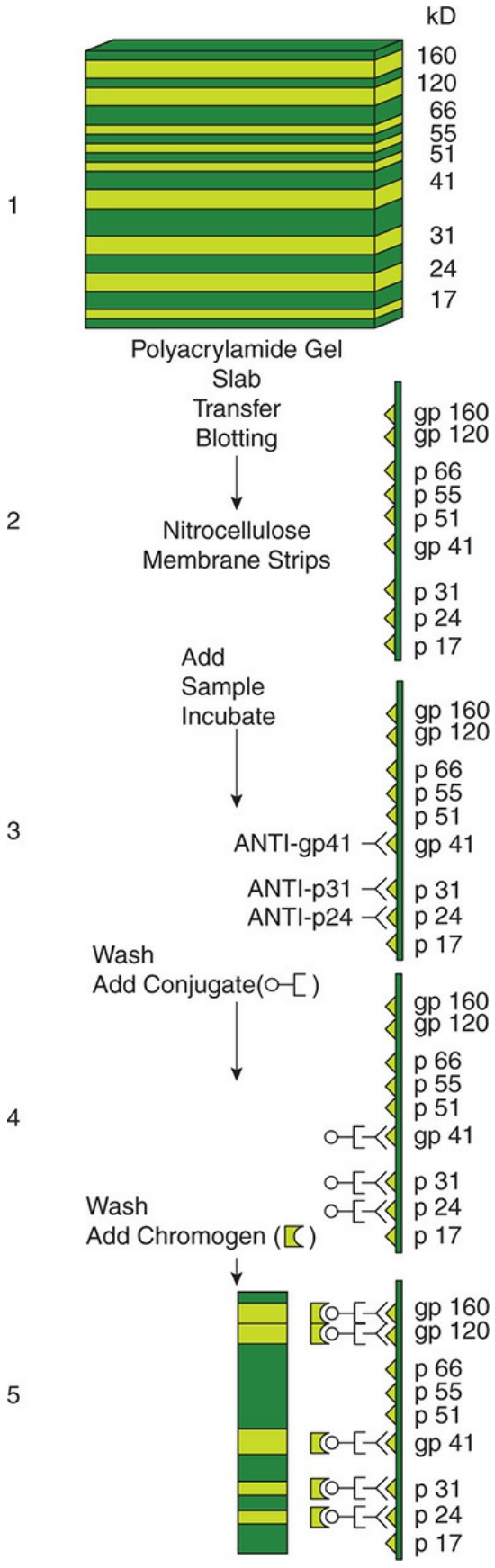
kD
160
120
66
55
51
41
31
24
17
1
Polyacrylamide Gel
Slab
Transfer
Blotting
2
Nitrocellulose
Membrane Strips
gp 160
gp 120
p 66
p 55
p 51
gp 41
p 31
p 24
p 17
Add
Sample
Incubate
3
ANTI-gp41
ANTI-p31
ANTI-p24
Wash
Add Conjugate(
)
4
Wash
Add Chromogen (
)
5

■ **FIGURE 3-5** Western blot technique for detection of anti-HIV-1 antibodies. In step 1, virus that is grown in tissue culture is solubilized, partially purified, and subjected to electrophoresis in a polyacrylamide slab gel. This separates the viral proteins and glycoproteins by their molecular weights. In step 2, the antigens in the gel are electrophoretically "transblotted" onto a nitrocellulose sheet, which is then cut into strips. The strips are then incubated (step 3) with the test sample (serum). After washing away unbound material, an enzyme-labeled conjugate is added (step 4). This material binds to antibodies from the serum sample that have bound to the strip. After another wash step, the enzyme's chromogen substrate is added (step 5), and colored bands appear on the strip at the sites of initial antibody reactivity. In this diagram, reactivity is shown with gp41, p24, and p31, confirming that the serum sample contains anti-HIV-1 antibodies. (Reprinted, with permission, from Sandler SG. Xxxxx. In: DeVita VI Jr, Hellman S, Rosenberg SA, eds. AIDS Etiology, Diagnosis, Treatment, and Prevention. 2nd Ed. Philadelphia, PA: Lippincott, 1988:128.)

appear on the strip in areas where an initial antibody–antigen reaction has occurred. The position of the bands and comparison of the patterns with positive control samples enable reactivity of a specimen with specific viral antigens to be assessed and interpreted.[128] The HIV-1 Western blot remains the "gold standard" confirmatory test for HIV-1 infection and has a specificity of over 99%.

Immunoblot techniques can also be used to assess specificities of antibodies against other agents. For example, many EIAs that are used to detect antibodies against herpes simplex viruses (HSVs) are not able to differentiate HSV-1- or HSV-2-specific antibodies. Newer, second-generation microtiter-based EIAs were introduced that used purified gG1 and gG2, HSV-1- and HSV-2-specific glycoproteins, to capture virus type–specific antibodies in serum specimens.[38,203] Current immunoblot methods that detect antibodies against several virus type–specific antigens have greater than 98% sensitivity and specificity in differentiating antibodies against HSV-1 and HSV-2.[109,176] Immunoblot technology has also been evaluated as a test format for syphilis diagnosis. Antibodies directed against immunodeterminant *Treponema pallidum* antigens with molecular weights of 15.5, 17, 44.5, and 47 kDa are diagnostic of acquired syphilis when an anti-IgG conjugate is used.[123] When developed with anti-IgM conjugate, the immunoblot procedure is a sensitive and specific assay for the diagnosis of congenital syphilis.[67] Because of the relative insensitivity of EIA and immunofluorescence techniques, immunoblot procedures have also emerged as a sensitive and specific method for serodiagnosis of Lyme disease caused by *Borrelia burgdorferi*, and diagnostic criteria based on immunoblot banding patterns have been established by the Centers for Disease Control and Prevention.[25] Immunoblot methods have contributed to the diagnosis of cat scratch disease by identifying immunodominant antigens of

Bartonella henselae, the agent of cat scratch disease, that are most useful for diagnosis. These antigens could be used for development of EIA or indirect fluorescent antibody (IFA) tests that would provide a sensitive and specific serologic diagnosis.[97,112,187]

Both EIA and immunoblot test systems for the detection of HIV-1 antibodies have undergone several modifications since their introduction in 1984.[135] Initially, test kit manufacturers used lysates prepared from HIV-1-infected cultured T-lymphocyte cell lines as the source for antigens in both the first-generation EIA and immunoblot procedures. Biological false-positive reactions were seen because of the reactivity of antibodies directed against HLA (human leukocyte antigen) proteins expressed by the lymphoid cell lines used to grow the virus. Subsequently, recombinant antigens were obtained by cloning viral genes into bacteria or yeast expression systems using plasmid vectors. Use of these antigens resulted in the development of second-generation EIAs with even greater sensitivity and specificity than the first-generation assays.[35] In these assays, cross-reactions with contaminating bacterial or yeast antigens, however, may be a cause of false-positive reactions. Purification and amino acid synthesis of retroviral antigens then led to the use of synthetic peptides as antigens in the EIA procedure (third-generation assays). These antigens can be produced in large quantities and show little lot-to-lot variability. Because of their purity, indeterminate or atypical reactions due to contaminating components in lysate- and recombinant-based tests are minimized. Although the first- and second-generation assays detected primarily

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IgG, the third-generation assays detect all classes of antibody (i.e., IgG, IgM, and IgA) to HIV-1 present in serum or saliva.[16] Third-generation HIV-1 EIAs also differ from earlier assays in their methodology; synthetic viral antigens fixed to the solid phase initially "capture" anti-HIV-1 antibodies, and the bound antibodies are detected by adding enzyme-labeled HIV-1 antigen instead of an enzyme-labeled antiglobulin. Fourth-generation HIV-1 EIAs, which are now approved by Food and Drug Administration (FDA), not only detect antibodies against HIV-1, but also have the ability to detect p24 antigen, which is present in blood during acute HIV-1 infection before the formation of antibodies.[141,142,181,189,190,191] These assays will help detect HIV-infected patients who present in the window period, the time between infection and the production of HIV-1-specific antibodies by 4 to 5 days. Western immunoblot–like assays have also been designed that use recombinant or synthetic peptides bound to nitrocellulose strips instead of transblotted viral lysate antigens.[110,116,140,183,201,202] These assays, termed *line immunoassays*, generally have fewer false-positive reactions than the standard HIV-1 Western blot because of the absence of cellular

proteins that are usually found associated with viral lysate–based immunoblot procedures. These novel immunoassays also include recombinant/synthetic peptides for serologic confirmation of HIV-2 infections (RIBA HIV-1/HIV-2 SIA, Chiron Corp., Emeryville, CA; Innolia HIV-1/HIV-2, Innogenetics, Antwerp, Belgium; Lia-Tek HIV III immunoblot assay, Organon Teknika, Turnhout, Belgium)

EIA Antibody-Capture Methods for IgM Detection

EIA methods for antibodies detect primarily IgG. However, specific IgM detection can be used to help differentiate recent from past infections because only IgM is present early in the course of most infections. Methods specific for IgM detection obviate the need for testing of acute and convalescent specimens for specific IgG. In congenital infections, detection of specific IgM in the fetal/neonatal blood indicates active infection rather than transplacental antibodies. Initially, EIA formats for IgM detection used an enzyme-labeled anti-IgM antibody as the conjugate. However, if IgG and IgM are present together in the same specimen, the smaller, more numerous IgG molecules effectively compete for binding sites on the antigen fixed to the solid phase, resulting in no binding of the anti-IgM conjugate and a false-negative IgM test result. In addition, rheumatoid factor in the specimen may interfere with the assay as well. Rheumatoid factors are antibodies primarily of the IgM class that are directed against IgGs of any specificity. If serum contains both IgG and rheumatoid factor, the smaller, specific IgG molecules bind to the antigen on the solid phase, and the rheumatoid factors, in turn, bind to the IgG. Addition of a labeled anti-IgM conjugate results in its binding to the rheumatoid factor, with the subsequent generation of a false-positive result on addition of the enzyme substrate.

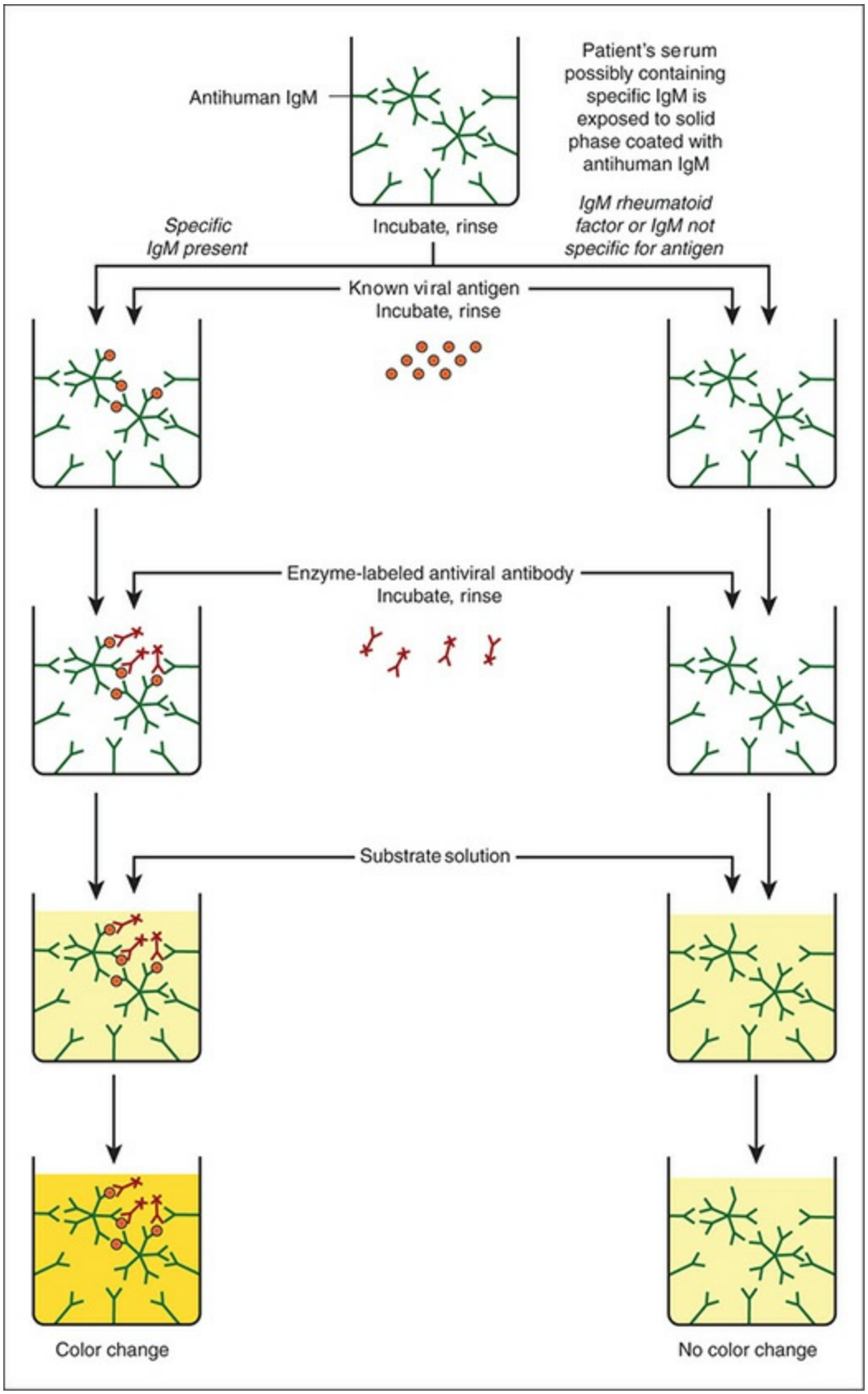
In order to detect IgM in patient sera, several methods have been developed to separate IgM from IgG. Ion exchange column chromatography and gel filtration separate Ig classes based on molecular size and charge. Using buffers of varying ionic strength, IgM can be eluted from these columns and separated from IgG. Sucrose gradient density centrifugation also separates the molecules based on size, with IgM molecules traversing further in the sucrose gradient than the lighter, smaller IgG molecules. Serum specimens may also be treated with anti-IgG antibodies, resulting in binding of IgG into immune complexes and effectively preventing these antibodies from reacting in IgM assays. Staphylococcal protein A, either mixed directly with the serum specimen or incorporated into a gel filtration column, may also be used to “bind up” IgG molecules in patient specimens. However, these separation methods pose other technical problems. None of the methods are totally effective in separating IgM from IgG; so IgG interference with IgM detection may still be a problem even after specimen pretreatment. In addition, all of these techniques result in dilution of the serum specimen. Because IgM may be present in very low levels, specimen dilution may result in false-negative IgM test results even when agent-specific IgM actually is

present. Accurate and reliable laboratory testing for IgM is of considerable importance for the diagnosis of congenital rubella syndrome, because the risk of serious damage to the fetus is substantial. Termination of pregnancy is the recommended approach if congenital rubella syndrome is diagnosed antepartum.

The IgM detection method of choice is an "antibody-capture" method (Fig. 3-6). In this modification, IgG directed against IgM is bound to the solid phase. Incubation of the specimen results in binding of all IgM in the specimen aliquot to the immobilized IgG. After a wash step, the specific antigen is added to the microtiter well and becomes bound to IgM antibody molecules having that particular antigenic specificity. Subsequent addition of an enzyme-labeled IgG antibody conjugate directed against another epitope of the antigen results in indirect, enzymatic labeling of the antigen-specific IgM, yielding a colored end product after the addition of enzyme substrate. Captured rheumatoid factor or IgM antibodies of other antigenic specificities do not bind the conjugate to produce a colored end product. In recent years, IgM-capture EIAs have become commercially available for a variety of viral infections.[1,153,198]

EIAs for Antigen Detection

EIA formats may be modified for detection of antigens rather than antibodies. In this case, a "capture" antibody is fixed to the solid phase (Fig. 3-7); in most EIA formats, the solid phase is a well of a microtiter tray. Incubation with a specimen containing the antigen results in binding of the antigen to antibody. After a wash step, incubation with enzyme-labeled (or biotinylated) antibodies against a second epitope of the antigen results in binding of that antibody. After washing, enzyme substrate (or enzyme conjugated to avidin) is added, and subsequent addition of enzyme substrate yields a colored end product. Detection of this end product is usually determined spectrophotometrically; cut-off values for positive and negative results are determined by positive and negative controls or calibrators run simultaneously. Antigen-capture EIAs are available for the detection of diverse microbial pathogens or virulence factors in several specimen types such as diarrheal stool (e.g., EIAs for Shiga-like toxins of enterohemorrhagic *E. coli,* organism-specific antigens of *Giardia*, *Cryptosporidium*, and *Helicobacter pylori,* rotavirus antigen, and *Clostridium difficile* antigens and toxins), urine (EIAs for *L. pneumophila* serogroup 1 antigen), and serum (*Aspergillus* galactomannan [GM] and HIV-1 p24 antigen). A diagram of a typical antigen-capture assay for detection of antigen is presented in Figure 3-7.


Antihuman IgM
Patient's serum possibly containing specific IgM is exposed to solid phase coated with antihuman IgM
Incubate, rinse
Specific IgM present
IgM rheumatoid factor or IgM not specific for antigen
Known viral antigen
Incubate, rinse
Enzyme-labeled antiviral antibody
Incubate, rinse
Substrate solution
Color change
No color change

■ **FIGURE 3-6** IgM-capture EIA. Patient's serum possibly containing IgM is exposed to antihuman IgM attached to a solid phase. Any IgM in the serum is "captured" or bound by the antihuman IgM. Subsequent steps in the assay (addition of known viral antigen, enzyme-labeled antiviral antibodies, and substrate) are performed to determine the specificity of the captured IgM. Captured IgM of the proper specificity will produce a final result of color change. Captured IgM of other specificity, including rheumatoid factor, will not produce an end result of color change. (Redrawn from Leland DS. Clinical Virology. Philadelphia, PA: Saunders, 1996.)

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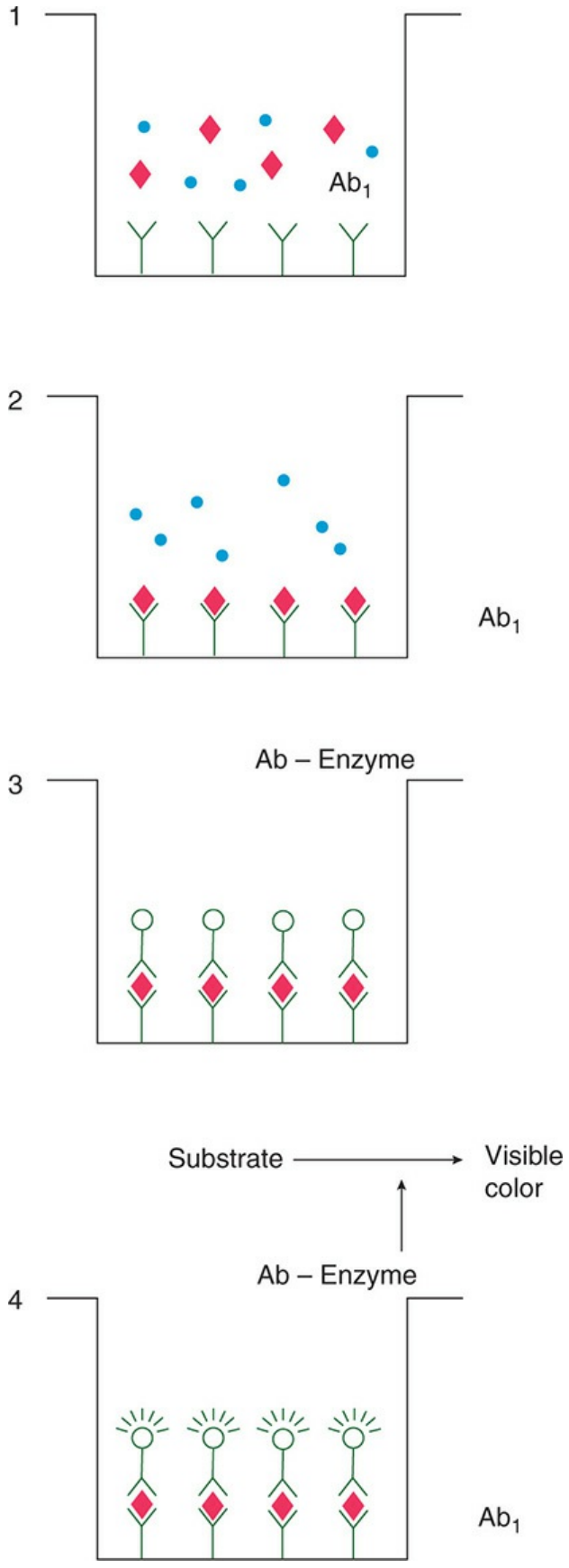
1
Ab1
2
Ab1
Ab – Enzyme
3
Substrate
Visible color
Ab – Enzyme
4
Ab1

■ **FIGURE 3-7** EIA antigen-capture technique for *H. pylori*. In this technique, antibody directed against the antigen to be detected is bound to the solid phase. A stool specimen is added to the well (step 1). Antigens present in the urogenital sample are "captured" by the solid-phase antibody. After a wash step, anti -*H. pylori* antibody that is conjugated to an enzyme is added (step 2) and reacts with the solid-phase antibody-bound antigen. After another wash step, enzyme substrate is added (step 3), and a visible color is detected.

Gastroenteritis is associated with bacterial, parasitic, and viral infections, and antigen-detection EIAs have been developed that address common agents in all of these organism groups. Food-borne enterocolitis and hemolytic-uremic syndrome in children are associated with *E. coli* O157:H7, but these syndromes can also be caused by non-O157:H7 *E. coli* that produce Shiga-like toxins. Sensitive and specific EIAs are available that detect *E. coli* O157:H7 (Premier *E. coli* O157 EIA, Meridian Bioscience, Inc.) or Shiga toxins 1 and 2 (Premier EHEC EIA, Meridian Bioscience, Inc.; ProSpecT Shiga Toxin *E. coli* (STEC), Alexon-Trend, Ramsey, MN) directly in stool specimens.[56,66,80,102] These EIAs can be performed directly on diarrheal stool, or on MacConkey broth enrichment cultures. The Premier EHEC EIA uses monoclonal antibodies directed against Shiga toxins 1 and 2 as capture antibodies, and polyclonal antibodies tagged with horseradish peroxidase for detection of bound toxins. The ProSpeCT STEC test uses polyclonal antibodies against Shiga toxins 1 and 2 as capture antibodies, and horseradish peroxidase–labeled monoclonal antibodies to detect the bound toxins. These assays have sensitivities of 79% to 87% when stools are tested directly and sensitivities of greater than 98% when stools are tested after overnight broth enrichment. Specificities of these assays with either specimen type exceed 97%. A microplate EIA is also available for detection of *Giardia lamblia*- and *Cryptosporidium*-specific antigens in stool specimens. The ProSpecT *Giardia* and *Cryptosporidium* microplate assays (Remel) have sensitivities of 70% to 80% for *Cryptosporidium* and over 90% for *Giardia,* with specificities of greater than 99% when compared with microscopy.[52,74,167,205] EIAs for these agents may not be positive if the numbers of parasites in the specimen are small. EIAs for detection of rotavirus in diarrheal stool have been in use in clinical laboratories for many years. When compared with IEM and reverse transcription PCR (RT-PCR) assays for rotavirus, the Premier Rotaclone assay (Meridian Bioscience, Inc.) has sensitivities and specificities of 95% to 100% and 99%, respectively.[39,96]

H. pylori is a common human pathogen that is associated with gastroduodenal syndromes, including dyspepsia, chronic gastritis, peptic ulcer disease, gastric cancer, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma.[170]Diagnosis of *H. pylori* infection is accomplished by invasive measures involving endoscopy and gastric biopsy, or noninvasive methods such as

the ^{13}C-urea breath test and stool antigen tests. These tests are used for diagnosis, and have also been assessed for their ability to detect persistent infections following eradication therapy. Although serology for antibody detection is often used to determine whether an individual has been infected with *H. pylori*, serology cannot be used to determine eradication following treatment, because declines in antibody titers vary from person to person depending on the intervals between follow-up visits.[179] EIAs detect antigens of *H. pylori* in fecal specimens and use either immunoaffinity-purified polyclonal anti- *H. pylori* rabbit antibodies (Premier Platinum HpSA, Meridian Bioscience, Inc.) or monoclonal antibodies (Amplified IDEIA Hp StAR, DakoCytomation/Oxoid Ltd., Cambridge, UK; FemtoLab *H. pylori*, Connex, Martinsried, Germany) adsorbed to the microtiter well solid phase. In evaluations, the Premier Platinum HpSA assay has demonstrated sensitivities ranging from around 80% to over 95%, with corresponding specificities ranging from 79% to 99%, when compared with the ^{13}C-urea breath test.[59,91,146,177,178] EIAs that use monoclonal antibodies for antigen capture are comparable or slightly more sensitive and specific than the polyclonal antibody

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assays.[23,107] In the United States, the Premier assay is more widely used than other microtiter EIA methods, and even this test is being replaced by lateral flow immunoassays for *H. pylori* antigen in many laboratories (see below).

Legionella infections are diagnostic dilemmas for the clinician and the laboratory. *Legionella* species are increasingly being recognized as agents of community-acquired, travel-associated, and nosocomial pneumonias in the United States and other countries.[18,24,119] *Legionella* species are responsible for 2% to 5% of all community-acquired pneumonias, and more than 90% of these cases worldwide are due to *L. pneumophila*. Of these infections, 70% to 80% are caused by *L. pneumophila* serogroup 1.[24,41,47] Immunosuppressed patients (i.e., organ transplantation, corticosteroids, hematological malignancies, bone marrow transplants) are also at risk. Many laboratories do not offer *Legionella* culture, as culture methods are laborious, requiring preanalytical specimen processing, inoculation of selective/nonselective media, and technical expertise for recognition and identification. Serologic diagnosis is retrospective and not clinically useful in the acute setting. Development and availability of EIAs that detect soluble antigens of *L. pneumophila* serogroup 1 in urine has changed the clinical and laboratory approaches to diagnosing *Legionella* infections. Urine EIA tests detect Pontiac MAB type 2 of *L. pneumophila* serogroup 1, but are less sensitive for other *L. pneumophila* serogroup 1 MAB types, and are insensitive for the detection of nonserogroup 1 *L. pneumophila* and other *Legionella* species. *L.*

pneumophila urine antigen EIAs use either microtiter (Biotest *Legionella* urinary antigen EIA [Biotest AG, Dreieich, Germany]; Binax *Legionella* urinary antigen EIA [Binax, Portland, ME]; Bartels ELISA *Legionella* urinary antigen EIA [Intracel, Frederick, MD]) or an immunochromatographic strip (Binax NOW *Legionella* urinary antigen ICT) formats. In a side-by-side comparison, the Binax assay demonstrated sensitivities of 63.7% and 88.9% when unconcentrated and concentrated (25-fold by ultrafiltration) specimens were tested, respectively.[43] Corresponding sensitivities for the Biotest EIA were 66.7% and 86.7%, respectively. Both assays were 100% specific. An evaluation of the Bartels EIA reported similar sensitivity and specificity consistent with the other two commercial EIAs.[61] A meta-analysis of all *Legionella* urinary antigen assay evaluations found a pooled sensitivity of 74% (range, 68% to 80%) and a specificity of 99.1% (range, 98.0% to 99.7%) for detection of *L. pneumophila* serogroup 1 antigen, with no statistically significant differences among the various brands.[162] These assays are best used to rule in rather than rule out *Legionella* infections.

The detection of *C. difficile* toxins A and B in stool specimens by antigen-capture EIA has been used in clinical laboratories for several years. These tests originally were validated against the cell-culture cytotoxin assay, which has sensitivities ranging from 80% to 100% and specificities of 98% to 99%, depending on the reference method employed.[118,136,152] *C. difficile* toxin microwell EIAs include the Meridian Premier Toxin A and B (Meridian Bioscience, Inc., Cincinnati, OH), TechLab Tox A/B (TechLab, Blacksburg, VA), and the VIDAS EIA (bioMérieux, Inc., Marcy l' Étoile, France), with the former two assays being most widely used. The sensitivities and specificities of the Premier assay range from 80% to 99% and 94% to 99%, respectively, when compared with the cell-culture cytotoxicity assay.[118,127] Similar sensitivities of 75% to 86% and specificities of 92% to 100% have been reported for the TechLab A/B assay.[111,155] Recent increased morbidity and mortality due to *C. difficile* infection and recognition of emergent strains of *C. difficile* that demonstrate significantly greater cytotoxin production (the NAP1/BI/027 *C. difficile* strain known by its restriction endonuclease pattern [BI], its pulsed-field gel electrophoresis pattern [North American PFGE type 1], and its PCR ribotype [027]) stimulated the development of more rapid and sensitive diagnostic approaches, including the use of toxigenic culture.[12,98,113,188] Toxigenic culture refers to isolation and identification of *C. difficile* from diarrheal stool specimens followed by in vitro detection of toxin production by the isolate using either EIA or the cell-culture cytotoxicity assay. Comparisons of EIA methods with toxigenic culture showed that the toxin EIAs were not as sensitive as once thought.[30,31,139] In order to optimize detection, the use of another EIA, the glutamate dehydrogenase (GDH) assay, was introduced. GDH is a cell wall enzyme that is found in both toxigenic and nontoxigenic *C. difficile* isolates.

This test was introduced as a screening test, so that only GDH-positive specimens would be tested by either toxin EIA or cytotoxicity assays. Unfortunately, GDH tests from different manufacturers vary in their sensitivity as well. The development, availability, and FDA clearance of commercial, highly sensitive molecular assays for *C. difficile* toxin gene detection have eclipsed the diagnostic utility of many *C. difficile* toxin A/toxin B EIAs.[136] Laboratories that lack molecular testing capabilities have formulated stepwise algorithms using EIAs for GDH followed by toxin A/toxin B EIAs to increase the sensitivity, specificity, predictive values, and accuracy of *C. difficile* disease diagnosis.[86,124,147,152,156,160]

In mycology, several EIA assays are now available to assist in the diagnosis of endemic and opportunistic fungal infections. These tests include a cryptococcal antigen EIA, *Histoplasma* antigen EIA, and the GM EIA for detection of invasive *Aspergillus* infection. The Premier cryptococcal antigen EIA (Meridian Bioscience, Inc.) uses a polyclonal-capture Ig and a monoclonal detection antibody and can be performed on both CSF and serum specimens with no pretreatment steps. This assay has demonstrated comparable performance with the cryptococcal antigen latex agglutination assays.[10,45,49,174] The cryptococcal antigen EIA is not affected by rheumatoid factor, syneresis fluid, or serum macroglobulins, and quantitative titers tend to be higher than those obtained with the latex agglutination test. The GM EIA (Platelia *Aspergillus* EIA, Bio-Rad, Redmond, WA) is a microtiter EIA for detection of GM of *Aspergillus* species in serum specimens of at-risk patients. The test uses a galactofuranose-specific rat MAB that recognizes the 1→5-β-D-galactofuranose side chains of the *Aspergillus* GM to capture and detect GM in serum specimens. This test was FDA cleared for use in the United States in 2003. When initially released, centers in Europe were using the cut-off value suggested by the manufacturer (positive: OD values >= 1.5); in the United States, the FDA approved an OD cut-off value of 0.5 for test interpretation. With lower cut-off values, test sensitivity was increased, but specificity was decreased.[138] In a European study of adult hematology patients, lowering the cut-off value increased the test sensitivity from 76.3% to 97.4%, but decreased the overall specificity from 97.5% to 90.5%.[105] Testing of two consecutive serum specimens using the lower cut-off value resulted in improved test specificity (97.5%) and predictive value of

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positive test results (87.5%). In general, sensitivity of the GM EIA ranges from 57% to 100%, with specificity ranging from 66% to 100%; both of these parameters are influenced by the use of prophylactic and empirical antifungal therapies in patients at risk for invasive aspergillosis.[70,108,114] Sensitivities and specificities exceeding 85% to

90% have been reported for the GM EIA among patients with hematological malignancies and recipients of allogeneic hematopoietic stem cell transplants.[79,106,138,171]

The Platelia GM EIA has also been applied to other clinical specimens, such as BAL fluid. In one study of 333 BAL specimens from 116 lung transplant patients (including 9 BALs from patients with proven invasive aspergillosis), the sensitivity and specificity of the GM assay were 60% and 95%, respectively.[70,71] The EIA also compared well with molecular assays for detecting evidence of *Aspergillus* in BAL fluids. In a study of 49 hematopoietic stem cell transplant patients with proven/probable invasive pulmonary aspergillosis and 50 patients without the disease, the sensitivity of the GM EIA was 76% (using an index cut-off of 0.5) and the specificity was 94%, while the sensitivity and specificity of PCR were 67% and 100%, respectively.[117] In the same study, the sensitivities of GM EIA and PCR with 22 culture-negative BAL specimens from patients with invasive aspergillosis were 59% and 36%, respectively. The GM EIA has some documented limitations that are a matter of concern. Several other filamentous fungi have been demonstrated to produce antigens that cross-react with *Aspergillus* GM. Giacchino and colleagues described three cases of invasive *Geotrichum capitatum* infections in leukemic patients where the GM EIA was positive.[58] Subsequently, Wheat and colleagues found false-positive GM EIA results with serum specimens obtained from patients with disseminated histoplasmosis.[193] In vitro studies using culture filtrates have demonstrated additional test cross-reactivity with antigens derived from cultures of *B. dermatitidis*, *Nigrospora oryzae*, *Paecilomyces lilacinus*, *Penicillium chrysogenum*, and *Trichothecium roseum*.[32] Kitasato and colleagues reported a false-positive GM immunoassay result in a patient with pulmonary actinomycosis, and lipoteichoic antigens from *Bifidobacterium* may cause false-positive GM EIA results.[82,115] In addition, false-positive GM EIA results have been reported because of cross-reactivity of mold-derived antimicrobial agents such as amoxicillin, amoxicillin-clavulanate, ampicillin, ampicillin-sulbactam, piperacillin, and piperacillin-tazobactam.[2,8,13,172,186] These cross-reactivities may have significant clinical implications for patients who are at risk for invasive aspergillosis and are receiving broad-spectrum therapy, particularly piperacillin-tazobactam.

HIV-1 p24 antigen-detection methods have been available since the mid-1980s and have demonstrated some clinical utility. During acute HIV-1 infection, p24 antigen is present in high levels prior to the development of anti-p24 antibodies. When antibodies appear, much of the p24 antigen is present as immune complexes, and so free p24 antigen levels decrease.[60] Heat treatment or other methods to dissociate and remove anti-p24 antibodies have been used to enable detection of antigenemia beyond the period immediately following acute infection.[65,157] Commercial tests for detecting p24 antigen use polyclonal or monoclonal antibodies fixed to microtiter wells for antigen

capture from dilutions of patient sera, and detection is accomplished by addition of a second anti-p24 antibody conjugated with an enzyme. In 2010, Parpia and colleagues reported a dipstick, lateral flow p24 antigen-detection assay that used heat shock to disrupt immune complexes attributable to maternal anti-p24 antibodies. Using blinded specimens archived from the NIH-sponsored Women-Infants Transmission Study (WITS), this assay demonstrated an overall sensitivity and specificity of 90% and 100%, respectively, for detection of p24. In a field study of 389 specimens from South African infants at risk for HIV, 24 (6.8%) of the 389 specimens were HIV RNA positive by RT-PCR. The dipstick p24 test demonstrated a sensitivity of 95.8% and a specificity of 99.4% in detecting p24 in these heat-dissociated specimens.[131]

Immunoconcentration and Immunochromatographic Immunoassays

The basic technology that underlies immunoconcentration and immunochromatographic test systems was described in the 1960s, and the first commercial applications were actually home pregnancy tests, which became available in 1988. Since then, this technology has been applied to a wide array of tests for clinical, veterinary, and industrial applications. Immunoconcentration or "flow-through" assays use a nitrocellulose membrane fixed in a cartridge device.[180] Depending on the assay, either antigen (for detection of antibody) or antibodies (for detection of antigen) are immobilized on the membrane in discreet spots. Following antibody or antigen capture from the clinical specimen and a flow-through wash step, sequential addition of an enzyme-labeled conjugate and substrate results in the appearance of a colored reaction product directly on the membrane. These assays also contain internal positive and negative control spots on the membrane along with the test area; these controls determine if the test was performed correctly, and expected control results must be observed on the membrane for the test to be considered valid.

An example of an immunoconcentration assay for antibody detection is the ImmunoCard *Mycoplasma* (Meridian Biosciences, Cincinnati, OH), which detects *Mycoplasma pneumoniae*– specific IgM in serum samples.[3,17,173] In this assay, patient serum (two drops) is added to each of the two sample ports of the test cartridge. The samples then migrate along the membrane into the two reaction ports. One reaction port (Control port) serves as a procedural control and has human IgM bound to the membrane. In the other reaction port (Test port), the detergent-extracted antigens of *M. pneumoniae* are bound to the nitrocellulose membrane. Three drops of antihuman IgM conjugated to alkaline phosphatase are then added to each of the two sample ports, and this conjugate then migrates into the two reaction ports. Alkaline phosphatase enzyme substrate is then added to the Control and Test ports. The anti-IgM conjugate binds to the IgM bound to the membrane in the Control port. The

patient specimen is added to the Test port. Next, the enzyme-labeled antihuman IgM conjugate is added, followed by substrate. A blue color in both the Control and Test ports indicates a valid reaction and the presence of anti- *M. pneumoniae* IgM in the patient specimen, respectively. If anti- *M. pneumoniae* IgM antibodies are not present in the specimen, no color will be seen in the Test port, but the Control port should still be blue. However, if anti- *M. pneumoniae* antibodies are present in the specimen, the IgM in the specimen reacts with the

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M. pneumoniae antigens on the membrane of the Test port. In one comparative evaluation of several serologic methods for detection of *M. pneumoniae* antibodies, including ImmunoCard *Mycoplasma*, the consensus sensitivity and specificity were 90% and 93%, respectively, and the test outperformed a microplate EIA, IFA testing, and CF.[3] This test has its greatest utility in the diagnosis of pediatric infections, since adults with *M. pneumoniae* infection may elaborate only an IgG response on primary infection and reinfection.[36,165]

Immunoconcentration assays for antigen detection have also been marketed and used in clinical laboratories. Second-generation rapid antigen tests for detection of group A streptococci in throat swab specimens utilized immunoconcentration flow-through technology when they were first introduced. An example of one of these was the TestPack Plus Strep A test (Abbott Laboratories, North Chicago, IL). Following extraction of a throat swab with nitrous acid, the extract is placed on a membrane and allowed to soak through. Anti–group A streptococcal antibody conjugated to alkaline phosphatase is then added to the membrane. Following a wash step, the alkaline phosphatase substrate is added, and the result is indicated by the appearance of a blue "+" or "–" sign, indicating the presence or absence of streptococcal antigen in the specimen, respectively. Immunoconcentration assays for group A streptococci have sensitivities ranging from 64% to 89.9%.[22,88,93,158,200] Most commercially available immunoconcentration assays have been replaced by immunochromatographic techniques.

Immunochromatographic or lateral flow immunoassays are essentially immunoassays that have been adapted to the test strip format (Fig. 3-8). A number of variations of the technology have been developed into commercial products, but they all operate according to the same basic principles. A typical test strip consists of an absorbent pad onto which specimen is applied, a conjugate or reagent pad that contains antibodies specific to the target analyte conjugated to colored particles (i.e., colloidal gold particles or latex microspheres), a reaction membrane onto which antitarget analyte antibodies are immobilized in a line across the membrane as a capture zone or test line, a control zone containing antibodies specific for the

conjugate antibodies, and a waste reservoir composed of another absorbent pad (wick assembly) designed to draw the sample across the reaction membrane by capillary action. The components of the strip are fixed to an inert backing material and may be formatted as a simple dipstick or inside a plastic casing with a sample port and a reaction window showing the capture (test) and control zones.

Lateral flow immunoassays used in clinical microbiology laboratories are usually double-antibody sandwich assays. For detection of antigens, the capture zone on the membrane contains immobilized antibodies. The specimen applied to the sample pad migrates from the sample pad through the conjugate pad where the antigen target binds to the conjugate. The sample then continues to migrate across the membrane until it reaches the capture zone where the target/conjugate complex will bind to immobilized antibodies,

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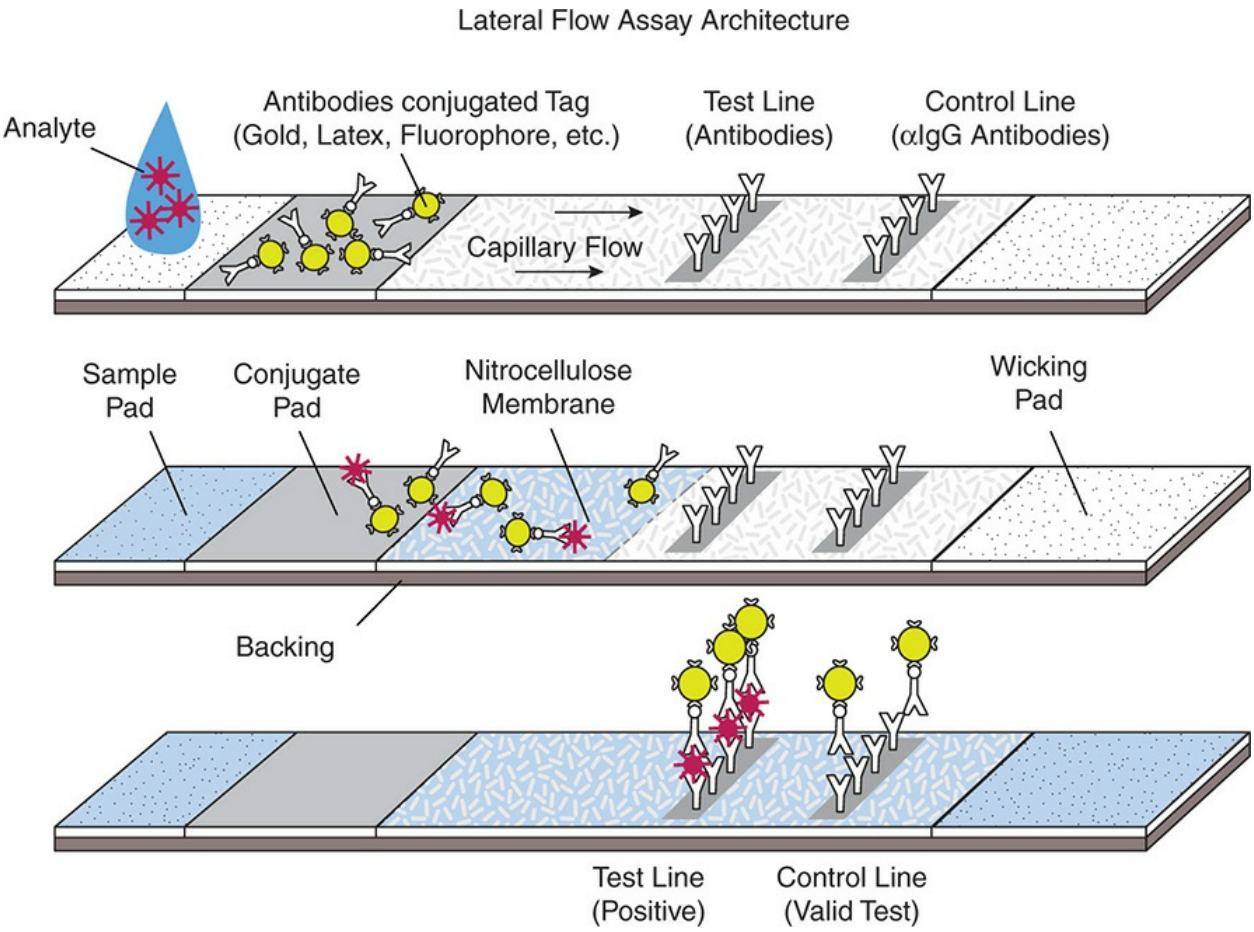
producing a visible line on the membrane. The sample then migrates further along the strip until it reaches the control zone where excess conjugate binds and produces a second visible line on the membrane. This control line indicates that the sample has migrated across the membrane as intended. A clear line in the control zone and in the test area on the membrane constitutes a positive result. A single line in the control zone is a negative result. Examples of commercially available lateral flow assays for antigen detection include tests for RSV in nasopharyngeal wash specimens (Binax NOW RSV, Inverness Professional Medical Diagnostics, Princeton, NJ; Remel Xpect RSV, Remel Laboratories) and for the detection of rotavirus (ImmunoCardSTAT! Rotavirus, Meridian Bioscience, Inc.), *G. lamblia* and *Cryptosporidium parvum* (ImmunoCardSTAT! *Cryptosporidium/Giardia*, Meridian Bioscience, Inc.), and *H. pylori* antigens (ImmunoCardSTAT SpSA, Meridian Bioscience, Inc.) in stool filtrates. The basic construction and principle of a lateral flow immunoassay are depicted in Figure 3-8.



■ **FIGURE 3-8** Lateral Flow Immunoassay. This figure shows a lateral flow immunoassay for detection of antigen. The specimen (e.g., serum, urine) containing the analyte to be detected is placed on the sample pad, which soaks up the specimen fluid. The fluid then migrates to the conjugate pad, which contains antibodies directed against the analyte. These antibodies are conjugated with gold, colored latex, or a chromophore. The analyte is captured by the antibodies on the conjugate pad and then migrates across a nitrocellulose membrane toward the "test" line. As more and more analyte–antibody complexes are captured at the "test" line, the line becomes visible on the membrane. Antibodies that are not analyte-specific are not captured and continue to migrate toward the "control line." The control line is composed of immobilized antibodies directed against immunoglobulin. As more and more uncomplexed antibody passes over the "control" line, the uncomplexed antibodies are captured and become visible at the "control" line. The presence of a "control" line only indicates that the test was performed properly and that the analyte is absent from the specimen. Lines at both the test and control areas indicate a positive test. A single line in the test area without a corresponding control line is an invalid test.

These rapid lateral flow antigen-detection immunoassays have shown generally good performance in clinical use. The Binax Now RSV and the Xpect RSV tests have

sensitivities ranging from 33% to over 80% and specificities exceeding 97%, resulting in positive predictive values of 57% to 100%, depending on the population and the specimen type being tested (i.e., nasopharyngeal washes or aspirates, BALs).[21,75] A 1999 evaluation of the ImmunoCardSTAT Rotavirus test demonstrated a sensitivity of 94% and a specificity of 100%.[39] Garcia and coworkers evaluated the ImmunoCardSTAT! *Cryptosporidium/Giardia* immunoassay and reported sensitivities of 98.8% and 93.5% for detection of *Cryptosporidium* and *Giardia,* respectively, with 100% specificity for both parasites.[54] Slightly lower sensitivities for detection of *G. lamblia* (81%) and *Cryptosporidium* (68%) were reported for the ImmunoCardSTAT! by Johnston and coworkers.[74] Evaluations of the ImmunoCardSTAT! HpSA (*H. pylori* stool antigen) assay have shown performance equivalent to or exceeding that of the *H. pylori* antigen microplate EIA.[76,77,197] Lateral flow immunoassays for antigen can also be "multiplexed" by configuring multiple capture zones on the detection pad so that multiple antigens in the same specimen can be detected simultaneously. For example, the Triage Micro Parasite Panel (Biosite Diagnostics, Inc., San Diego, CA) is a lateral flow immunoassay that is performed on fecal specimens and has immobilized antibodies directed against *G. lamblia*, *E. histolytica/E. dispar*, and *C. parvum*.[53,161] In an evaluation of this product by Garcia et al., the sensitivity of the Triage assay for detection of *G. lamblia*, *E. histolytica/E. dispar*, and *C. parvum* was 95.9%, 96.0%, and 98.3%, respectively, with over 97% specificity for each of the three parasites.[53]

However, not all lateral flow EIA tests have demonstrated the accuracy necessary for clinical use. For example, initial evaluations of the Clearview *Chlamydia* assay (Wampole Laboratories, Cranbury, NJ) compared this lateral flow EIA to culture and reported a sensitivity of 72.9% for the device.[20,84] Subsequent evaluations in which the Clearview assay was compared with nonamplified probe and then NAATs for *Chlamydia trachomatis* reported sensitivities ranging from 50% to 95%, depending on the tests being compared, the specimen types being evaluated, and the prevalence of *C. trachomatis* infection in the study population.[29,69,89] Similar poor performance owing to the lack of sufficient sensitivity for detection of *C. trachomatis* has been reported for the Quickview Chlamydia Test (Quidel Corp., San Diego, CA).[149,182]

The availability of recombinant and synthetic antigens with sensitive and specific reactivity in regular microtiter EIA formats has led to the development of rapid, immunoconcentration and immunochromatographic tests for the detection of HIV-1 and HIV-2 antibodies. FDA-cleared rapid HIV tests include the OraQuick Advance Rapid HIV-1/2 antibody test (Orasure Technologies, Inc., Bethlehem, PA), the Reveal G3 Rapid HIV-1 antibody test (MedMira, Inc., Halifax, Nova Scotia, Canada), Uni-Gold Recombigen HIV (Trinity Biotech, Bray, County Wicklow, Ireland), the Multispot HIV-1/HIV-2 Rapid Test (Bio-Rad Laboratories, Hercules, CA), the

Clearview HIV-1/HIV-2 STAT-PAK, the Clearview Complete HIV-1/HIV-2 (Inverness Medical Professional Diagnostics, Princeton, NJ), and the INSTI HIV-1 Antibody Test (bioLytical Laboratories, Inc., Richmond, BC, Canada). The OraQuick Advance assay can be performed on whole blood, plasma, and oral fluid, which is collected by swabbing completely around the upper and lower outer gums with a small absorbent pad. The Reveal G3 and the Multispot tests can be performed on either serum or plasma, whereas the Uni-Gold test, both Clearview tests, and the INSTI test can be performed on whole blood, serum, or plasma. Whole blood for these latter tests is collected by finger stick using test kit–specific devices (e.g., lancets, specimen collection "loops").

The Reveal G3 Rapid HIV-1 Antibody Test, the Multispot HIV-1/HIV-2 Rapid, and the INSTI HIV-1 Antibody Test use membrane immunoconcentration "flow-through" technology, whereas the OraQuick Advance Rapid HIV-1/HIV-2 Antibody Test, the Trinity-Biotech Recombigen HIV, and the Clearview HIV-1/2 STAT-PAK and Complete HIV-1/HIV-2 antibody tests employ lateral flow immunochromatographic technology. The Reveal G3 Rapid HIV-1, the Multispot HIV-1/HIV-2, and the INSTI tests are configured as single-use cube-shaped cartridges containing an immunoreactive membrane. In the Reveal G3 assay, the membrane contains a spot composed of synthetic peptides corresponding to conserved regions of HIV-1 structural proteins and a procedural/reagent control line composed of protein A. A small aliquot of specimen (serum or plasma) is placed in the center of the reaction membrane and is absorbed. A cap containing a protein A/colloidal gold conjugate is placed on the reaction cartridge, and 12 drops of buffer are applied to the center of the cap. Once all the fluid is absorbed, the cap is removed and the test is read. A reactive test results when the protein A portion of the conjugate binds to the captured HIV-1 antibodies, producing a red "dot" in the test zone and a vertical, red line in the control zone of the membrane. A negative test, resulting from absence of the HIV-1 antigen/antibody complex, is indicated by the presence of only the red vertical line in the control area of the membrane.

In the Multispot HIV-1/HIV-2 Rapid Test, the cartridge membrane contains a procedural control spot, an HIV-1 peptide (immunodominant HIV-1 gp41 envelope glycoprotein) spot, a recombinant HIV-1 antigen (recombinant HIV-1 gp41 envelope glycoprotein) spot, and HIV-2 peptide (immunodominant epitope of the HIV-2 gp36 envelope glycoprotein). These spots are composed of microparticles coated with these antigens. A dilution of the specimen (serum or

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plasma) is applied to a prefilter covering the cartridge, and, after specimen absorption onto the membrane, the prefilter is removed. If antibodies against HIV-1 or HIV-2 are present, these antibodies bind to the antigens on the microparticles. A conjugate

(alkaline phosphatase–labeled goat antihuman IgG) is dropped onto the reaction membrane and binds to the human antibody/antigen complexes immobilized on the reaction spots of the cartridge membrane. After a wash step to remove unbound conjugate, color development reagent (i.e., a colorimetric alkaline phosphatase substrate) is added to the cartridge. A purple color appears on the test spots for HIV-1 or HIV-2 if antibodies to these agents were present in the specimen. A purple color develops at the procedural control spot on the membrane if the assay was performed correctly, regardless of the presence or absence of HIV antibodies. The INSTI HIV-1 Antibody Test membrane contains both HIV-1 and HIV-2 antigens, but the test has only been validated for detection of HIV-1 antibodies.

The OraQuick Advance Rapid HIV-1/HIV-2 Antibody Test, the Uni-Gold Recombigen HIV, and the Clearview HIV-1/HIV-2 STAT-PAK and Complete HIV-1/HIV-2 tests use lateral flow immunochromatographic technology. In these rapid (10 to 20 minutes) assays, a small aliquot of the specimen (oral fluids, whole blood, serum, or plasma, depending on the kit) is added to a specimen/sample well of the lateral flow cartridge. A running buffer is then added to facilitate the lateral migration of the specimen through the reaction cartridge by capillary action and to reconstitute the conjugate fixed to the membrane. If present, the anti-HIV antibodies bind to a colloidal gold dye-conjugated antibody binding protein. The dye-conjugated immune complex migrates further along the nitrocellulose membrane and is captured by the HIV antigens that are immobilized in the "test" area, producing a pink-to-purple-colored line and indicating the presence of antibodies to HIV-1 and/or HIV-2. The sample continues to migrate along the membrane and produces a pink-to-purple line in the control area, which contains IgG antigens. This internal procedural control verifies that the specimen and buffer have been properly applied to the cartridge and establishes the validity of the test.

The development of these rapid HIV antibody assays has provided the medical community with essential tools for the efficient detection of HIV infection in both developed and developing countries.[7,28,135] Depending on the Clinical Laboratory Improvement Amendments requirements of the various tests (waived vs. nonwaived), these point-of-care tests enable HIV testing to be offered in hospital emergency departments and clinics, physicians' offices, community-based clinics, outreach programs, and in field work and serosurveys. Evaluations of these rapid HIV tests generally have shown exquisite sensitivity and specificity in diverse populations worldwide.[37,90,101,125,126,129,132] Since IgG is the antibody detected, these tests are not suitable for the diagnosis of neonatal infections, because of the presence of maternal antibodies up to about 12 months of age. A recent Australian prospective evaluation of five of the rapid HIV antibody test kits licensed in Europe with a cohort of 200 known HIV-positive individuals showed reduced sensitivity of three of the FDA-cleared tests when result obtained with whole blood obtained with finger stick

was compared with matched serum specimens.[132] The sensitivities of finger-stick blood versus serum were 94.5% versus 97.5% for the OraQuick Advance Rapid HIV-1/HIV-2 antibody test and 94.7% versus 100% for the Uni-Path HIV-1/HIV-2 assay. Only the INSTI HIV-1 Antibody Test showed equivalent performance with whole blood and serum, with a sensitivity of 99%.[132] In this study, use of oral fluid specimens produced the lowest sensitivity (86.5%) compared to 94.5% and 97.5% for finger-stick whole blood and serum, respectively. The rapid HIV antibody tests also vary in their ability to identify recently infected persons.[99] Just as the fourth-generation EIAs for HIV-1 and HIV-2 detect both specific antibodies as well as HIV-1 p24 antigen, a lateral flow immunoassay for simultaneous detection of antibodies and HIV-1 p24 antigen is also available (Alere Determine HIV-1/HIV-2, Alere San Diego, CA) and is FDA cleared. An evaluation of this assay demonstrated 100% sensitivity and specificity for detection of HIV-1 and HIV-2 antibodies, and a sensitivity of 86.6% for detection of p24 antigen, as documented by testing 10 different seroconversion panels.[15]

Immunofluorescence Techniques

Immunofluorescence provides an alternative to EIA as a means for detecting and localizing antigens in making the diagnosis of bacterial, fungal, parasitic, and viral diseases. This technique may also be used to detect antibodies for retrospective diagnosis of infectious diseases. Fluorescence is defined as the radiation of energy when light of a shorter wavelength (the "excitation" wavelength) incites the electrons of a molecule to a higher energy state for a very short time. As the electrons return to the preexcitation or ground state, the energy is released as light of a longer wavelength. In fluorescence immunoassays, specific antibody is conjugated to a compound capable of photoexcitation and subsequent fluorescence (usually fluorescein isothiocyanate [FITC] or rhodamine derivatives), resulting in a sensitive tracer with unaltered immunologic reactivity. The conjugated antiserum is added to cells or tissues on a slide and becomes fixed to antigens, forming a stable immune complex. Nonreacting materials are removed by washing, and the preparation is then dried and observed with a fluorescence microscope equipped with an appropriate light source and barrier filters. Antigens bound specifically to a fluorescent antibody (IF) can be detected as bright apple-green or orange-yellow objects against a dark background depending on the fluorochrome being used. Immunofluorescence techniques may be direct or indirect. Direct immunofluorescence tests are usually used for antigen detection; the indirect method can be used for both antigen and antibody detection (i.e., serology).

Immunofluorescence Techniques for Antigen Detection

Direct immunofluorescence techniques (also called **direct fluorescent antibody** [**DFA**] techniques) (Fig. 3-9) involve application of the labeled conjugate to the material being examined, followed by a 15- to 30-minute period of incubation in a humid environment at 35° to 37°C to allow the

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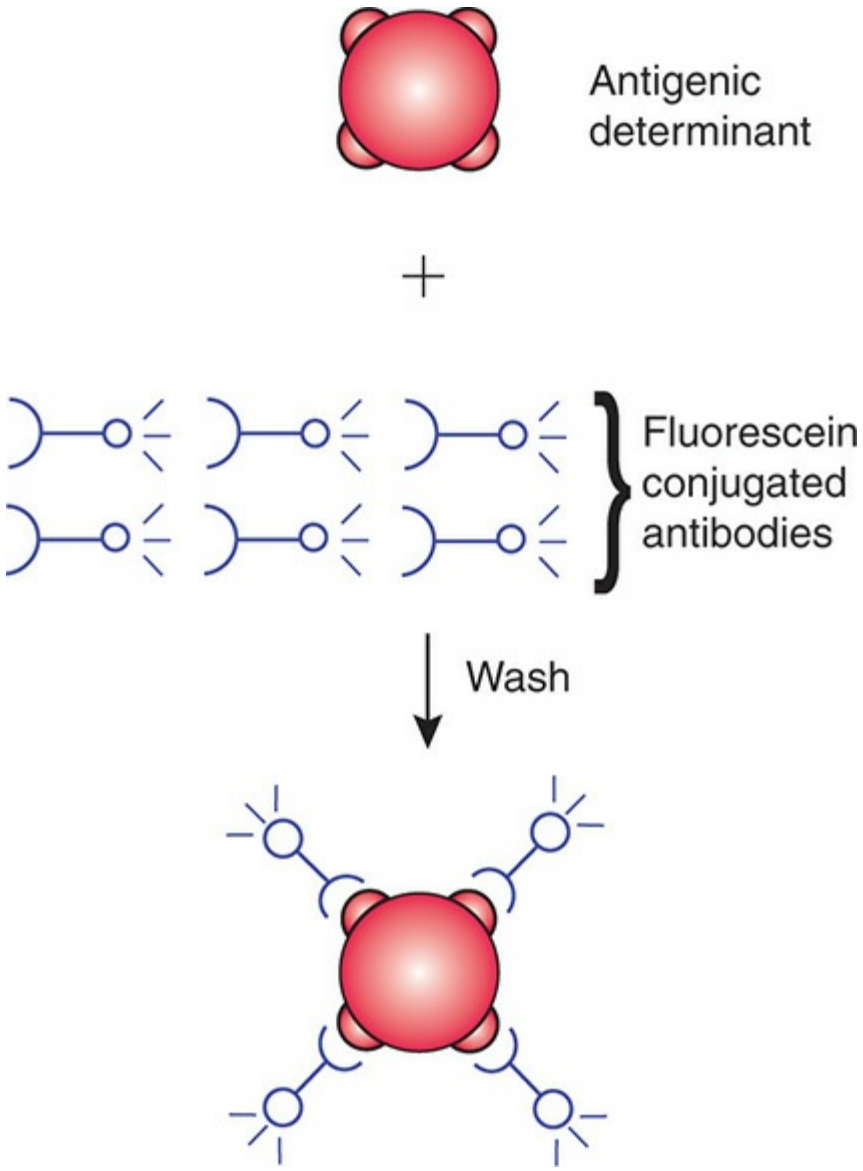
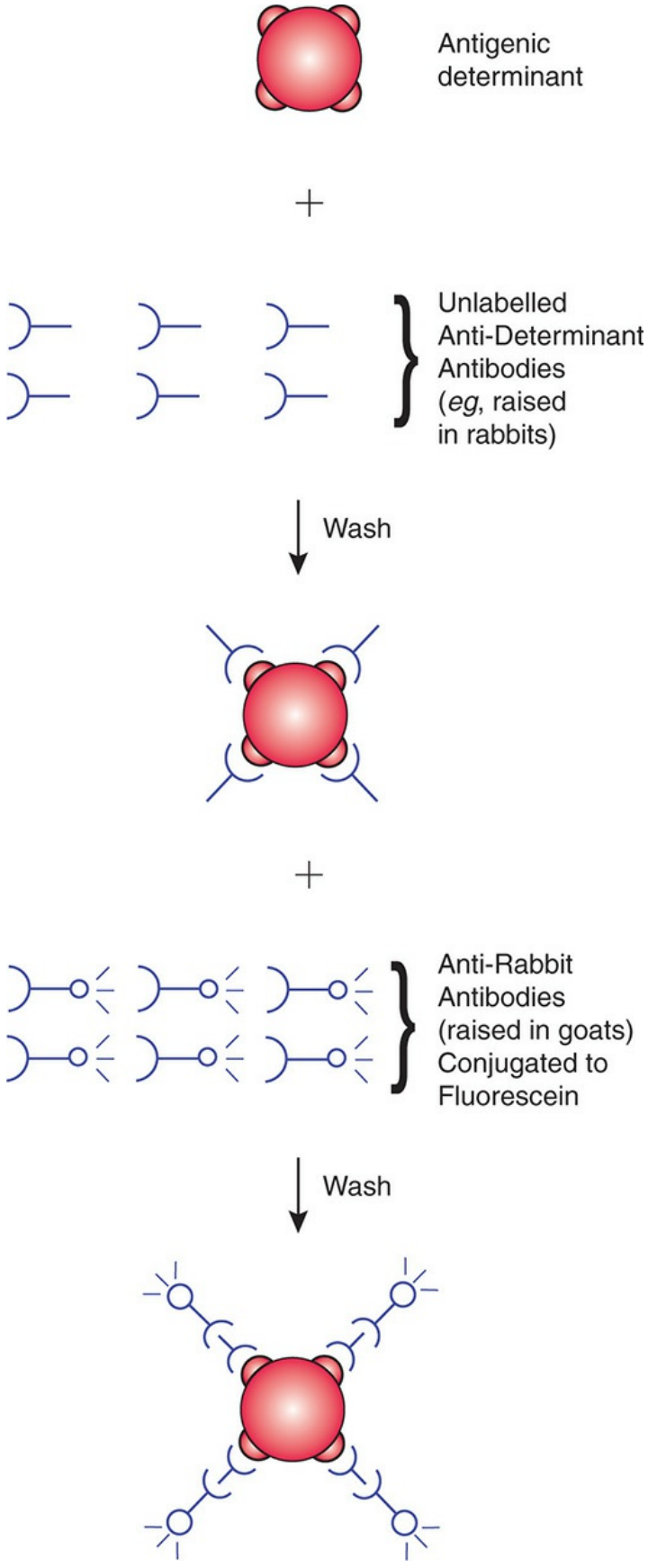


■ **FIGURE 3-9** Schematic diagram of a direct immunofluorescence assay (DFA). In the DFA method, the antigen (e.g., respiratory specimens for RSV) is placed in the well of an FA slide and reacted directly with a fluorescein-conjugated monoclonal antibody directed

against the antigen. After incubation and washing, the slide is examined for characteristic fluorescence.

antigen–antibody reaction to occur. After a wash step to remove unbound conjugate, the preparation is air dried and mounted for observation under a microscope fit with an appropriate fluorescent light source and barrier filters. In the **IFA** procedure (Fig. 3-10), the material to be examined is first overlaid with an excess of unlabeled immune serum directed at the antigen and allowed to react for 30 to 45 minutes at 35° to 37°C. The specimen is washed with phosphate-buffered saline solution and then reacted with tagged antiserum against the species of Ig used in the initial reaction (e.g., fluorescein-conjugated antihuman antibody raised in goats). After washing the background free of extraneous material, the presence of microscopic fluorescence indicates the presence of antigen. DFA tests are simple and rapid to perform with fewer nonspecific reactions; however, they may be less sensitive than indirect procedures. IFA methods are more sensitive and produce brighter fluorescence; however, they may be less specific and are subject to increased cross-reactivity.

Reagents for either DFA or IFA detection of antigens are commercially available for a variety of agents, including HSVs, VZV, respiratory viruses (i.e., influenza, RSV, parainfluenza viruses), *Pneumocystis jirovecii*, *G. lamblia*, and *C. parvum.* Chan and colleagues evaluated the SimulFluor DFA for HSV-1, HSV-2, and VZV and found the assay to be slightly less sensitive than immunoperoxidase staining of shell-vial cultures for HSV detection (80% sensitivity versus 87% sensitivity) and cytospin DFA staining for VZV (87.1% sensitivity). However, the SimulFluor assay provided same-day results for HSV detection, and no difference in turnaround time for detection of VZV compared with cytospin DFA detection.[26] The Merifluor *Pneumocystis* DFA test (Meridian Diagnostics) is more sensitive than other


Antigenic determinant
+
Unlabelled Anti-Determinant Antibodies (*eg*, raised in rabbits)
Wash
+
Anti-Rabbit Antibodies (raised in goats) Conjugated to Fluorescein
Wash

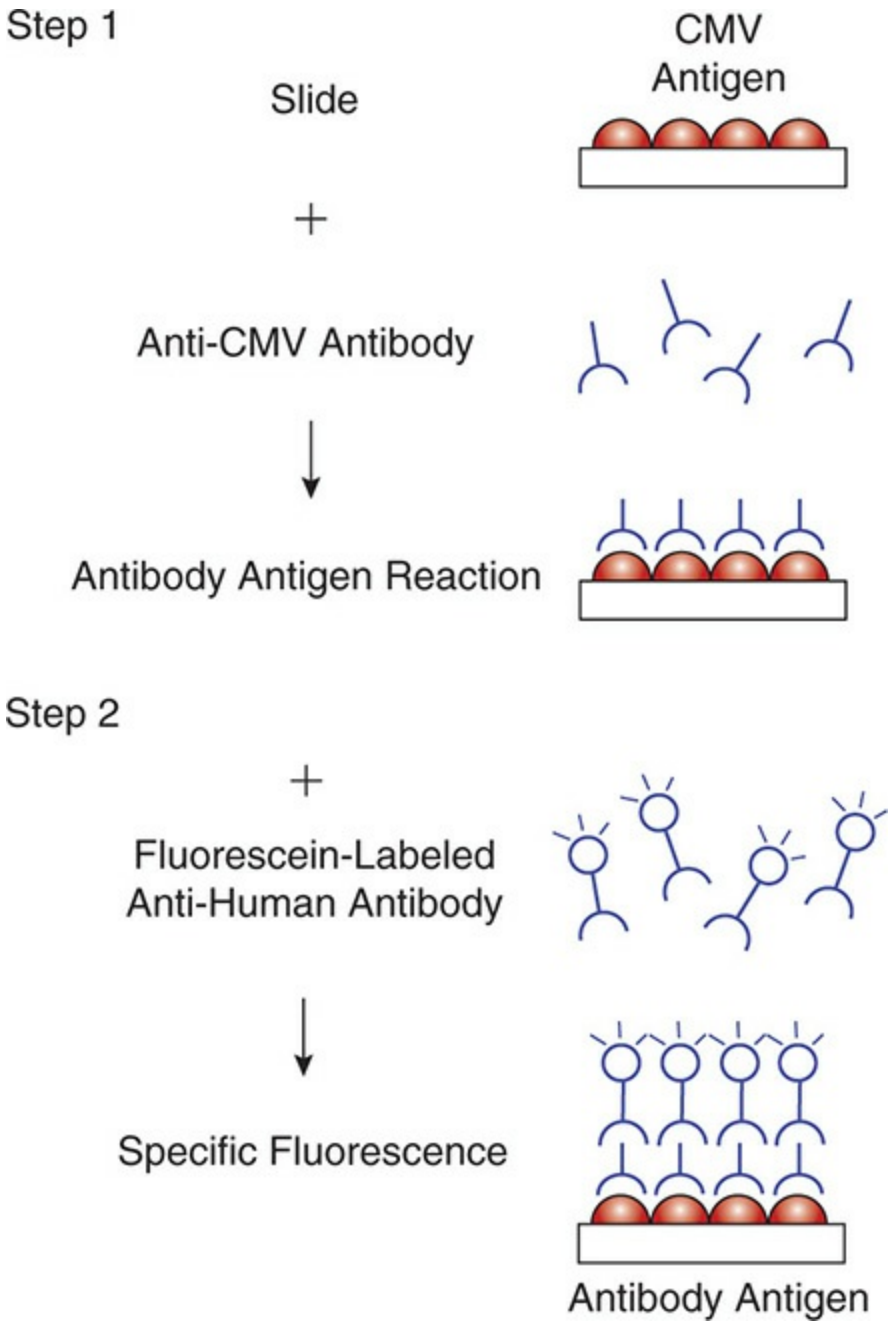
■ **FIGURE 3-10** Schematic diagram of an indirect immunofluorescence assay (IFA) for antigen detection. In this method, the specimen (e.g., sputum for *Legionella*) is reacted with an excess of unlabeled antibodies directed against the antigen. After a wash step, fluorescein-conjugated antibodies directed against the species of antibody used in the initial reaction (e.g., fluorescein-labeled antirabbit IgG raised in goats) are overlaid on the FA slide. After washing, the slide is examined for specific fluorescence. With *Legionella*, for example, unlabeled rabbit antibodies against a large number of serotypes can be used for the first step in the procedure, but only a single fluorescein-conjugated goat antirabbit Ig is required for the second step. If *Legionella* organisms were to be detected by a DFA method, separate fluorescein-labeled conjugates for each serotype would be required.

staining methods for *P. jiroveci*, (i.e., calcofluor white, GMS, Diff-Quik), but it appears to be less specific (i.e., more false-positives); some evaluations have suggested that positive immunofluorescent results be confirmed with another method to increase the specificity and positive predictive value of the DFA.[120,121,145,169] The Merifluor *Cryptosporidium/Giardia* DFA test (Meridian Diagnostics, Inc.) detects both organisms and is very sensitive and specific; in some studies, this DFA test outperformed other detection methods, including reference parsitologic methods and EIAs.[4,55,74,205] Some immunofluorescent reagents, particular for viruses such as HSV-1, HSV-2, and VZV, can be used for both direct detection of organisms in clinical specimens (e.g., cells scraped from the base of vesicular lesions, sputum, BAL) and for confirmation of isolates recovered in culture (e.g., respiratory viruses, HSVs, VZV).

Immunofluorescence Techniques for Antibody Detection

IFA assays can be used for detection of antibodies in much the same way as the CF or HAI tests. IFA test is often used for retrospective diagnosis of viral infections by looking for seroconversion or for a fourfold increase in antibody titer between acute and convalescent specimens. In the IFA procedure for viral antibodies, slides with virus-infected tissue culture cells fixed in discrete FA test slide wells are overlaid with serial dilutions of patient serum. After incubation and washing, a fluorescein-labeled goat or rabbit antibody directed against human Ig (conjugate) is added to each of the slide areas containing the viral antigens. After washing, the slides are inspected with a fluorescence microscope and an endpoint, that is, the highest dilution of serum producing positive immunofluorescence, is determined. Changes in titer may be determined by inspection of slides reacted with serial dilutions of acute- and convalescent-phase sera that are performed together in the same test. Indirect assays

can also be performed using enzyme-labeled conjugates (e.g., horseradish peroxidase) instead of fluorochrome-tagged reagents. Enzyme conjugate tests produce a colored precipitate that is read with a light microscope instead of a fluorescence microscope. Most laboratories that use IFA for serology will screen sera at a dilution of 1:8 or 1:10. If a positive reaction is obtained at this dilution, further twofold dilutions are prepared and each dilution is tested by IFA until an endpoint dilution is reached. The reciprocal of the highest serum dilution showing fluorescence is the titer of that specimen for the antibody in question. IFA can be used to document infections with bacterial agents that are difficult to culture in vitro, such as *B. henselae*, the major cause of cat scratch disease.[34,151,184,185,204] Figure 3-11 shows an IFA procedure for detection of antibodies to CMV.



■ **FIGURE 3-11** IFA method for antibody detection. In this method, antigen (e.g., CMV-infected tissue culture cells) is fixed onto a FA slide and reacted with patient serum. If anti-CMV antibodies are present, they bind to the antigens on the slide. After a washing step, a fluorescein-conjugated goat antihuman antibody is overlaid on the slide. This results in labeling of the CMV-infected cells on the slide and indicates the presence of anti-CMV antibodies in the initial serum specimen. Titers can be determined by performing the assay with twofold dilutions of serum and reading for the highest serum dilution, resulting in a proscribed degree of fluorescence.

The diagnoses of certain viral infections such as Epstein–Barr virus (EBV) infection rely totally on serologic detection of antigens or antibodies because these agents do not grow in cell-culture lines used in routine clinical microbiology laboratories. EBV is a member of the family *Herpesviridae* and causes infectious mononucleosis. The specific immune response to EBV infection is characterized by the sequential appearance of certain antibodies, such as anti-VCA (viral capsid antigen) IgM and IgG, and antibodies against Epstein–Barr nuclear antigen (EBNA) and early antigen (EA) (see Chapter 23). Although anti-VCA IgM and then IgG appear during acute infection, anti-EBNA antibodies are absent during acute infection, appear during convalescence, and are maintained for life. Therefore, the presence of anti-EBNA in serum is indicative of past rather than current infection. Because EBNA is present in low levels in EBV-infected cells, the sensitive **anticomplement immunofluorescence (ACIF) test** is a reference method used for its detection (Fig. 3-12).[40,133] In ACIF, an EBV-infected lymphoblastic cell line is used as the source of antigen. Raji cells are usually used for this purpose because these cells express only EBNA antigens; VCA is not expressed and EA is rarely detectable in this cell line. These cells are fixed to a slide and reacted with patient serum. Specific anti-EBNA antibodies, if present, bind to the nuclear antigen. After a wash step, complement is overlaid on the cells. If specific anti-EBNA antibodies have reacted in the first step, complement binds to the immune complexes. After another wash step, fluorescein-conjugated anticomplement antibodies are overlaid on the cells. After incubation, the slide is again washed and then observed under a fluorescence microscope. The presence of

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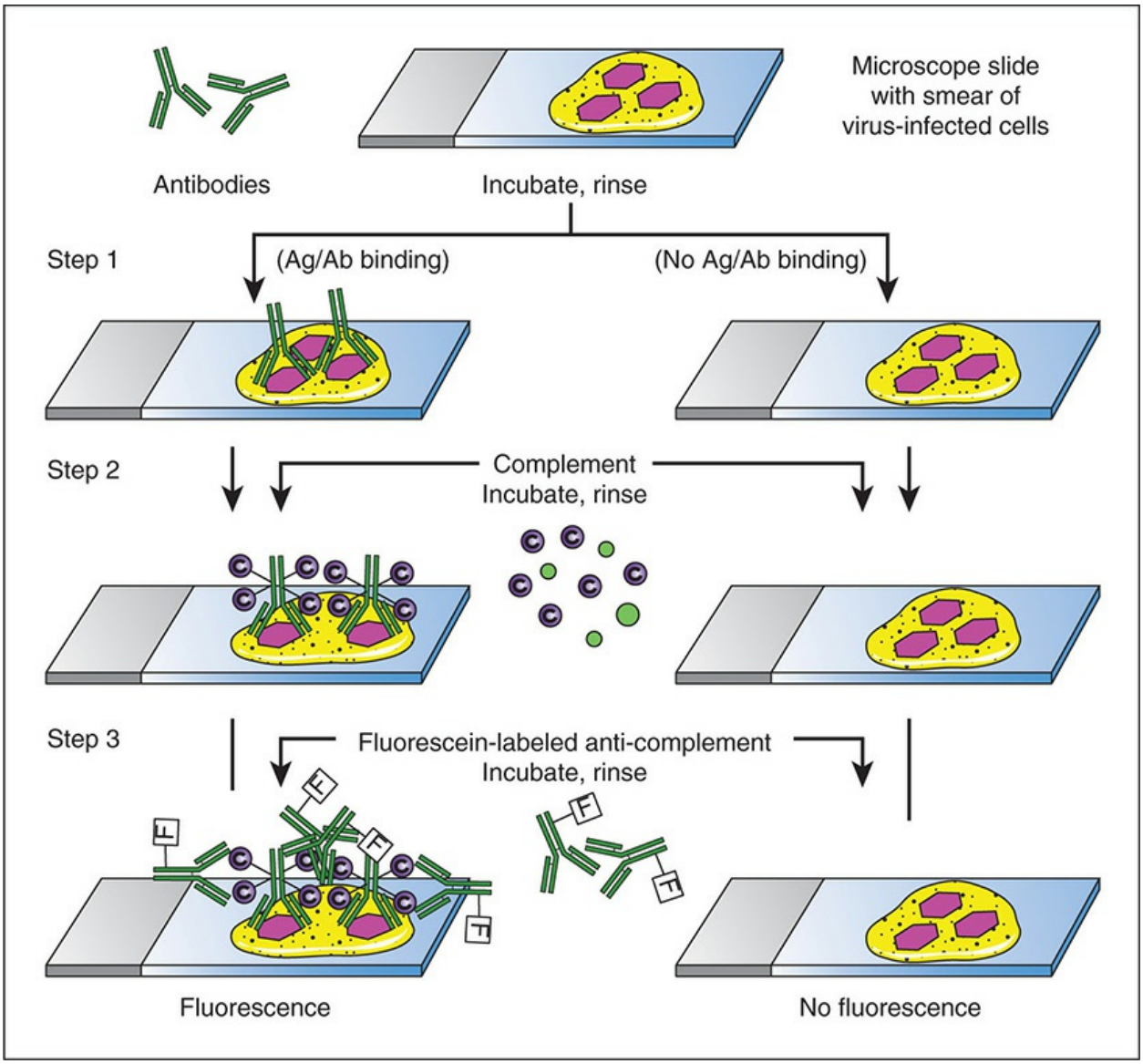
nuclear fluorescence indicates the presence of EBNA and indicates past infection with EBV. ACIF has also been used for determining immune status (e.g., presence of protective antibodies to VZV), detection of antibodies to CMV, and for demonstrating seroconversion following immunization with viral vaccines (e.g., varicella zoster vaccine).[11,51,81,144,150]



■ **FIGURE 3-12** Anticomplement immunofluorescence. In step 1, antibodies are exposed to virus-infected cells fixed on a microscope slide. In step 2, complement is added and binds to antigen–antibody (Ag/Ab) complexes formed in step 1. In step 3, fluorescein-labeled anticomplement is added and binds to complement that bound in step 2, and fluorescence is seen. If antibodies do not bind in step 1, complement cannot bind in step 2, fluorescein-labeled anticomplement cannot bind in step 3, and no fluorescence is seen. (Redrawn from Leland DS. Clinical Virology. Philadelphia, PA: Saunders, 1996.)

Another immunofluorescence assay called the **fluorescent antibody to membrane antigen (FAMA) test** is the test of choice for determining immune status for certain viruses such as VZV largely because of its exquisite sensitivity.[51,196] In this assay, patient serum is serially diluted in microtiter wells, and cultured cells infected with

VZV are added to the diluted serum. After incubation, the microtiter tray is centrifuged to force the cells to the bottom of the wells and washed twice, with intervening centrifugation steps. A working dilution of fluorescein-conjugated antihuman IgG is added to the wells and incubated. After a few wash/centrifugation steps, the cells are harvested, fixed to the wells of an FA slide, and examined with a fluorescence microscope. The FAMA test is more sensitive than CF or EIA, but it requires live, virus-infected cells for its performance.

Immunofluorescence and EIA approaches each have their own advantages and disadvantages. EIA methods for antigen detection are advantageous in high-volume laboratories where many samples are examined daily for a single determinant (e.g., rotavirus, RSV). Although DFA techniques are more labor intensive, the ability to directly observe the background cellular elements in certain direct antigen procedures to determine the adequacy of the specimen is a distinct advantage. For example, since *C. trachomatis* preferentially infects cervical columnar epithelial cells, the presence of squamous epithelial cells and/or segmented neutrophils, erythrocytes, and mucus indicates that the specimen is inadequate for diagnostic purposes. Adequate specimens show a preponderance of intact cuboidal and columnar epithelial cells. This assessment can be made with the direct FA test and incorporated into the laboratory report, allowing the physician to weigh clinical evidence of chlamydial infection along with the possibility that a negative report may actually reflect an inadequately collected endocervical specimen. These determinations cannot be made with nonvisual tests such as EIA and most molecular assays.

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CHAPTER 4

Molecular Microbiology

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Introduction

Nucleic Acids—The Basics of DNA and RNA

- Structure of DNA
- Structure of RNA
- Function of DNA—Information Storage
- Function of RNA—Information Transfer
  - Reading (Transcription) and Interpretation (Translation) of the Genetic Code

Signal Amplification Methods

- Nucleic Acid Probes
  - Clinical Applications
- Hybrid Capture
  - Clinical Applications
- Branched DNA
  - Clinical Applications

In Situ Hybridization
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Introduction

Microbiologists have always searched for and continue to search for more rapid and efficient ways to detect and characterize microorganisms. The molecular techniques are some of the most powerful tools that may be used to this end. More recently, the widespread adoption of mass spectrometry has changed the way we practice clinical microbiology. These assays have revolutionized the microbiology laboratory and have changed the way we detect, characterize, and quantify microorganisms directly in clinical specimens and from cultures. Over the past 20 years, these techniques have progressed from once cumbersome, highly technical, labor-intensive assays to user-friendly tests that are so rapid that it is, in some instances, possible to achieve test results within an hour from the start of the assay, directly from the clinical specimen.

The molecular assays used by the early molecular microbiologist were often used for the detection of only fastidious or uncultivable microorganisms or to determine the cause of significant outbreaks. However, as these assays became easier to use and less expensive, they have been exploited for the detection and characterization of more common microorganisms.

Molecular microbiology may be separated into three categories. The first is the detection of microorganisms without the use of nucleic acid amplification. These applications rely on the amplification of a signal generated, usually light or a color, which is the result of the successful hybridization of a nucleic acid probe with the target nucleic acid molecule. Second, nucleic acid amplification methods are used for the detection, characterization, and, in some instances, quantitation of microorganisms. Finally, molecular methods have also been used extensively to determine the relatedness among microorganisms (i.e., strain typing), which are critical tools for the hospital and public health epidemiologist. These technologies, in one way or another, all use nucleic acid chemistry. Therefore, we will begin this chapter with a review of the basics of nucleic acids.

It would be impractical and beyond the scope of this book to review all the molecular assays that have been used in the assessment of microorganisms. Therefore, tests that

have proven most useful in the clinical laboratory, tests that are commercially

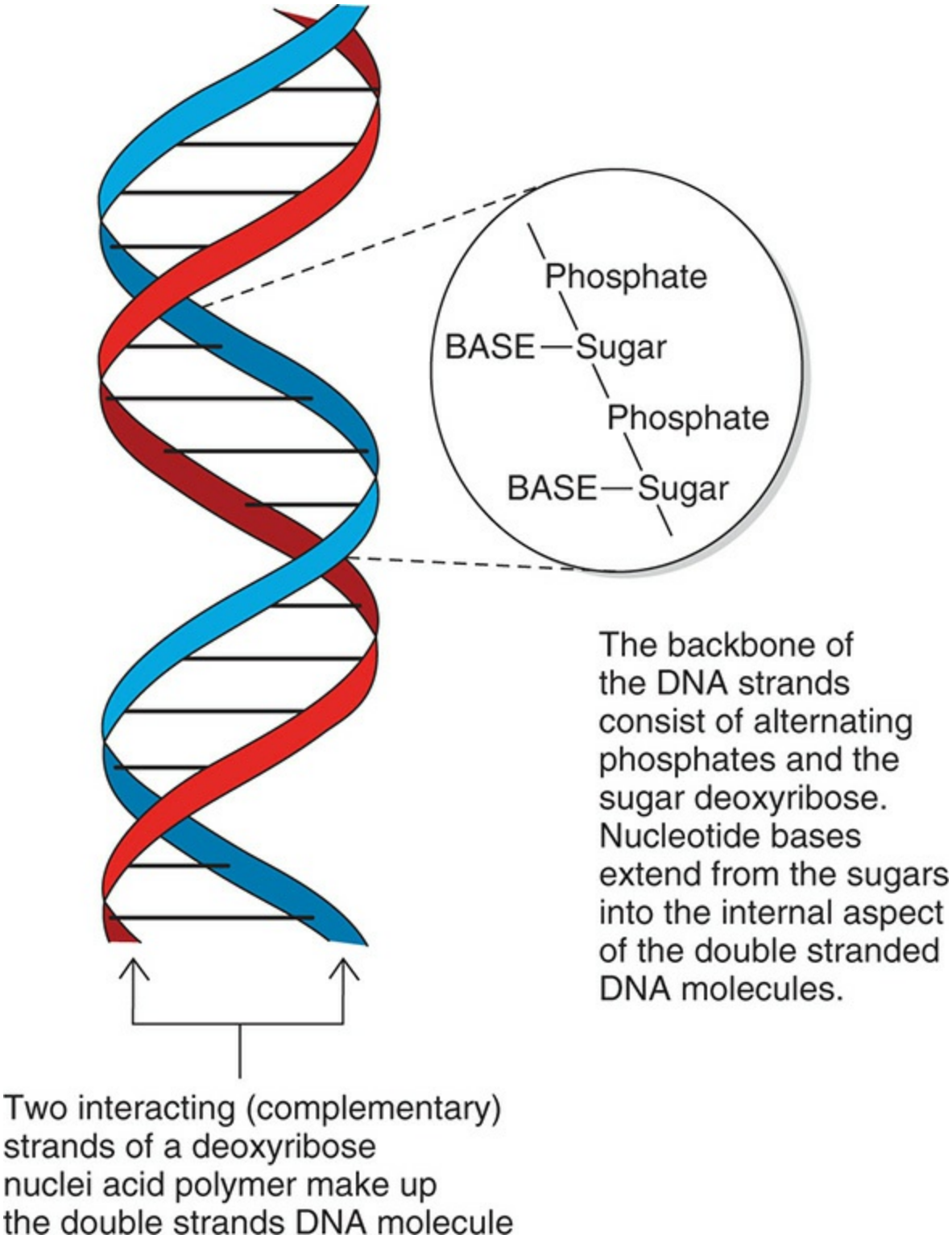
available, and some newer assays that hold particular promise are covered in greater detail. There are now many different types of nucleic acid amplification available. The *polymerase chain reaction* (PCR), which is the forerunner of the other types of nucleic acid amplification assays and may on occasion be used in a generic sense in this textbook, is a surrogate term for nucleic acid amplification. This is done for practicality and for the flow of the text, but the reader should know that the use of other types of nucleic acid amplification chemistries is also feasible.

Finally, since the last edition of this text, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has become commercially available and is becoming widely distributed. The basics of this technology and pertinent clinical applications are reviewed.

Nucleic Acids—The Basics of DNA and RNA

Structure of DNA

DNA is a long molecule composed of two strands. Each strand of DNA is a polymer, which means that each strand is composed of repeating similar subunits. The repeating subunits, or the building blocks, of DNA are nucleotide monophosphates. The two strands that compose the DNA molecule have direction determined by their organic chemistry, and they are oriented in directions opposite to one another. The two strands of an intact DNA molecule interact through hydrogen bonds and are termed *complementary*. The basic configuration of DNA is depicted in Figure 4-1.



■ **FIGURE 4-1** Structure of DNA. The structure of DNA has been described as a double helix. DNA is a double-stranded polymer that consists of nucleotides as the basic repetitive unit; nucleotides, in turn, consist of a sugar (deoxyribose), a phosphate, and one of four bases.

There are four kinds of nucleotide monophosphates that make up DNA; they are named according to the type of base they contain. Each nucleotide monophosphate contains a sugar (i.e., deoxyribose), a phosphate, and a nucleotide base (Fig. 4-2). The backbone of each of the two strands of the DNA molecule consists of alternating deoxyribose sugar and phosphate molecules, which are covalently bonded to one another. The nucleotide bases extend from the sugar molecule into the central aspect of the double-stranded DNA molecule. The positions and the orientation of nucleic acids are determined by convention according to the numbering of the carbon molecules of the sugar. The nucleotide base is attached to the sugar at the 1′ carbon position. The bridging phosphate molecules are attached at the 3′ and 5′ carbons, which gives the strand its particular orientation, the importance of which will become clear later in the text. The four nucleotide bases present in DNA are adenine (A) and guanine (G), both of which are purine molecules, and thymine (T) and cytosine (C), both of which are pyrimidine molecules. The purine adenine present on one strand of DNA forms two hydrogen bonds with the pyrimidine thymine on the opposite strand of DNA. These nucleotides are said to be complementary. Similarly, the guanine on one DNA strand forms three hydrogen bonds with the complementary cytosine on the opposite strand of DNA.

The structure of DNA is often depicted as a ladder, for simplicity, wherein the rungs of the ladder are the complementary bases of the two strands of DNA and the sides of the ladder are understood to represent the alternating sugar phosphate molecules (Fig. 4-3). In life, DNA does not exist, except perhaps temporarily, as a linear molecule. Rather, it is supercoiled; the entire length of the DNA molecule is often greater than the length of the cell that contains it. DNA, therefore, must be supercoiled so that its extensive length can be packed into very small spaces. The actual structure of DNA is the classic double helix described by James Watson and Francis Crick in 1953 (http://nobelprize.org/medicine/laureates/1962/). The two strands of DNA held together by hydrogen bonds, as previously described, exist as a left-handed, helical molecule with major and minor grooves. A knowledge of the basic structure of DNA and the replication of this molecule was pivotal in the development of what are now commonly used nucleic acid amplification-based tests.

Structure of RNA

There are many types of RNA molecules in the cell, but we will confine this discussion to the most commonly described forms, which are messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). The building blocks of RNA are similar to those of DNA. RNA is also a polymer, but it is single stranded. The backbone of this molecule also consists of alternative sugar and phosphate molecules, but for RNA the sugar is ribose. Like DNA, nucleotide bases are covalently bonded to the sugar molecule. In RNA, however, the nucleotide base thymine is replaced by the base

uracil.

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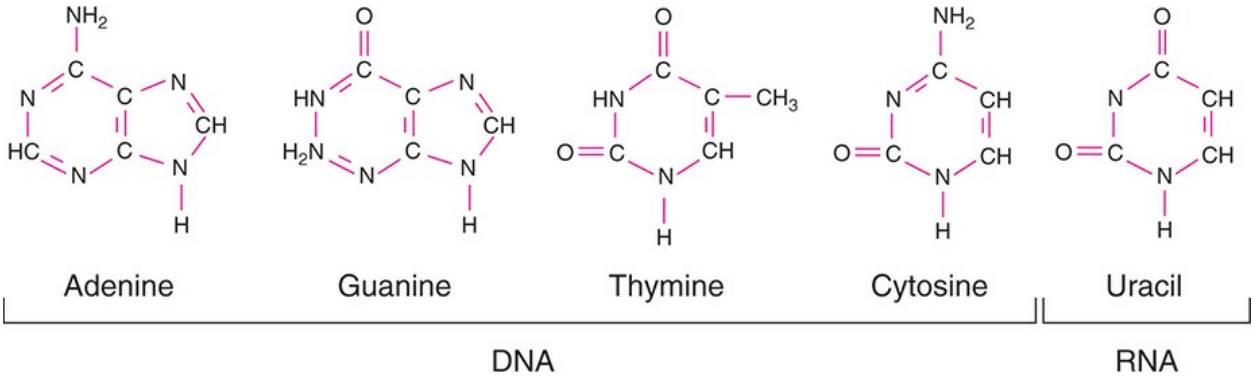
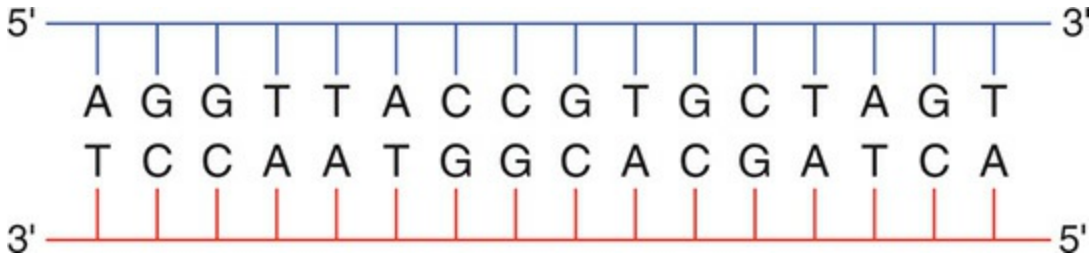


■ **FIGURE 4-2** Nucleotide bases of DNA and RNA. The four nucleotide bases present in DNA are adenine (A) and guanine (G), both of which are purines, and thymine (T) and cytosine (C), both of which are pyrimidines. Uracil (U) is present in RNA in place of thymine.



■ **FIGURE 4-3** A simplified drawing of DNA. The complex structure of DNA is often simplified to that of a ladder-like structure, wherein the rungs of the ladder are depicted as the complementary nucleotide bases. This is often simplified even further, wherein only the one strand of DNA is depicted in a 5′ to 3′ direction and the presence of the complementary strand is understood.

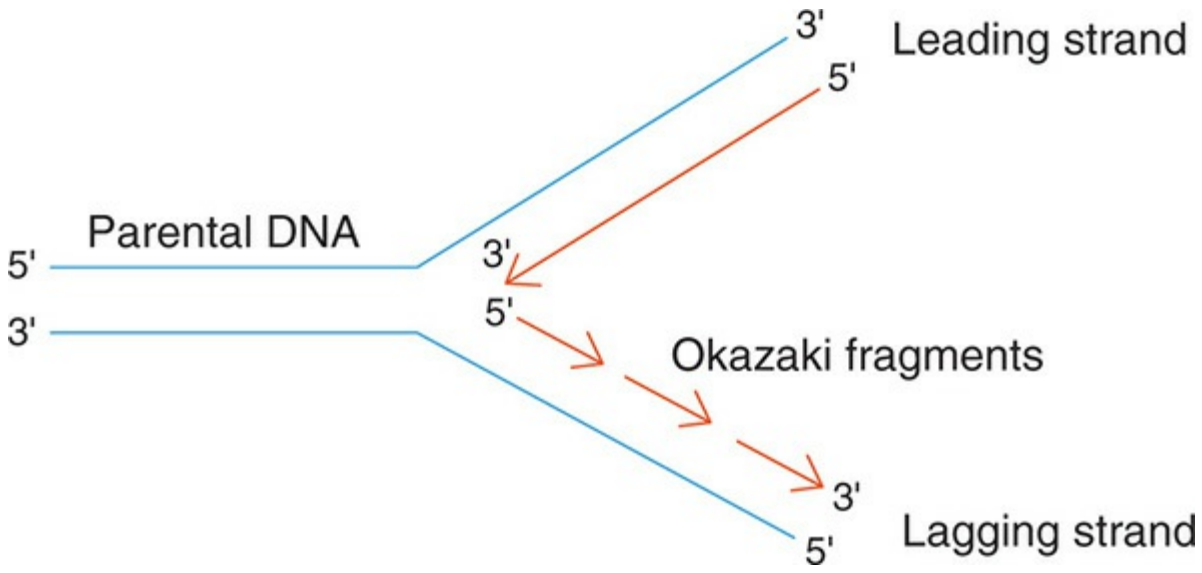
Although most RNA molecules are single stranded, they may fold back on themselves and form complementary hydrogen bonds, thereby creating a secondary structure.

Function of DNA—Information Storage

The genes that encode for the vast majority of the structural and functional proteins of the cell are contained in the chromosomal DNA of the cell. If the cell requires more protein for its cytoskeleton or if it needs more of an enzyme to metabolize a sugar, it

can make more by opening, reading, and using the message in the DNA (see the next section). The DNA has, therefore, been referred to as the blueprint of the cell, which is an apt analogy. When a cell divides, the new cell must also have a copy of the DNA blueprint in order to preserve its structure and perform its functions.

It is important to understand the basics of DNA replication, since many of these principles are used in the PCR and in some other methods of nucleic acid amplification. DNA replication begins with the relaxation of the supercoiled DNA molecule. Then, the more linearized portion of the double helix is at least partially separated into its two component strands. It is as if the rungs of the schematic DNA ladder were sawed down the middle and the two sides of the ladder pulled apart (Fig. 4-4). DNA helicase is an enzyme important in separating the two strands of DNA. One limitation in DNA synthesis, which is performed by DNA polymerase, is that it occurs only from the 5′ to 3′ direction. Therefore, only one strand of DNA can be made in a continuous manner in the 5′ to 3′ direction. This strand, because it is synthesized continuously and more rapidly than the opposing strand, is termed the *leading strand*. The lagging strand is replicated in sections, since the 5′ end becomes available for synthesis only after DNA helicase has performed its function. The discontinuous portions of DNA that are synthesized on the lagging strand are ligated or annealed one to another by DNA ligase, which makes that final lagging strand a continuous strand of DNA. The end result in the replication of DNA is the creation of two daughter strands of DNA from one parent DNA molecule (Fig. 4-5). Each of the new daughter molecules of DNA consists of one strand from the parent DNA molecule and one newly synthesized strand that is complementary to the parent strand. Each of the newly synthesized double-stranded DNA molecules is a perfect match of the parent DNA molecule, unless an error occurs in replication. DNA replication must precede cell division so that each new cell formed has a full complement of DNA.



■ **FIGURE 4-4** DNA replication. DNA replication occurs in a 5′ to 3′ direction. Therefore, one strand, the leading strand, is continuously synthesized, whereas the opposite strand is segmentally synthesized in Okazaki fragments.

Of practical interest, each of the enzymes described above has been utilized in current or former methods of nucleic acid amplification. Helicase has been used for helicase-dependent amplification (HDA), DNA polymerase for the PCR, and ligase for the ligase chain reaction (LCR).

Function of RNA—Information Transfer

Reading (Transcription) and Interpretation (Translation) of the Genetic Code. The nucleic acids and proteins of the cell interact in the formation of new proteins, which form the structure and perform the functions of the cell. The first step in protein synthesis is the "rewriting," or transcription, of the genetic message so that it can be transported to the ribosome. The message, which is encoded by the DNA, is transcribed in the form of an RNA molecule, which is aptly termed *messenger RNA*. This occurs in a manner similar to DNA synthesis, but uses a different set of enzymes. Briefly, the DNA strand is unwound and a single-stranded RNA molecule is created that is complementary to the gene being transcribed. This may or may not undergo additional processing (transcriptional processing). The message is then read, or the genetic code is translated by the cellular machinery. This involves other types of RNA molecules, such as tRNA, rRNA, and a variety of proteins.

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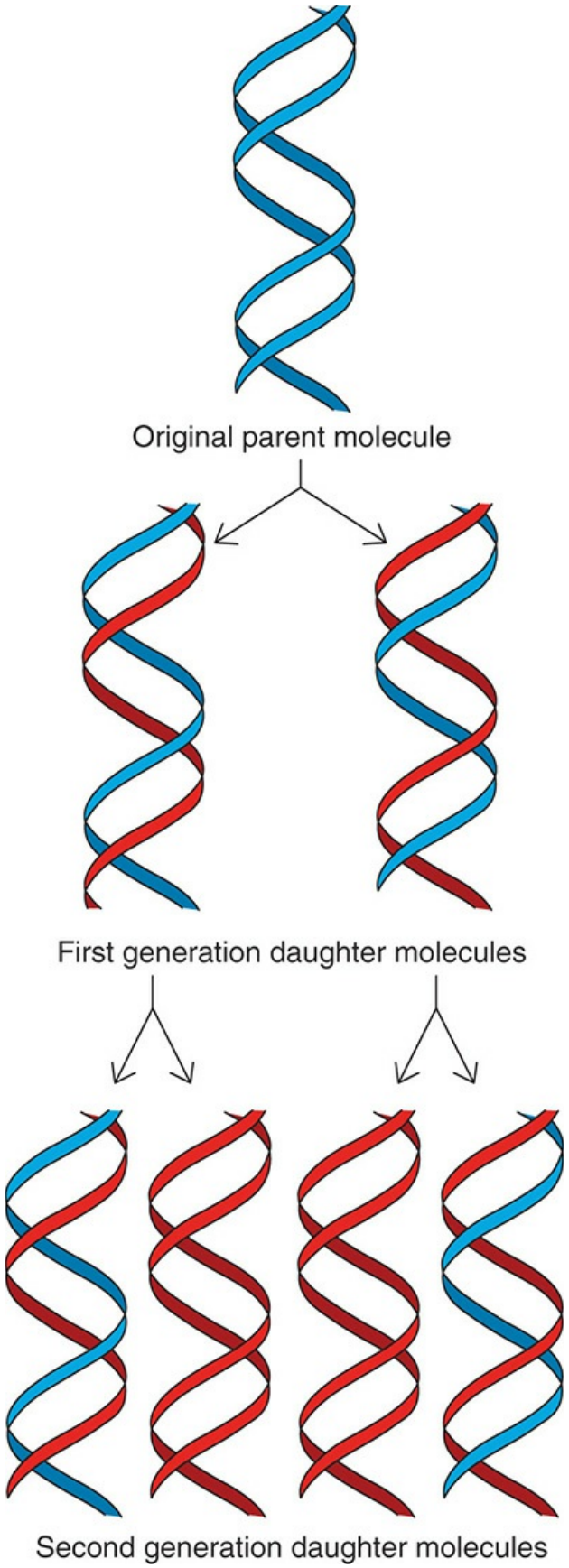
Original parent molecule
First generation daughter molecules
Second generation daughter molecules

■ **FIGURE 4-5** Products of DNA replication. Each of the first-generation daughter molecules of DNA contains one strand from the parent molecule and one newly synthesized strand. Two of the daughter molecules of the second generation of DNA replication contain one of the original parent strands and two consist of entirely newly synthesized DNA.

The translation of the message into a protein occurs on a complicated superstructure composed of proteins and rRNA termed the *ribosome*. The mRNA becomes associated with the ribosome and is processed three bases at a time. These three-base-pair segments are called **codons**, and they encode for particular amino acids. The codons on the mRNA are complementary to anticodons on a portion of the tRNA. Particular tRNAs transport or transfer particular amino acids to the ribosome, the site of protein synthesis. The amino acid molecules are situated so that peptide bonds may be enzymatically formed between them. Eventually, a chain of amino acids is formed (i.e., a peptide), which through continued extension, secondary structure folding, and posttranslational modifications becomes a protein (Fig. 4-6). The reader who is interested in obtaining a more thorough understanding of these processes is directed to textbooks of biochemistry and molecular biology.[5,345]

Signal Amplification Methods

The signal amplification methods described combine some type of nucleic acid, usually a probe, with a generation of a signal. The signal is often amplified through an enzymatic reaction. The signal from fluorescence in situ hybridization (FISH), however, may be directly observed, following the hybridization of the fluorescently labeled probe to its complementary nucleic acid target. A variety of chemical and enzymatic modifications may be applied to FISH or chromogenic in situ hybridization (CISH), if further amplification of the signal is necessary, but are beyond the scope of this book. We will discuss four types of signal amplification in this section: nucleic acid probes, Hybrid Capture (HC), branched-chain DNA, and in situ hybridization (ISH).

Signal amplification is usually considered to be less sensitive than nucleic acid amplification methods. One exception may be FISH, wherein a single copy of a target molecule may be identified in a large population of other cells. This application of FISH is more useful in anatomic pathology specimens or research, wherein a minor subpopulation of cells may be of clinical importance. Signal amplification technologies have several advantages over nucleic acid amplification methods. These procedures are far less likely to produce false-positive results secondary to contamination compared with traditional nucleic acid amplification assays. However, signal contamination or the bleed over of a strong signal from one reaction chamber into an

adjacent well has been reported.

Nucleic Acid Probes

Nucleic acid probes were the first molecular assays to become commonplace in many laboratories. The probes, termed *AccuProbes*, are commercially available for the detection of numerous microorganisms (Hologics, San Diego, CA). These are DNA probes that contain a chemiluminescent label and target the rRNA of the microorganism of interest, which is a useful target since the rRNA is present in greater quantities than the rDNA genes that encode for it. This is an example of biologic amplification, or the amplification of a nucleic acid that occurs during the normal course of cellular events or secondary to microorganism growth. This proprietary chemistry allows for the differentiation of the hybridized from the nonhybridized probe. When the stable DNA–RNA hybrid is treated with the detection reagents provided, light is produced secondary to a chemical reaction. The presence of a certain amount of light that exceeds a predetermined threshold value is considered a positive reaction and indicates the presence of the microorganism to which the probe was designed to hybridize.

Clinical Applications. AccuProbe genetic probes are available for the direct detection of bacteria in clinical specimens or as a rapid and specific method of identifying microorganisms in culture. When used on direct specimens, these probes are less sensitive than enhanced culture for Group A *Streptococcus* and is distinctly less sensitive than PCR.[41] Former products utilizing this technology for *Neisseria gonorrhoeae*

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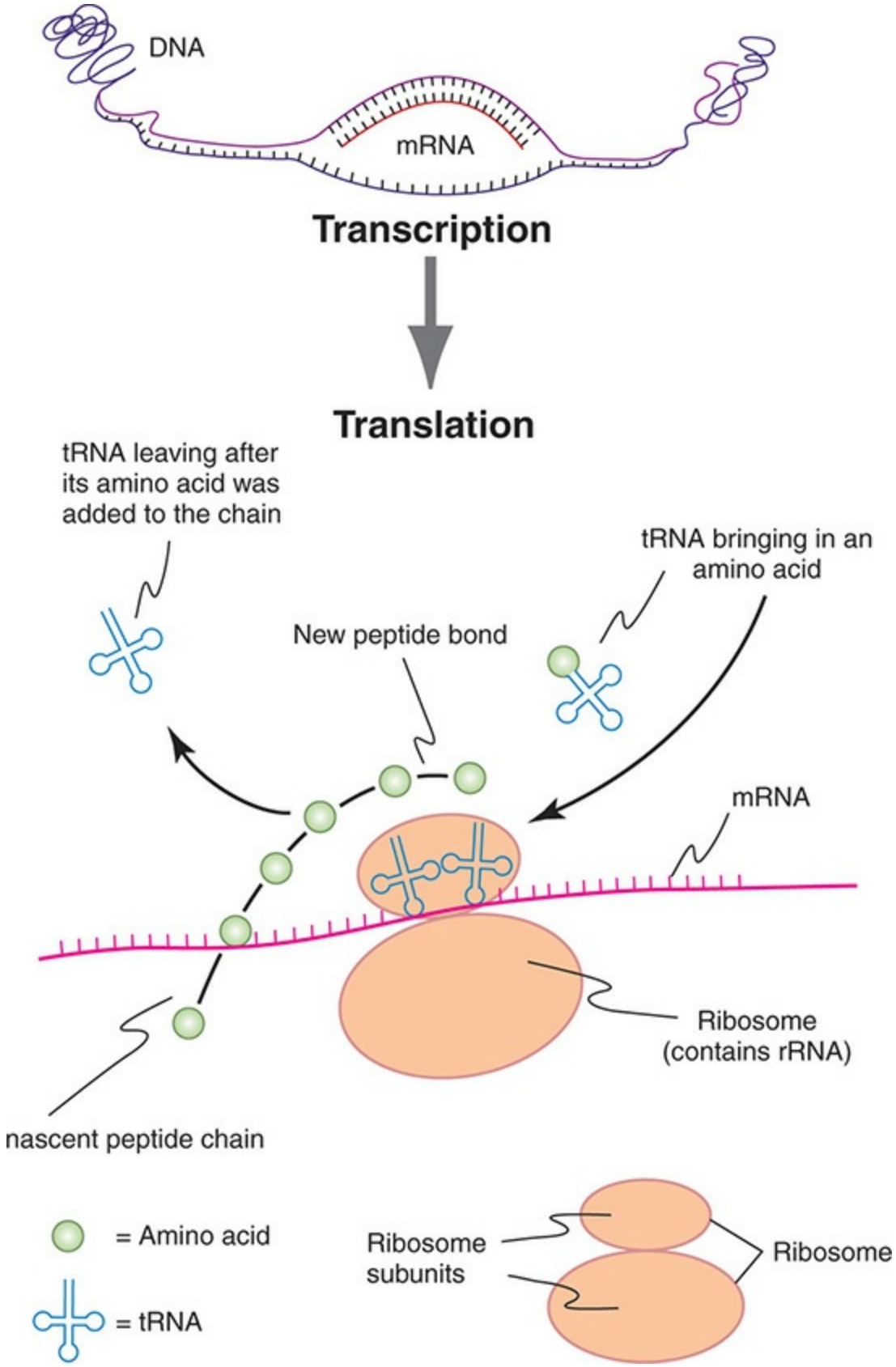

DNA
mRNA
Transcription
Translation
tRNA leaving after its amino acid was added to the chain
tRNA bringing in an amino acid
New peptide bond
mRNA
Ribosome (contains rRNA)
nascent peptide chain
= Amino acid
= tRNA
Ribosome subunits
Ribosome

■ **FIGURE 4-6** Schematic of transcription and translation. In the most basic representation, the genetic message in DNA is rewritten or transcribed (transcription) into messenger RNA (mRNA), which is used as the substrate for translation (i.e., the forming of proteins from the genetic code).

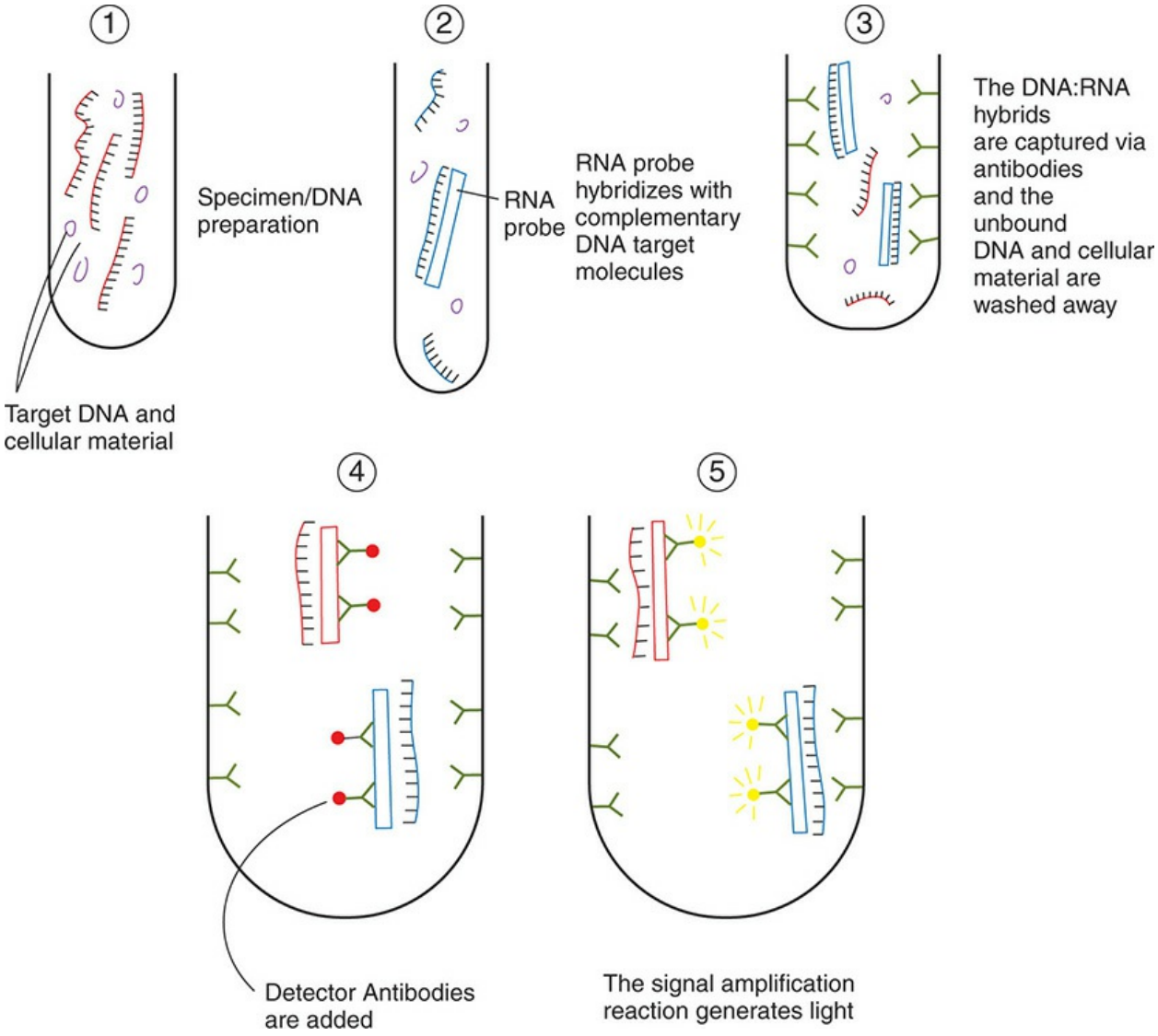
and *Chlamydia trachomatis* represented a significant advance in sensitivity compared with culture for these fastidious pathogens, but have subsequently been replaced by nucleic acid amplification methods.[41,48,119,202]

Today, genetic probes remain a useful option for the rapid identification of a variety of microorganisms in culture, many of which would otherwise require complicated and time-consuming tests for identification. The most useful of these are assays for the identification of mycobacteria and the dimorphic fungi. Genetic probes are commercially available for the *Mycobacterium tuberculosis* complex, *M. kansasii*, *M. gordonae*, and the *M. avium/M. intracellulare* complex.[75,115,243] Genetic probes are also available for *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides* spp.[131,156] The use of these probes for the characterization of cultures that contain mycobacteria or fungi suspected to be dimorphic fungi significantly diminishes the time to identification for the clinical laboratory when compared with routine, traditional methods. Although more expensive, these have overall cost savings, when cost of both materials and labor is considered.

A variety of AccuProbes are also available for the identification of certain bacteria in culture. These include assays for *Campylobacter*, group A and group B *Streptococcus*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *N. gonorrhoeae*.[63] These assays, when performed from cultures, have sensitivities and specificities in the high 90s to 100%. The advantage of using these assays is time savings. The disadvantage is higher cost.

Hybrid Capture

Hybrid Capture (Qiagen, Gaithersburg, MD) is a signal amplification technology that consists of the retention (capture) of a DNA–RNA molecular complex (a hybrid) in a microtiter plate (Fig. 4-7). Initially, the specimen is processed to prepare the



■ **FIGURE 4-7** HC technology. HC technology is a signal amplification assay. It has become a popular way to detect high-risk HPV subtypes.

DNA. The RNA probes used are complementary to the DNA molecule from the microorganism of interest. The detection of a DNA–RNA hybrid complex is useful, since this complex exists only transiently in nature, primarily during transcription. If the complex is not present, the specimen is considered negative. The DNA–RNA hybrid, if present, is introduced into a tube or the well of a microtiter plate, the walls of which have been coated with a monoclonal antibody that recognizes a DNA–RNA hybrid. This antibody captures and immobilizes the complex and allows the other constituents of the processed clinical specimen to be washed away. Next, another antibody is added and that also recognizes the DNA–RNA complex, but this antibody is labeled with a reporter molecule and able to generate a signal in the form of light.

Light above a certain threshold is considered a positive reaction.

Clinical Applications. There are HC assays commercially available for the detection of high-risk human papillomavirus (HPV) subtypes, a subtyping product to determine if HPV types 16 or 18 are present, *N. gonorrhoeae* and *C. trachomatis.* In the past, an assay for cytomegalovirus (CMV) was an option, but this has been discontinued with the migration of users to quantitative PCR for CMV. The high-risk HPV assay has become standard of care in monitoring women for the cervical dysplasia and carcinoma. A meta-analysis showed that the HCII assay had a greater sensitivity and equivalent specificity compared with a repeat Pap smear for the detection of women with cervical intraepithelial neoplasia type 2 (CIN 2) lesions or higher, when the initial Pap smear was interpreted as atypical squamous cells of undetermined significance (ASCUS).[9] In addition, it has been added to the recommendations of the American College of Obstetricians and Gynecologists as an adjunct to cytologic screening for women 30 to 65 years old; updated guidelines can be found at http://www.uspreventiveservicestaskforce.org.[2,8] The strength of this assay is its high sensitivity and its high negative predictive value.[154] It has been found by some to even be comparable to PCR; however, others have found it slightly less sensitive than nucleic acid amplification-based methods.[30,49] Although this is a highly useful assay, both the sensitivity and the specificity have been questioned.[68,283] de Cremoux et al. showed that some of the false-positive reactions with this assay were due to probe cross-reactivity with low-risk HPV subtypes, while others were likely due to "signal contamination" or bleed over of the fluorescent signal from strongly positive wells into an adjacent wells

that contained negative specimens.[68] Although this technology is time tested, more recently released nucleic acid amplification-based assays are available, which are more automated and have excellent sensitivity and specificity.

The HC has also been used successfully for the detection of *N. gonorrhoeae* and *C. trachomatis.*[64,65,236,316,362] This assay has been reported to be more sensitive than culture, which is not surprising given the fastidious nature of these pathogens, and more sensitive than genetic probe analysis.[65] It has also been used to detect CMV and hepatitis B virus (HBV).[257,361,379] When CMV HC was compared with PCR, the HC technology was 1.5 to 2.0 log less sensitive than PCR; the specificities of PCR and HC in this comparison were 100% and 93.8%, respectively.[34] These uses of HC have essentially been replaced by more sensitive nucleic acid amplification assays.

Branched DNA

Branched-DNA (bDNA) technology is or has been used for the detection and quantitation of pathogens such as HIV, HBV, and hepatitis C virus (HCV). This technology consists of a variety of bDNA probes and signal amplification reporter molecules. In brief, the organism-specific bDNA oligonucleotide probes hybridize to the target nucleic acid molecule, and this complex is captured onto a solid substrate. bDNA oligonucleotide reporter molecules, which are conjugated to reporter enzymes, are added. These, in turn, generate a chemiluminescent signal after the addition of the appropriate substrate. The amount of signal, as with the HC assay, is proportional to the amount of target nucleic acid, which means quantitative results can be generated when this assay is performed with quantitative standards.[312,360]

Clinical Applications. Although a very useful and highly reproducible technology, a large number of clinical assays that use this technology have not been developed. This technology has been used most successfully for the detection and quantification of the HIV, HBV, and HCV.[45,84,265,387] It has been used to monitor the viral loads of patients infected with HIV. The VERSANT HIV-1 RNA 3.0 (Siemens, Malvern, PA) has been found to be a highly reproducible assay with wide linear range (75 to 500 copies of HIV-1 RNA) that is useful for quantitative HIV-1 viral load measurements, even when compared with reverse transcriptase (RT)-PCR.[83,113] Similarly, multicenter evaluations of the VERSANT hepatitis B DNA 3.0 assay and the VERSANT HCV RNA 3.0 assay found that these assays and this technology were reproducible, had a wide dynamic range, and demonstrated results comparable with other commonly used assays.[82,265,387] Although the sensitivity of the bDNA assays may be slightly less than that for PCR, there is overall a good correlation between viral loads generated by these different assays.[62,240] Although useful, the applications of this technology too are being steadily replaced by nucleic acid amplification methods.

In Situ Hybridization

In situ hybridization has been used in the molecular pathology for many years to detect chromosomal translocations (i.e., t9;22) and gene amplifications (i.e., Her2) and to identify infectious agents.[224] These once complex methods are becoming easier to use, and semiautomated platforms are available. ISH may be separated into FISH, wherein the oligonucleotide probe is labeled with a fluorophore that is detected by direct fluorescence microscopy, and CISH, wherein the oligonucleotide probe is labeled in such a way that an enzymatic reaction generates a color that can be viewed using traditional light microscopy. Several studies have demonstrated the feasibility and potential advantages of in situ assays in the clinical microbiology laboratory.[152,164,177,249,250,296]

Some of the first ISH assays used were for the detection of viruses through direct hybridization of an oligonucleotide probe and the viral nucleic acids. These have been used to identify the particular virus associated with a certain cytopathic effect (i.e., CMV as a cause of a Cowdry type A intranuclear inclusion), as well as to differentiate viruses that produce identical cytopathic effects (i.e., herpes simplex virus [HSV] and varicella zoster virus).[28] This technology may also be used to differentiate the high-risk and low-risk HPV subtypes, which is important in the assessment of squamous cell carcinomas of the head and neck.[334]

ISH has also proven useful as a technology that allows for the demonstration of the certain viruses with neoplasia, such as HPV with dysplasia/squamous cell carcinoma of the uterine cervix, Epstein–Barr virus (EBV) with posttransplantation lymphoproliferative disorders and Burkitt's lymphoma, and human herpesvirus 8 (HHV-8) with Kaposi's sarcoma.[39,203,334,367,] The direct visualization of the virally infected cells in the focus of pathology helps support the etiologic role of the detected organism with the disease. Although the detection of viruses using ISH has proven useful in the molecular pathology laboratory, these are often replaced by immunohistochemical methods; it is uncommon to find these technologies used in clinical virology laboratories.

ISH may also be used for the detection of bacteria, mycobacteria, fungi, and parasites. When ISH is used to detect these organisms, the microbial rRNA may be used as the target for the hybridization probe. This is advantageous over a DNA target for two reasons. First, there are many more copies of rRNA in the cell than there are copies of the genes that encode for the ribosomes (rDNA), which increases the sensitivity of the assay. Second, the presence of rRNA helps denote the presence of a viable organism, wherein the detection of DNA, particularly by methods such as PCR, may occur even after the organism is nonviable (i.e., killed by antimicrobial therapy).

Prior to the introduction of FISH into the clinical microbiology laboratory, these technologies have been used by environmental microbiologists to identify and enumerate *Legionella*, *Escherichia coli*, and other bacteria in water samples and biofilms.[215,216,274] Veterinary microbiologists have also used these techniques to study a variety of diseases.[192,207] The molecular pathologist has used the ISH to study *Helicobacter pylori* in gastric biopsies, *Chlamydia* in a variety of disease states, and *Legionella pneumophila* in fixed respiratory specimens.[16,18,23,109,127,165]

The ability to rapidly characterize mycobacteria as tuberculosis or nontuberculous mycobacteria has been the goal of many assays. The mycobacteria are excellent candidates for identification by molecular methods, since some of these cause severe disease and are slow-growing in culture (i.e.,

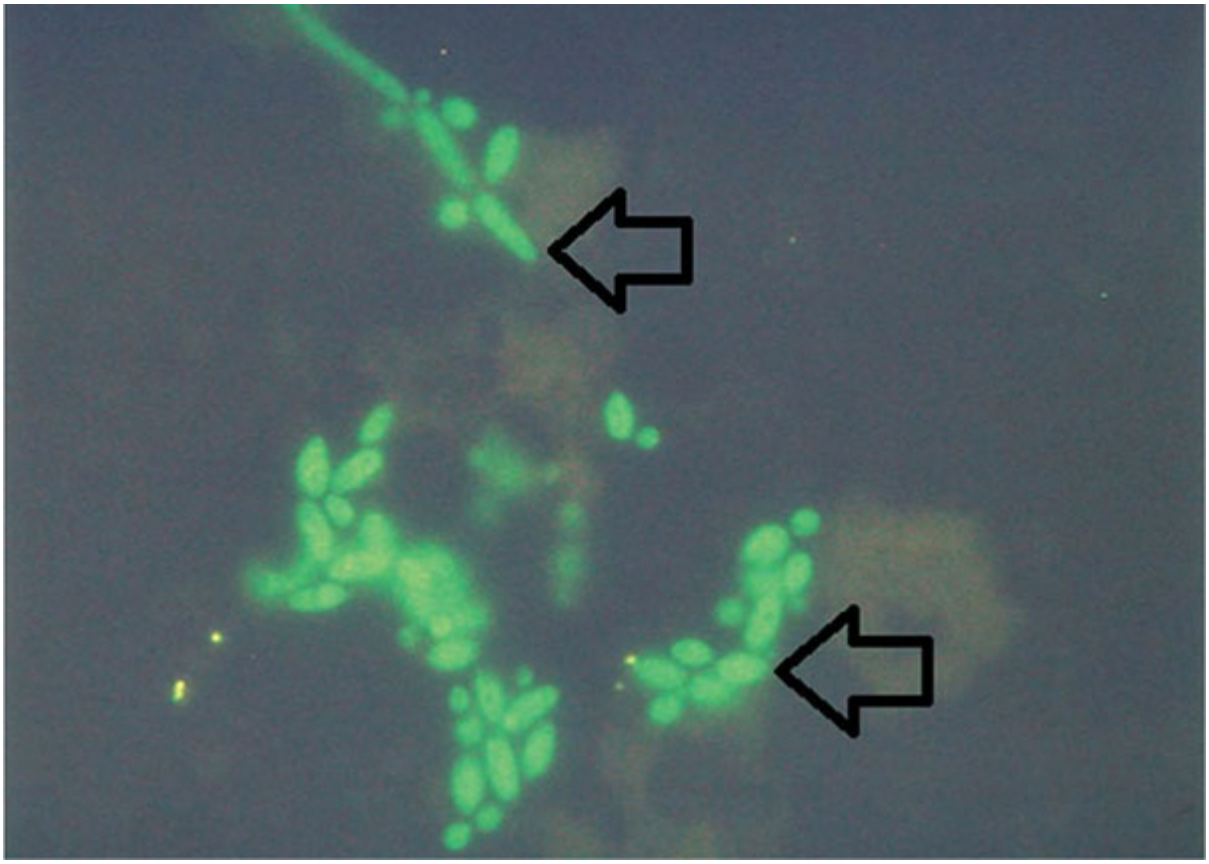
M. tuberculosis), and some are fastidious (i.e., *M. genavense* and *M. haemophilum*) or cannot be cultivated on artificial media (i.e., *M. leprae*). FISH using peptide nucleic acid (PNA) probes has been used successfully for the rapid identification of *M. tuberculosis* in smears from mycobacterial cultures and directly in respiratory samples that contained acid-fast bacilli.[254,342,343] Traditional in situ applications have also been used in histologic sections to definitely identify *M. leprae*.[10]

Fungi and parasites have also been characterized by ISH. ISH assays have been described for the identification of *Aspergillus* species, which is important given the increasing importance of non- *Aspergillus* hyaline septate molds, such as *Fusarium* and *Pseudallescheria*, that are pathogenic to humans and have antifungal susceptibility profiles that are different from most *Aspergillus* species.[138,258,393] These methods are especially important when the excised material was not submitted for culture or if the mold fails to grow in culture. CISH, using a tyramide signal amplification method, has also been applied to the difficult histologic differential diagnosis of morphologically similar yeast and yeast-like forms in human tissues.[145] These methods were less sensitive than the methenamine silver stain for the detection of some of the fungi, but they demonstrated 100% specificity with species-specific identification.[145] Another fungus that has been studied using ISH is *Pneumocystis jiroveci*.[144] The use of ISH for the detection of this fungus may hold some advantages, since both the trophozoite and cyst forms may be detected, rather than only the cyst form, which is the case with some histochemical stains, such as Gomori's methenamine silver (GMS) stain.

Clinical Applications. The use of ISH in clinical microbiology has predominantly focused on the rapid identification of microorganisms in positive blood cultures. Jansen et al.[164] and Kempf et al.[177] simultaneously published articles investigating the use of ISH for the rapid identification of organisms present in positive blood culture bottles. A variety of probes were used, all of which were directed against specific target sequences present in bacterial rRNA. Some probes were genus- and family-inclusive probes (i.e., all staphylococci or all *Enterobacteriaceae*, respectively), whereas others were specific for particular species (i.e., *S. aureus* or *E. coli*). The majority of these probes were highly accurate for the identification of the bacteria responsible for the positive blood cultures. One attractive feature of these assays was that the selection of the ISH probes to be used could be directed based on the findings of the Gram's stain. Another attractive feature was the time to detection, which may be as little as 2 to 3 hours. Kempf et al. compared the time to identification for ISH with traditional methods for 115 positive blood cultures they examined. They found an approximate time savings of 26 hours for the identification of staphylococci (62 of 62 identified by ISH), 46 hours for streptococci (19 of 20 identified by ISH), and 40 hours for gram-negative bacilli (28 of 30 identified by ISH).[177] Overall, they concluded that with a

limited number of ISH probes, the cause of 96.5% of positive blood cultures could be identified within 2.5 hours, whereas conventional culture took between 1 and 3 days.[177]

ISH has also been used for the direct detection of pathogenic bacteria directly in clinical specimens. Hogardt et al. used ISH to detect and identify the bacteria likely to be present in the respiratory specimens of patients with cystic fibrosis.[152] They tested for *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *H. influenzae*, and *S. aureus* using ISH. The ISH assays performed directly on the clinical specimen demonstrated 90% sensitivity and 100% specificity compared with culture, even in the complicated milieu of respiratory specimens from patients with cystic fibrosis.[152] It is conceivable that a battery of ISH probes may be useful for the detection of pathogens that are commonly associated with particular diseases, such as bacterial meningitis and community-acquired pneumonia.



■ **FIGURE 4-8** *Candida albicans* PNA FISH. The yeast and pseudohyphae *(arrows)* fluoresce apple green and are easily detected against the red background using the *Candida albicans* PNA FISH (AdvanDx).

Another method of ISH, which uses a PNA probe rather than a DNA probe, has been developed and is commercially available. PNA probes have properties that may be advantageous over DNA probes with regard to penetration, when hybridization is performed on intact organisms, and for greater differentiation, particularly of single nucleotide polymorphisms (SNPs).[341] AdvanDx (Woburn, MA) is a supplier of PNA ISH kits for the rapid detection of *S. aureus* or coagulase-negative staphylococci, *E. faecium* and other *Enterococcus* species, and a number of gram-negative bacilli. Several studies have examined these assays and found them to be useful for identifying and differentiating morphologically similar bacteria in positive blood cultures.[42,249,250]

ISH has also been shown to be useful for the rapid identification and differentiation of clinically important yeast in positive blood cultures. Kempf et al. differentiated *Candida albicans*, *C. glabrata*, *C. krusei*, and *C. parapsilosis* using four ISH probes.[177]Similarly, the *C. albicans* PNA FISH assay (AdvanDx) has been shown to differentiate *C. albicans* from non- *C. albicans* yeast in positive blood cultures[251,296] (Fig. 4-8). The original *C. albicans* only assay has been changed to a two-color assay, using a green fluorophore for *C. albicans* and a red fluorophore for *C. glabrata.* Interestingly, this company subsequently created what could be viewed as a fungal pharmacogenomics assay. It is a three-color probe set marketed as a "traffic light." Green fluorophores are used for *C. albicans* and *C. parapsilosis,* indicating that it is acceptable to "go" ahead and use fluconazole. A yellow fluorophore

is used for *C. tropicalis.* Red fluorophore-labeled probes are used to hybridize with *C. glabrata* and *C. krusei*. The red color is to provide a warning concerning the use of fluconazole, since the susceptibility of *C. glabrata* to this drug is not predictable and many isolates are resistant, and *C. krusei* is innately resistant.[355]

Traditional and PNA ISH has also been used for the rapid differentiation of mycobacteria. The mycobacteria are obvious targets for differentiation by molecular methods, since members of this group cause severe disease (i.e., *M. tuberculosis*), many are slow-growing, and one at least, *M. leprae*, has not been cultivated on artificial media. ISH has been used to differentiate *M. tuberculosis* from nontuberculous mycobacteria in cultures that become positive, as well as directly from positive acid-fast smears and in histologic sections.[343,392] The confirmation of *M. leprae* in histologic sections has also been achieved with ISH.[10]

There has been limited use of ISH when studying parasites and parasitic diseases, but some uses have been described.[162,261,358,394] There has been even less use in the clinical parasitology laboratory. ISH has been used to detect the most common

microsporidia, *Enterocytozoon bieneusi*, which causes gastroenteritis in patients with AIDS.[368] PNA FISH has been used to detect circulating trypanosomes of patients with African sleeping sickness.[285]

ISH is a molecular diagnostic tool that has been integrated into many clinical microbiology laboratories. The rapid time to detection and the ability to selectively choose probes to be used based on the findings of the Gram's stain or the characteristics of the patient population under consideration makes this technology attractive as a diagnostic tool. However, there are new technologies (see Microarrays) that may replace the FISH tests that are commonly used in clinical microbiology.

Nucleic Acid Amplification

Basics of the PCR

The PCR, first described by Kary Mullis in 1983, exploits the basic biochemistry of DNA replication, with the end goal of amplification of a particular portion of DNA.[241,242] The portion of DNA that is amplified usually contains diagnostically or therapeutically useful information. The PCR reaction mixture consists of the target DNA for amplification and the master mix. The master mix, in turn, consists of oligonucleotide DNA primers, the four nucleotide triphosphates, thermostable DNA polymerase, magnesium chloride, and water or buffers. The traditional PCR consists of three phases, which are repetitive, which is the basis for the "chain reaction" portion of the name. These three phases are (1) DNA denaturation—or the separation of the two strands of DNA, (2) primer annealing, and (3) primer extension—the portion of the reaction wherein DNA synthesis occurs.

Denaturation of the template DNA molecule or separation of the two individual strands that compose the parent double-stranded DNA molecule is the first step of PCR. This is accomplished by elevating the temperature of the reaction mixture to approximately 95°C. At this temperature, the double-stranded DNA is physically separated into single strands. This occurs because the elevated thermal energy breaks the hydrogen bonds that hold the two strands of DNA together at lower temperatures. Thermal denaturation of DNA is favored over chemical denaturation, since it is readily reversible by cooling.

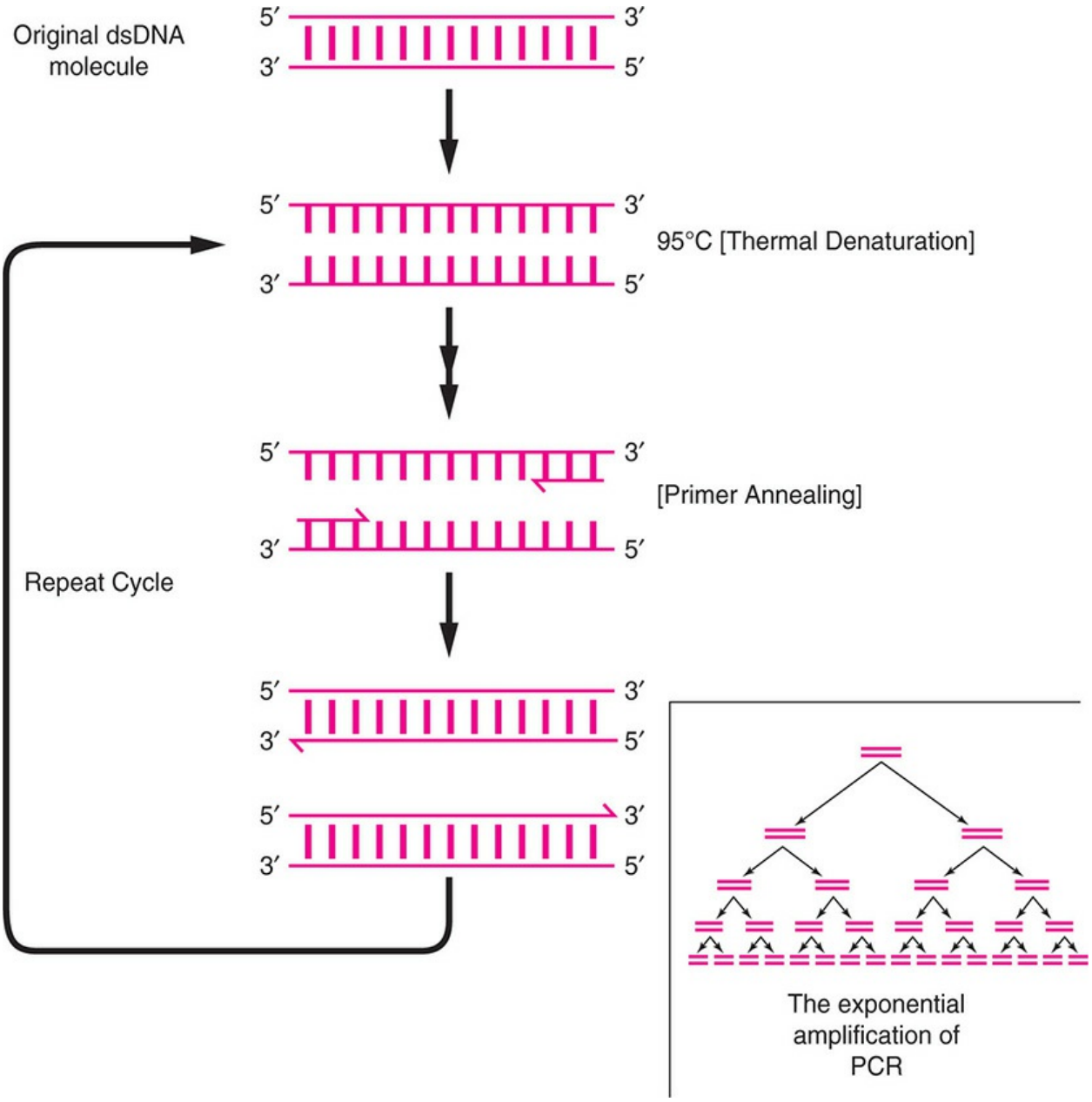
The next step in PCR, primer annealing, begins when the reaction mixture is cooled and the oligonucleotide primers, which flank the area to be amplified, hybridize with the single strands of the template DNA molecule. The oligonucleotide primers are short pieces of DNA, usually between 12 and 20 nucleotides in length, which are necessary to start or prime the DNA synthesis reaction. One primer is the same sense as the upper strand of DNA and is termed the *forward primer*, as denoted (Fig. 4-9);

the other is the same sense as the opposite strand of DNA and is termed the *reverse primer*. The primers hybridize to the target through traditional Watson and Crick base pairing; so the primer annealing temperature should be less than or equal to the T_M, or melting temperature, of the primers used; for simplicity, the T_M is the temperature at which the primers anneal.

When choosing a set of primers, number of technical factors must be considered, many of which are now included in primer design software programs. These are dealt with by industrial scientists when we purchase commercially available PCR kits. Most of the details of primer design are beyond the scope of this text; however, a few points deserve mention. If a single DNA-amplification product, also known as an amplicon, is desired, then the primers should be chosen to hybridize with a single region of the DNA molecule of interest. It is important that the primers do not hybridize with other areas of the molecule from the same organism, or with the DNA of another species that may be present in the specimen preparation. For example, if the molecular microbiologist is attempting to detect *L. pneumophila* in clinical specimens, then he or she should choose a region unique to this microorganism that is not present in the human genome. This has been done successfully by targeting the macrophage infectivity potentiator (*mip*) gene, which contains regions unique enough to differentiate *L. pneumophila* from other bacteria, and is not a constituent of the human genome.[295,382] Cross-reactivity with the human genome would, at best, nonspecifically exhaust the primers and nucleotide triphosphate bases in the reaction mixture, and could, at worst, result in a false-positive PCR reaction, depending on the detection system used.

The presence of the products of PCR amplification is often first assessed by examining the products following gel electrophoresis and staining with ethidium bromide. The presence of a single band in the gel is good preliminary evidence of the specificity of the reaction. The presence of multiple bands suggests that the primers are nonspecific and/or the stringency conditions of the reaction are not optimal (Fig. 4-10). In some instances, the specificity may be improved by changing the reaction conditions, such as salt concentration or primer annealing temperature. The easiest of these to change is the primer annealing temperature. The specificity of the binding of the primers to the target DNA molecule will increase as the annealing temperature used approaches the T_M of the primers (i.e., a more stringent condition).

The synthesis of a new strand of DNA that is complementary to the parent strand of DNA is accomplished by DNA polymerase after primer annealing is complete. The



■ **FIGURE 4-9** PCR. The PCR is a repetitive cycling of thermal denaturation, primer annealing, and extension of the primers through the action of DNA polymerase (not shown here). The reaction results in the exponential production of new DNA molecules; the beginning and end of which are determined by the positions of the forward and reverse primers.

discovery of a thermostable DNA polymerase enzyme afforded the cycling of this reaction in a closed tube, without the need to add new enzyme after each thermal nucleic acid denaturation step. DNA polymerase synthesizes the new complementary strand at a rate of approximately 25 bp/sec. This is important to determine how long

the extension phase of the reaction should last, which is based on the length of the amplicon. After the appropriate amount of time has elapsed to allow for complete extension of the amplicon, the reaction mixture is again cycled to 95°C for another cycle of the PCR.

In this manner, the target portion from one molecule of DNA is amplified exponentially to form millions of amplicons. This exponential increase of the target region of DNA explains the exquisite sensitivity of the PCR reaction and the ability of diagnostic tests that use such methods to detect a very low number of pathogens. This extreme sensitivity also explains the great caution that must be taken when working with amplified DNA, since each of the amplified molecules, if released into the environment, could contaminate a clinical specimen (i.e., amplicon contamination) and result in a false-positive reaction. Although PCR sounds as if a single copy of target DNA should be detectable, in practice, there are numerous PCR inhibitors in clinical specimens that limit the sensitivity of these assays. Although nucleic acid extraction is not covered in detail here, the preamplification processing of clinical specimens, to purge them of inhibitors and prepare them for nucleic acid amplification, is likely as important as the amplification reaction itself.

Clinical Applications. The introduction of PCR into the diagnostic laboratory represented a significant advance in medicine. PCR-based assays have allowed laboratory workers to detect organisms that could not be cultured, as well as those that were fastidious and/or slow-growing. The early methods of detecting the products of PCR, however, were laborious and time-consuming (see Postamplification Analysis). Early PCR-based assays, therefore, were reserved for only the most significant of pathogens. The development of automated and semiautomated PCR platforms and easier, more rapid methods of detection increased the use of these technologies, which continues today, for a wider variety of pathogens. Almost every microorganism of clinical interest has been detected and studied by PCR.

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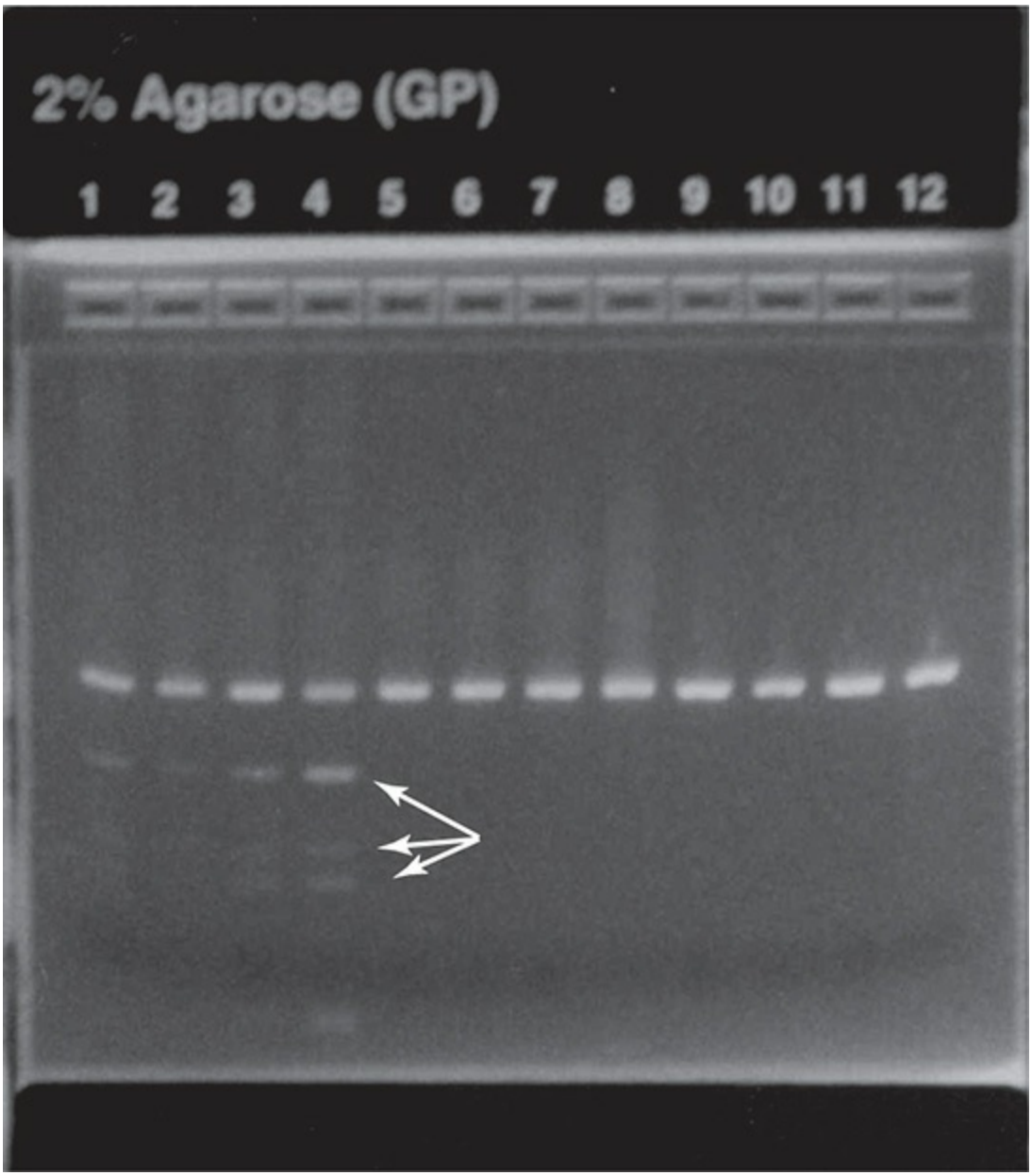

■ **FIGURE 4-10** Nonspecific nucleic acid amplification. Nonspecific nucleic acid amplification may occur during the PCR or other nucleic acid amplification assays if the primers hybridize to an alternative site or bind with one another and form primer dimers. Nonspecific amplification and primer dimers may be minimized through careful assay design and optimization of the reaction conditions. Lanes 1 to 5 all demonstrate nonspecific amplification bands, but these are most prominent in lane 5 *(arrows)*. The solid band in all lanes represents the intended amplification target.

Apart from detection, PCR provided researcher with a tool to examine the presence or absence of particular genetic elements within populations, and, when coupled with

DNA sequencing, allowed researchers to examine the composition and variability of genes of interest. RT-PCR (see below) allowed researchers to detect RNA viruses and to measure the genetic response (i.e., the mRNA) of organisms and eukaryotic cells to a variety of stimuli.

Most PCR assays require nucleic acid extraction prior to amplification, although some assays are so robust that the amplification reaction can be performed following a simple lysis. There are many extraction options available from manual to semiautomated to highly automated (Image Plates 4-1A and B). Because of the possibility of amplicon and specimen contamination, the PCR master mix is prepared in a dead-air box (Image Plate 4-1C). There are now a wide variety of commercial assays that have received Food and Drug Administration (FDA) approval. Some of the first of these targeted two of the most common sexually transmitted bacteria, *N. gonorrhoeae* and *C. trachomatis*.[183,200] Although extremely useful because of an exquisite sensitivity that far exceeds culture, these assays are not perfect.[135] The specificity of these assays, although extremely high, is not 100%, which means in a population of low prevalence the positive predictive value is suboptimal and false-positive results may occur, and confirmatory testing has been recommended.[73,349,363] Furthermore, one of the first *N. gonorrhoeae* PCR assays, as well as an early assay that used strand displacement amplification (SDA) (see below), was shown to have the capacity to cross-react with certain nongonococcal *Neisseria* species, which could cause a false-positive reaction.[4,87,89] This demonstrates that just because an assay is FDA approved and just because it is "molecular" does not make it perfect. The laboratory director and team must still vigorously validate these assays and use them in the most appropriate manner for their patient population.

FDA-approved PCR assays are commercially available for the rapid identification of *M. tuberculosis* in acid-fast bacillus smear–positive respiratory specimens.[101,293] Most recently, the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA), which detects the presence/absence of *M. tuberculosis* and, if present, the genetic elements responsible for rifampin resistance, has been FDA approved[38] (Image Plate 4-1D).

There are a number of FDA-approved assays that use rapid-cycle PCR or loop-mediated amplification (LAMP) available for the rapid detection of group B *Streptococcus* (Image Plate 4-1D and E). Similarly, there are a number of commercial assays that detect methicillin-resistant and/or methicillin-susceptible *S. aureus* (see Real-Time Nucleic Acid Amplification).

FDA-approved assays that use RT-PCR (see below) are available for the detection and quantification of RNA viruses, such as HIV and HCV (Image Plate 4-1F). Additionally, RT-PCR is available in many commercially available assays for the detection of influenza A and B viruses, respiratory syncytial virus (RSV), and other RNA respiratory viruses. There are also FDA-approved assays for CMV, HSV, and other important pathogens.

In addition to FDA-approved assays, there are a wide variety of commercially available analyte-specific reagent and research-use-only products that are available for the study of a variety of human pathogens. These assays may be performed on a variety of rapid-cycle PCR instruments (Image Plates 4-1G and H).

Other Methods of Nucleic Acid Amplification

There are several different methods of nucleic acid amplification that have been devised, many originally devised to avoid licensing fees associated with PCR. Commercial diagnostic kits that utilize some of these technologies are available for several microbial pathogens. One of the first of these to be marketed, which is no longer available, used the LCR. This method was the first commercially available nucleic acid amplification assay to become available and widely used for the detection of *N. gonorrhoeae* and *C. trachomatis*. The non-PCR methods that have been marketed include the nucleic acid sequence-based amplification (NASBA) (bioMérieux, Durham, NC) and transcription-mediated amplification (TMA) (Hologic, San Diego, CA), which are basically the same technology, and SDA (BD Diagnostic Systems, Sparks, MD).

The LCR in its purest form is really a signal amplification reaction that relies on the ligation or connection of two probes that anneal contiguously on the template DNA strand. This technology, when combined with limited extension by DNA polymerase, was the basis of the Abbott products (LCx *Neisseria gonorrhoeae* or LCx *Chlamydia trachomatis* assays,

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Abbott Park, IL) for the detection of *N. gonorrhoeae* and *C. trachomatis*. The use of the two-probe system necessary for the ligase contributed to its excellent sensitivity. The drawback of this assay was the lack of an internal control. Given the absence of this technology from the commercial market, it will not be discussed further.

Both NASBA and TMA are examples of TMA. These isothermal assays use three enzymes: RT, RNase H, and T7 DNA-dependent RNA polymerase. In short, the RT enzyme makes a cDNA copy of the target molecule, which is usually RNA. The primer that is used to create the strand of complementary DNA to the target RNA molecule contains the T7 RNA polymerase binding sequence on the 5′ end of the molecule. The RNA portion of the DNA–RNA hybrid is then hydrolyzed by RNase H. The second primer then binds and creates the complementary strand of the DNA molecule, thereby completing the cDNA molecule. This cDNA molecule has the T7 RNA polymerase binding sequence incorporated into it and serves as the template for T7 RNA polymerase, which transcribes numerous copies of RNA, which may be detected by a variety of methods.

SDA is an isothermal reaction that relies on the ability of the DNA polymerase to displace one strand of DNA at the site of a single-strand nick and proceed with DNA replication or amplification in this assay. This technology, although efficient, requires special conditions and endonucleases that will generate a nick in only one of the strands of the double-stranded DNA molecule, rather than producing a cutting through both strands of DNA, which is the mode of action for most restriction endonucleases.

Rolling circle amplification (RCA) is a method of unidirectional, isothermal nucleic acid amplification. This method has both a linear phase and an exponential phase of amplification. There are a variety of applications demonstrated or proposed for RCA.[8] Loop-mediated isothermal amplification is another method of isothermal amplification. This chemistry uses multiple primers and a DNA polymerase with strand displacement activity. After the initial phases of linear amplification, a double stem-looped structure is formed through the incorporation of primers with specially incorporated complementary sequences. Complex exponential amplification ensues through a variety of intermediate structures. A by-product of amplification, pyrophosphate, binds with magnesium ions in the reaction mixture, which precipitate and afford detection. This technology has been commercialized in a user-friendly, cost-effective format, which is discussed later.

Clinical Applications. There are FDA-approved commercial assays available for the detection of *N. gonorrhoeae* and *C. trachomatis* that use TMA and SDA.[4] Akduman et al. examined over 3,500 urine specimens using the BDProbeTec-SDA assay (BD Bioscience) and reported a sensitivity of 99.2%, a specificity of 99.3%, a positive predictive value of 84.9%, and a negative predictive value of 99.9%. With regard to different amplification assays, these authors, like others, recommend the need to confirm positive results, given the positive predictive value. The BDProbeTec-SDA assay has an internal amplification control, which is useful for identifying potentially false-negative reactions secondary to amplification inhibition.

The APTIMA (Hologic) line of products is FDA approved, commercially available TMA assays for the detection of *N. gonorrhoeae* and/or *C. trachomatis*. These are now available on the newer, smaller, random-access Panther instrument, as well as on the traditional Tigris. Gaydos et al. studied the APTIMA II combo (GenProbe) and compared the sensitivity and specificity of this assay from endocervical swabs versus urine specimens.[107] For *C. trachomatis*, they found a sensitivity and specificity from endocervical swabs of 94.2% and 97.6%, respectively, compared with a sensitivity and specificity from the first-catch urine of 94.7% and 98.9%, respectively. For *N. gonorrhoeae*, they found a sensitivity and specificity from endocervical swabs of 99.2% and 98.7%, respectively, and a sensitivity and specificity from first-catch urine specimens of 91.3% and 99.3%, respectively. The APTIMA assays do not have an internal control for amplification, but rather use a target capture system, which they

claim virtually eliminates inhibition and negates the need for an internal control. In short, these assays are significantly more sensitive than culture-based methods for the detection of these fastidious pathogens.

The Nuclisens HIV-1 QT assay was an FDA-approved assay that used the NASBA technology for measuring HIV viral loads. Similarly, the Nuclisens CMV pp67 has been compared with a CMV antigenemia in a study of bone marrow transplant recipients, and found to be a suitable replacement for the more labor-intensive antigenemia assay.[111] This technology was particularly useful for the detection of RNA viruses, such as enteroviruses and the West Nile virus, but like TMA, NASBA has also been used to detect bacteria, by directly targeting rRNA or other specific mRNAs.[13,78,193,206] Landry et al., in a comparison with RT-PCR for the detection of enterovirus from clinical specimens, found the NASBA assay to be slightly more sensitive and that it had a shorter turnaround time.[194]

This technology is covered largely for completeness and historical purpose, as NASBA is no longer in common use.

LAMP has been commercialized by Meridian Biosciences, Inc. in the form of *Illumigene*. These assays are easy to use and require only a simple, inexpensive instrument. These assays represent a cost-effective solution to community hospital laboratories with limited experience with molecular diagnostics. There are assays available for *Clostridium difficile,* group A *Streptococcus* and group B *Streptococcus.*

Modifications of PCR

A number of modifications have been made to the standard PCR reaction, which have expanded the use of this test, and broadened the spectrum of the microorganisms that may be detected using these molecular methods.[85,264,373] Several of these modifications are well standardized and have been introduced as routinely used techniques in the clinical laboratory. Of the modifications, the techniques that will be described here are RT-PCR, broad-range PCR, multiplex PCR, and nested PCR. Many of these modifications are performed in a real-time PCR format, which will be covered under a separate heading.

Reverse Transcription-PCR. Retroviruses are RNA viruses that as a part of their life cycle make a DNA copy of their RNA genome. This feat is accomplished by the RT

enzyme. The addition of an RT step prior to PCR makes it possible to amplify and detect RNA targets. This may be accomplished in either a two-step or single-step reaction. A separate RT enzyme has been used prior to the addition of the DNA

polymerase in the two-step RT-PCR reaction, whereas it is now far more common to use a single thermostable DNA polymerase that also possesses significant RT activity and is used in the single-step reaction.[244] The cDNA created by RT is most commonly formed using specific oligonucleotide primers, but random oligonucleotide hexamers have also been used. The PCR portion of the reaction then proceeds as previously described.

RT-PCR is particularly useful for detecting RNA viruses, but it may also be used to detect other microorganisms by targeting the rRNA.[26,173,388] The detection of mRNA is useful for studying the genetic expression of both microorganisms and human host cells. As with PCR, quantitative results may be obtained with RT-PCR, which forms the basis for HIV and HCV viral load testing with this technology.[310] Other nucleic acid amplification technologies that preferentially amplify RNA, such as NASBA and TMA, are technologies competitive with RT-PCR.

Clinical Applications. The detection of RNA viruses has likely been the most important of the clinical applications for RT-PCR to date. Quantitative RT-PCR assays have become commonplace for the detection of HCV and HIV, and there are several FDA-approved assays.[110,265] The quantitative nature of nucleic acid amplification, when used in conjunction with external quantitative standards, is commonly used to determine the amount of these viruses present in the blood of a patient, which is commonly known as the "viral load." Viral load data are important for monitoring the response of the individual patient to therapy. For instance, a patient infected with the HIV virus should demonstrate an increasing CD4 T-lymphocyte count and a diminishing HIV viral load, when treated with appropriate antiretroviral therapy.

RT-PCR has also been used for the detection of the viral causes of meningitis and meningoencephalitis, such as enteroviruses and the West Nile virus.[130,149,155,161,176,370] Several studies have demonstrated the advantages of the rapid detection of enteroviruses from the cerebrospinal fluid (CSF) of patients with meningitis.[286,340] These have examined the advantages of performing these assays in a rapid manner to avoid unnecessary hospitalizations, unnecessary antibiotic use, and unnecessary ancillary procedures—to improve patient care and reduce costs.

The dengue viruses, the Hanta virus, human metapneumovirus, and the severe acute respiratory syndrome (SARS) coronavirus among others have been detected using RT-PCR.[80,175,270,313] The use of RNA-based amplification techniques for the detection of rRNA in bacteria, parasites, and fungi may be advantageous over detection of the rDNA gene by traditional PCR, since the presence of RNA is more likely associated with the presence of viable organisms.

Broad-Range PCR. The specificity of a primer set is determined by a variety of factors. Some of these factors are technical, such as the degree to which the primers

hybridize with their target (i.e., avidity), which is determined by the composition of the reaction mixture (i.e., salt concentration) and the complementarity of the primers with respect to their target sequence (i.e., 100% complementarity = a perfect match). The specificity of a PCR may also reflect the uniqueness of the complementary sequence with which the primers hybridize. In many instances, it is desirable to choose a highly unique (signature) sequence, if one is attempting to detect a single microorganism. For example, it has been shown that the gene that encodes for the *Coccidioides*- specific antigen is a useful target for the detection of *Coccidioides* and the differentiation of this organism from other fungi.[256] Failure to produce a species-specific assay may result in undesired cross-reactivity with closely related species. This is the case with two of the FDA-approved, commercially available nucleic acid amplification assays designed to detect *N. gonorrhoeae*, which are known to be capable of cross-reacting with certain commensal *Neisseria* species.[87,89]

Alternatively, there may be interest in detecting the presence of a larger group of microorganisms, rather than a single species. The primers of such an assay are designed to detect all the organisms in the group of interest while attempting to exclude as many organisms as possible that are not in this group. This would be considered a "broad-range" PCR assay. For example, a primer set that detected only one of the many enteroviruses capable of causing meningitis would be of little value for diagnosing or excluding enteroviral meningitis. In this case, the regions that are conserved among all the clinically relevant enteroviruses would be potential sites for primer and probe annealing. Both specific and broad-range primers are useful, with their utility being dictated by the particular clinical question that is being addressed by the assay. There are many advantages of using broad-range primers, but there are limitations too.

The main reason and principal advantage of using a set of broad-range PCR primers is that any of the members of a large group may be detected in a single reaction. When a positive reaction is obtained, the amplicon may be assessed by multiple methods to determine which member of the particular group is present. This may be done by the use of multiple probes in a Southern blot or enzyme immunoassay (EIA) format, by sequencing of the amplicon or by microarray analysis.[100,134,221]

Postamplification melt-curve analysis is a means of achieving limited differentiation following a broad-range real-time PCR (see Real-Time Nucleic Acid Amplification).[325] The limitation of this technology is that the larger the group the assay is designed to detect, the more likely it is that the reaction will also detect organisms that are phylogenetically related to, but not necessarily part of, the group of interest. For example, we have used a broad-range PCR for the detection of all clinically relevant mycobacteria, but it will also amplify the same segment of DNA from many other G-C (guanine-cytosine)–rich bacteria that are closely related to this group (e.g., *Corynebacterium* spp.). These limitations can be dealt with successfully by using

highly specific probes, but some limitations of this type of application remain. When multiple microorganisms are amplified (i.e., desired targets and undesired targets), there is an early exhaustion of the PCR primers. In addition, it is often difficult to obtain useful information from DNA sequencing because a meaningless mixed sequence will result, unless one is able to identify a sequence that is conserved in the group of interest but is not present in the contaminating organisms.

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Clinical Applications. Any group of related microorganisms, the members of which are of clinical interest, is a candidate for broad-range PCR. As previously mentioned, broad-range primers have proven useful for detecting the enteroviruses that cause aseptic meningitis.[282,286,340] One limitation of some broad-range assays for the detection of enterovirus is cross-reactivity with rhinovirus.[194] This should not be a significant problem if only CSF is examined, but may result in false-positive reactions if respiratory specimens are examined. This, in fact, was an issue in the recent enterovirus D68 outbreak in the United States; one commercial assay, which was claimed to not cross-react with enteroviruses, was found to cross-react in practice.[223]

Possibly the most commonly used targets for broad-range PCRs have been the genes that encode for the bacterial, mycobacterial, fungal, and parasite ribosomal subunits (rDNA).[131,141,320] These genes are particularly useful for both broad-range PCR and taxonomic categorization of microorganisms because they possess both highly conserved and variable regions. The highly conserved regions are excellent sites for broad-range primers, whereas the variable regions that lie between the primer sites may be used as target sites for species-specific probes or may be assessed by DNA sequencing. These applications have been used to help determine the cause of bacterial meningitis, infective endocarditis, bacteremia in patients with infective endocarditis, and other bacterial infections.[106,278,290,306,320] As with culture, the presence of contaminating bacteria is an issue for the interpretation of the results from a broad-range PCR.[19,118]

Not surprisingly, this type of approach has been used by many different groups for the identification of mycobacteria.[52,132,137] The most common type of postamplification analysis has been DNA sequencing, but multiple probes and DNA microarray analysis have also been used successfully for identification.

The rDNA target sites form the basis of many of the sequence-based identification systems currently used. The MicroSeq Microbial Identification System is a commercially available microbial identification system that uses broad-range primers to target the bacterial 16S rDNA and fungal D2 rDNA region (Thermo Fisher, Scientific, Inc., Foster City, CA). After PCR amplification of the unknown bacterium or fungus,

the sequence is obtained and submitted and matched against a database that is maintained and updated by the provider. This database has been evaluated by a number of groups regarding its usefulness in the molecular identification of a variety of pathogens from bacteria to dermatophytes.[52,132,134,228,246,278,384]

A number of other genetic targets, which like the 16S rDNA gene contain both highly conserved and variable regions, have been used for microorganism identification. These alternative targets for identification via broad-range PCR include the RNA polymerase (*rpoB*), heat-shock protein (*hsp*), the elongation factor Tu (*tuf*), and a translocating ATPase (*secA*).[76,180,221,391] Characterization of microorganisms, particularly mycobacteria, by the *rpoB* gene is particularly of interest, since analysis of this gene also provides information regarding the resistance of mycobacteria to rifampin.[12,69,237,324]

Multiplex PCR. Multiplex PCR is an alternative method to broad-range PCR for the detection of multiple pathogens.[21,108,299] In the simplest of multiplex assays, there are multiple primer sets used in a multiplex PCR reaction, each of which usually targets a particular pathogen. More complex multiplex assays could use combinations of broad-range and species-specific primer sets, for example. The advantage of multiplex reactions is that numerous pathogens may be detected in a single reaction, even if these pathogens are from taxonomically different groups. In some instances, one of the primer sets included in the amplification reactions may be directed against a human gene, such as β-globin, or a nucleic acid construct that has been added to the specimen; these are internal amplification controls for the reaction.[238] The amplification of an internal control is important to ensure that the amplification reaction was not inhibited, which helps to ensure the accuracy of the negative results. The quantitative competitive PCR assay is one method of obtaining a quantitative result and is a type of multiplex reaction.[268,386] The limitations of multiplex PCR reactions are largely secondary to interactions between the different oligonucleotides, which compromises the sensitivity of the reaction, particularly when compared with the individual amplification reactions. The design of highly efficient multiplex assays that have minimal oligonucleotide interactions is a complicated endeavor.

Clinical Applications. Multiple multiplex assays are usually designed to detect different microorganisms that cause the same types of diseases (i.e., a syndromic approach to diagnosis). For example, multiplex assays have been developed to detect *S. pneumoniae*, *H. influenzae*, and *N. meningitidis*, the most common causes of bacterial meningitis.[15,331] A number of multiplex assays have been described for the detection of the viral agents of meningitis and meningoencephalitis.[217,280,292] Multiplex PCR reactions are particularly useful when the number of possible pathogens is limited. Multiplex assays have also been used to detect and differentiate the

polyomaviruses that infect humans, the bacteria that cause middle ear infection, the agents of atypical and typical bacterial pneumonia, and causes of viral respiratory tract infections.[56,91,124,147,271,353]

A number of multiplex assays are commercially available. The first of these were available through Prodesse (Waukesha, WI), now owned by Hologic (Bedford, MA). Their products include the ProFlu+ for influenza A and B viruses and RSV; an assay to determine the type of select influenza A virus strains (ProFast+) and an assay to detect a myriad of medically important Adenoviruses (ProAdeno+).[328]

Two or the newest and most innovative products available use a combination of multiplex PCR/RT-PCR with other methods. The BioFire FilmArray (bioMérieux, Durham, NC) uses a combination of nested, multiplex, and individual PCR reactions to detect a variety of pathogens (see Nested PCR) (Image Plates 4-2A and B). The eSensor technology from GenMark Dx utilizes multiplex PCR and/or RT-PCR to amplify a variety of nucleic acid targets. These are then added to a bioelectric microarray, which is used for detection (see Microarrays).

Nested PCR. Nested PCR is a modification of PCR designed to increase the sensitivity of the assay reaction. This modification consists of two primer sets directed against the same target.[85,139] The first set of primers is developed in the usual manner, whereas the second set of primers is

situated internally or nested with respect to the first set of primers. The traditional approach to nested PCR was to perform a number of PCR cycles, usually less than a full PCR run would contain, and then open the reaction vessel and add the second, nested, set of primers.[93,139,299] As one may imagine, the major problem with this approach is amplicon contamination in the laboratory and a consequential loss of specificity of the assay as a clinical test. More recently, assays described as single-step nested PCR reactions have been reported, wherein both sets of primers are added to the initial reaction vessel and an extended PCR is performed. These have been performed in a real-time or homogeneous format (see below) and have demonstrated an increased sensitivity without amplicon contamination.[389] The advantages of these types of applications are being realized, as FDA-approved, commercially available assays have been introduced. These are constructed in a closed system, which essentially negates the issue of amplicon contamination.

Clinical Applications. Nested PCR has been used in many PCR assays in an attempt to improve the sensitivity of the assay. Nested PCRs have proven valuable for the detection of microorganisms that may be in low quantity in the blood and tissues, such as *Rickettsia*, *Bartonella*, and similar organisms.[222,389] Not surprisingly, these

have also been designed for the detection of herpesvirus and enterovirus from the CSF and have been used in the direct examination of the blood as a means of determining the cause of bacteremia.[72,99,128,181,333] Nested PCR primers may also be used in a multiplex reaction and have been used in this manner to detect different herpesviruses, such as HSV and CMV.[252] This approach has also been used to detect *M. tuberculosis*, which may be an advantageous approach when the bacilli are present in very low quantities.[253]

The BioFire FilmArray (bioMérieux) is a commercially available system that employs nested, multiplex, and singleplex PCR reactions for the detection of a variety of pathogens. The FilmArray pouch, wherein amplification and detection occur, includes all the necessary reagents and materials in a self-contained pouch in a freeze-dried format. All that must be added is the specimen and a hydration solution. The solution will be moved throughout the pouch once it is placed within the instrument. Different areas of the pouch will be activated and fluids mobilized at select times, moving them through the system.

The first step in the system, after the specimen has been added, is nucleic acid purification. This is followed by the performance of a nested, multiplex PCR for the microbial targets within each particular assay. It should be noted that this is performed in a closed system, which minimizes the risk of amplicon contamination secondary to nested PCR. The products from this first reaction are then mobilized into secondary chambers wherein a number of singleplex reactions occur designed to detect select agents of disease using end-point melt-curve analysis (see Real-Time PCR) (Image Plates 4-2 A and B).

A number of panels are commercially available for this easy-to-use system, which brings the capability of molecular diagnostics into laboratories of all sizes. The gastrointestinal panel detects all of the common and several of the less common bacterial causes of gastroenteritis, as well as four parasites and five types of viruses. Similarly, the respiratory panel includes not only the viral respiratory pathogens, but also *Bordetella pertussis*, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae.* The blood culture identification panel detects the most commonly occurring and medically important causes of bloodstream infections, as well as select genetic markers of resistance. These three products are all FDA cleared. In development is a meningitis panel that similarly will detect the most important causes of bacterial, viral, and fungal meningitis/meningoencephalitis.

The FilmArray is a clear advance in the syndromic approach to the molecular diagnosis of infectious diseases. It has proven reliable in a number of clinical studies.[287,335] The limitations to this approach are the higher cost of the individual assays. However, when compared with the cost per test for performing each test contained within the assay individually, the cost is quite reasonable and possibly less expensive than traditional methods. The most significant limitation is the throughput,

since each instrument can only process one pouch at a time. This may be ideal for smaller laboratories, but becomes a challenge for larger operations, which may need multiple instruments.

Postamplification Analysis

After completion of the traditional nucleic acid amplification reaction, the amplified product must be analyzed. The methods of analyzing the amplified product range from simply determining the size of the amplicon and the presence of any other products of amplification through gel electrophoresis to determine each nucleotide that composes the amplicon through DNA sequencing. Some methods of postamplification analysis such as hybridization protection assays and single-stranded conformational polymorphisms are not covered here, whereas others such as restriction fragment length polymorphism (RFLP) analysis are covered below in the section on microbial typing.

Traditional Methods of Detection

Gel Electrophoresis/Southern Blot Analysis. Gel electrophoresis is one of the simplest ways of gaining information about the amplicon. Different types of gels may be used for gel electrophoresis, but a simple agarose gel is often used. The agarose gel is rectangular and has wells near one end. The gel is placed into a gel box and covered with buffer; newer, single-use gel electrophoresis systems are available that do not require the addition of buffer. To perform gel electrophoresis, the products of amplification are mixed with a loading dye, which is a dense, viscous, and colored liquid. This helps the user visualize the placement of the mixture into the well when pipetting. After loading, an electrical current is passed through the contents of the gel box and the negatively charged DNA migrates toward the anode. The larger pieces of DNA are retarded by the gel matrix, compared with the smaller pieces of DNA. Therefore, the separation of DNA in gel electrophoresis is largely due to size. A set of DNA molecules of different known sizes, which is a commonly referred to as a ladder, is often placed in the first lane and run in concert with the samples. Through visual comparison of the migration of the amplicon(s) in the

sample lanes (the area beneath the well) and the migration of the DNA molecules of varying sizes in the ladder, the user can estimate the size of the DNA molecule(s) amplified.

The presence of a single band of the expected size is good preliminary evidence of

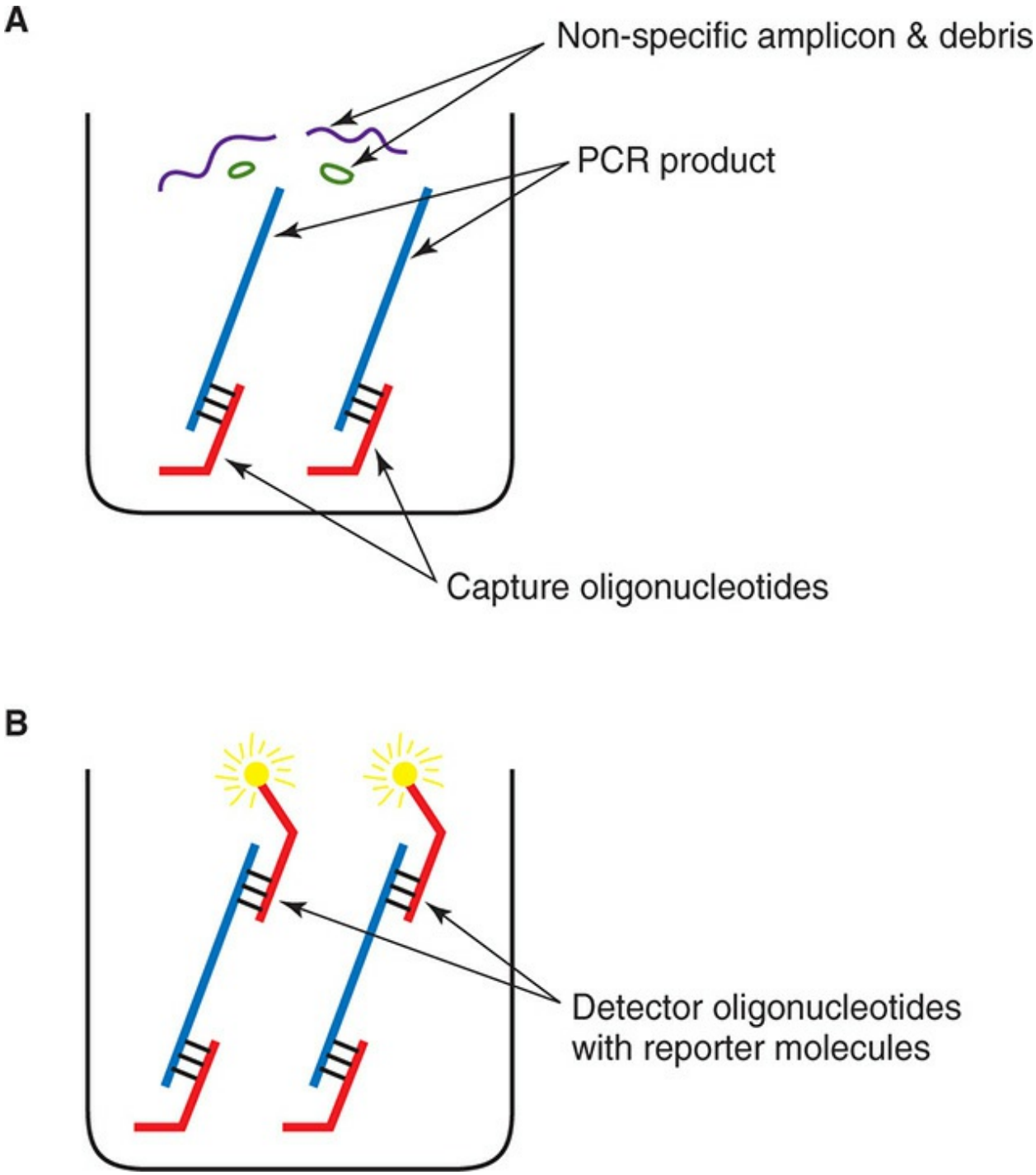
the specificity of the amplification reaction, but the question remains: How can one actually be sure that the amplified product is the desired product and not the product of nonspecific amplification that just happened to be of or near the same size? The answer to this question using traditional methods was achieved by using specific oligonucleotide probes and performing a Southern blot.

In short, after gel electrophoresis, the amplified product was transferred to nitrocellulose paper and a radiolabeled oligonucleotide probe was added. After an appropriate amount of hybridization time and subsequent wash steps to remove unbound probe, the nitrocellulose paper was exposed to x-ray film. If the expected amplicon was present, the radiolabeled probe would hybridize to its complementary sequence on the amplicon, producing an area of exposure on the x-ray film. If the amplicon was the result of nonspecific amplification, the probe would not hybridize and would be washed away, and the x-ray film would not contain an area of exposure on development. This procedure often took 2 to 3 days to complete, was technically complicated, and did not fit well into the clinical laboratory.

Enzymatic Detection of Amplified Products. The use of EIA technology as a method to detect the product of amplification represented a major advance in the rapid detection of specific amplified products.[350] The enzymatic reaction for the detection of the amplified products was often performed in a 96-well plate. Different methods of labeling and detecting the hybridized oligonucleotide probe may be used. The end result, however, is that the hybridized probe that has not been removed by washing generates a signal of either light or, more commonly, color[214] (Fig. 4-11). The use of an oligonucleotide probe increases the specificity of the reaction, whereas linkage with an enzymatic reaction increases the sensitivity of the reaction.[350] The amount of signal generated is proportional to the amount of amplicon present. When used with calibrated standards, quantitative information may be obtained. This methodology was used for an extended period, prior to the introduction of real-time PCR. It is no longer widely used in clinical laboratories.

Reverse Hybridization

The Southern blot, although effective, is time-consuming and labor intensive. In addition, multiple probes or sequencing of the amplicon would be necessary if the product of amplification was secondary to a broad-range PCR. An alternative, highly effective approach is to immobilize all the probes of interest on a nitrocellulose strip and then apply the amplicon to the strip and determine which probe hybridized with the amplicon. This is termed *reverse hybridization*, since the amplicon is applied to the probe that is immobilized in the nitrocellulose strip, which is opposite to how a Southern blot is performed. The assay also has the advantage of using a chromogenic reaction, rather than using radioactivity to detect hybridization (Fig. 4-12).



■ **FIGURE 4-11** There are many methods of detecting an amplicon using enzymes, although this approach is uncommonly used in the era of real-time PCR. **A.** Here, a capture probe is used to retain the specific amplicon so that any nonspecific amplified material and cellular debris may be removed by washing. **B.** The retained amplicon is then detected with another oligonucleotide, and a signal is generated using one of many signal amplification systems.

Clinical Applications. Reverse-hybridization technology is commercially available as line probe assays (LiPAs) (Fujirebio [formerly Innogenetics], Gent, Belgium). This type of technology has been used to differentiate the various genetic subtypes of HCV (i.e., HCV genotyping), a test that provides important prognostic and therapeutic information.[54,66,262,277] The INNO-LiPA HCV II was found to be comparable to DNA sequencing in a direct comparison.[247] Reverse hybridization has also proven useful for screening for the most common mutations present in the HIV genome that confer resistance to antiretroviral agents.[32,70,317,346] The LiPA is technically much easier to perform than DNA sequencing, with simpler posttest analysis. However, unlike sequencing, the use of reverse hybridization allows for an assessment of only the sequences for which there are probes, and does not afford the opportunity to evaluate new mutations, which may be either silent or clinically relevant.[291] Reverse hybridization has also been used to detect HPV and identify the subtypes that are associated with a high risk of dysplasia and subsequent carcinoma.[182,201,225,263,281,365] In addition to virologic applications, reverse-hybridization technology has been used to identify mycobacteria, medically important fungi, and mutations associated with mycobacterial resistance to rifampin.[1,17,37,69,151,220,226,230,233,305,315,347,354] Although highly accurate, the mycobacterial, fungal, and rifampin LiPAs are expensive and are not available in the United States.

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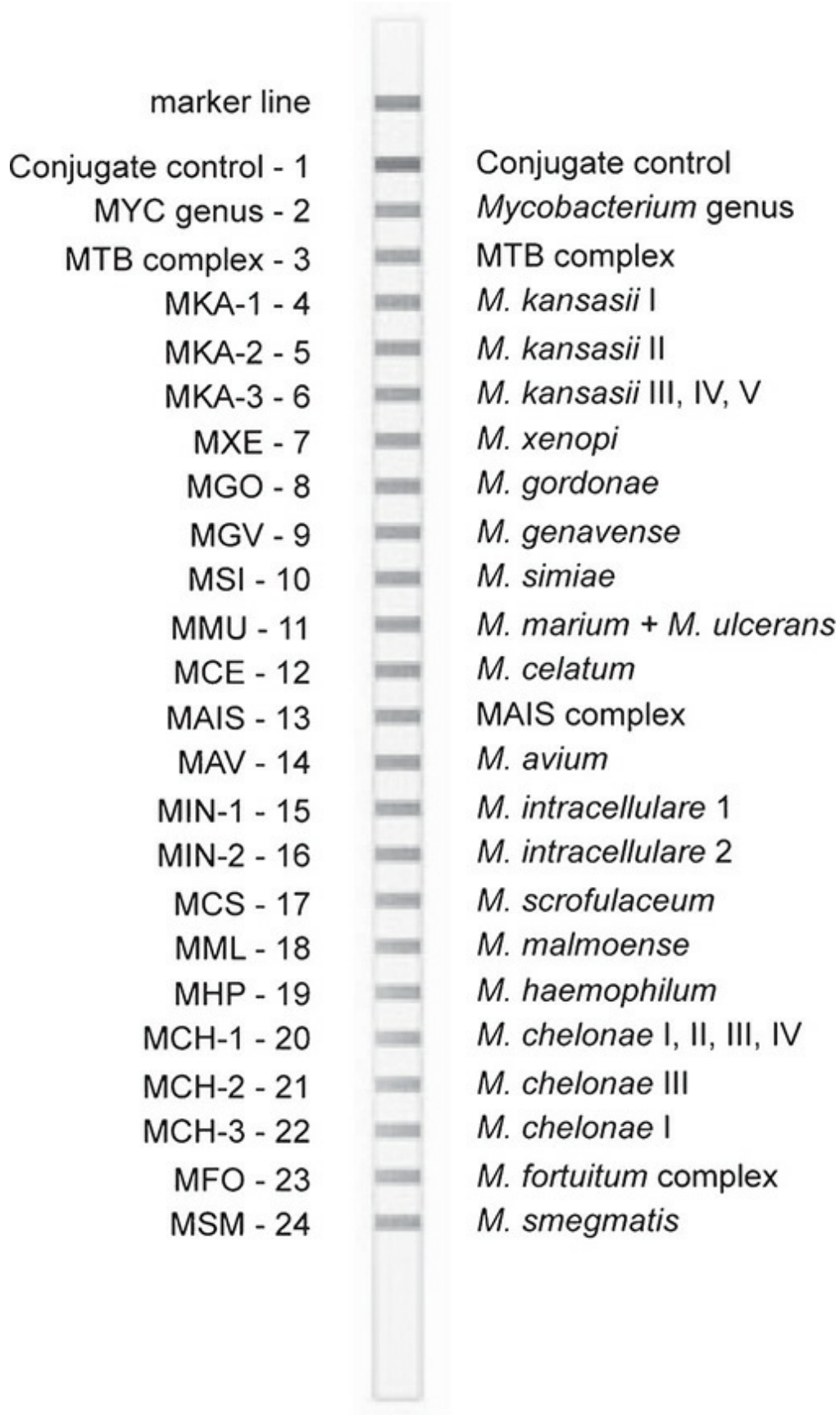

marker line
Conjugate control - 1
MYC genus - 2
MTB complex - 3
MKA-1 - 4
MKA-2 - 5
MKA-3 - 6
MXE - 7
MGO - 8
MGV - 9
MSI - 10
MMU - 11
MCE - 12
MAIS - 13
MAV - 14
MIN-1 - 15
MIN-2 - 16
MCS - 17
MML - 18
MHP - 19
MCH-1 - 20
MCH-2 - 21
MCH-3 - 22
MFO - 23
MSM - 24
Conjugate control
Mycobacterium genus
MTB complex
M. kansasii I
M. kansasii II
M. kansasii III, IV, V
M. xenopi
M. gordonae
M. genavense
M. simiae
M. marium + M. ulcerans
M. celatum
MAIS complex
M. avium
M. intracellulare 1
M. intracellulare 2
M. scrofulaceum
M. malmoense
M. haemophilum
M. chelonae I, II, III, IV
M. chelonae III
M. chelonae I
M. fortuitum complex
M. smegmatis

■ **FIGURE 4-12** The reverse-hybridization strip demonstrates that most clinically important mycobacteria could be identified using PCR followed by reverse hybridization of the amplicon. Similar assays are useful for determining the most frequently encountered HIV and HCV genotypes. (Photo from INNO-LiPA Mycobacteria V2 reverse-hybridization assay package insert, Innogenetics, Ghent, Belgium.)

DNA Sequencing

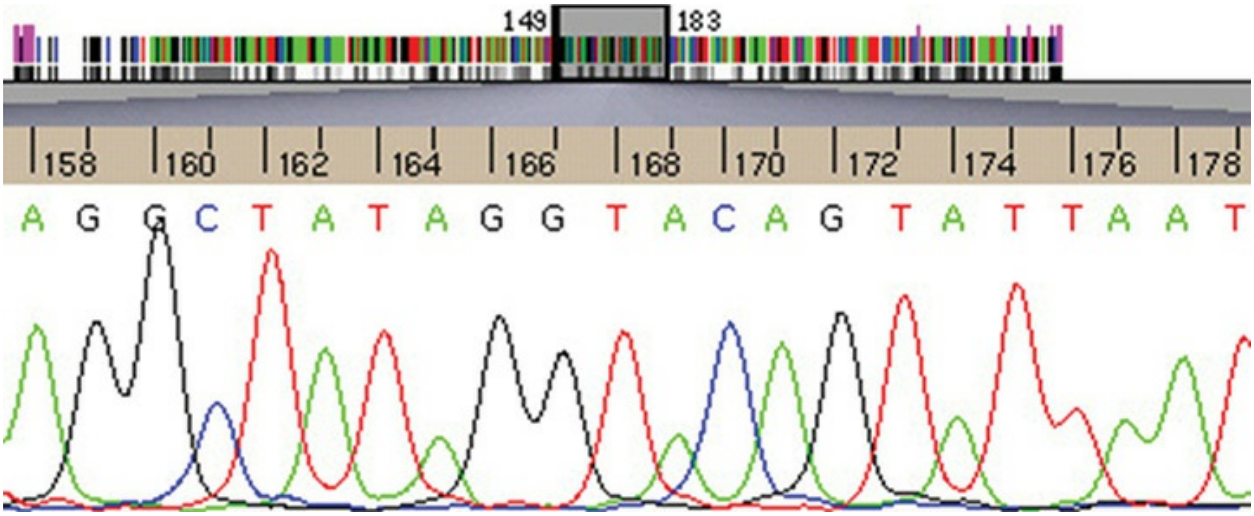
DNA sequencing for the analysis of an amplified product is now a common method of postamplification analysis. Although useful, this technology is more complicated than simple probe hybridization and often requires the user to have experience with sequence alignment and editing software, and genetic databases. DNA sequencing is particularly useful when analyzing a group of microorganisms to determine the areas of conservation and variability within the amplicon obtained by broad-range PCR. Analysis of these variable regions then becomes a powerful tool for the identification of microorganisms.[29,106,114,122,195,205,289,320,371,380,385] It has also proven critically useful for the analysis of genetic mutations within viral genomes, which may often be SNPs and may confer resistance to antiviral drugs.[53,136,186,209] Finally, it may be used for strain typing, by comparing the sequence of genes that contain stain-level variations (e.g., *Spa* typing).

Traditional DNA Sequencing. Traditional DNA sequencing, once a tool used solely in research laboratories, has also become commonplace in many molecular pathology and molecular microbiology laboratories (Image Plates 4-2C). This technology, which uses traditional Sanger sequencing or sequencing by termination, works through the incorporation of dideoxynucleotides into a growing strand of DNA. When a dideoxynucleotide is incorporated into the DNA strand, it can be extended no further. The analysis of the DNA fragments that result, which are as numerous as the nucleotides that compose the strand, provides the sequence information. In the past, this was accomplished with large gels poured between two glass plates and the use of radioactive reporter molecules. Today, these methods have been modified to use fluorescently labeled reporter molecules, with either a more user-friendly gel system or more commonly with capillary electrophoresis (Fig. 4-13). The sequence of long regions of DNA, up to hundreds of base pairs in length, may be readily determined by these methods.

Clinical Applications. HIV genotyping is one of the principal applications of DNA sequencing in the molecular microbiology laboratory.[47,301] This is done to determine the presence of acquired mutations in the genome of HIV for a particular patient that may confer resistance to antiretroviral agents. This assay is complicated and consists of extraction of the HIV RNA, followed by four-to-five RT-PCR assays, each of

which is followed by Sanger sequencing. Although nucleotide determination is largely automated in many of these systems, some manual sequence analysis of the generated sequences is still required. DNA sequencing has also been used to demonstrate resistance-associated mutations in CMV and to determine the HCV genotype.[136,143,186,209,247]

DNA sequencing has also been used successfully for the identification of bacteria, mycobacteria, *Nocardia*, and fungi. The genes that encode for the ribosomal subunits of these organisms are the most commonly used genetic targets of sequence-based identification. One of the most common uses of this approach has been for the identification of the causes



■ **FIGURE 4-13** Sequence from HIV. A DNA sequence from the HIV following RT-PCR is demonstrated here and was obtained by using sequencing by termination (i.e., traditional Sanger sequencing). The most commonly used methods of sequencing by termination utilize capillary electrophoresis rather than gel-based methods.

of bacterial endocarditis.[106,114,123,179,231,279] Sequence-based identification, however, has also been used to determine the causative agent of infection in a variety of other infections, including sinusitis and urinary tract infections.[40,125] This technology has revolutionized the way in which we identify slow-growing microorganisms such as mycobacteria and nocardiae.[51,132,178,228,260,266] It has also been used for the identification of fungal pathogens.[133,134,275] Although rDNA genes are commonly used as the genetic targets for sequence-based identification, other genes that have proven useful for characterizing microorganisms include the *rpoB, hsp*, and *tuf* genes, among

others.[76,77,97,178,191,259,298]

Sequencing by Synthesis (Pyrosequencing). Pyrosequencing is an alternative method of DNA sequencing, which is sequencing by synthesis, in contrast to sequencing by dideoxynucleotide termination or Sanger sequencing[248,302,303] (Image Plate 4-2D). This technology has been used extensively in the analysis of SNPs associated with genetic diseases and neoplasia; it has also been used for the identification and differentiation of microorganisms.[3,6,103,248] Unlike traditional sequencing by termination, this sequencing technology is based on nucleotide incorporation into the newly synthesized strand of DNA.

In short, the PCR-amplified DNA molecule, one strand of which contains a terminal biotin that was covalently bonded to the primer, is immobilized in the well of a microtiter plate. Processing with sodium hydroxide creates a single-stranded molecule. The sequencing primer and reagents are added, followed by the automated addition of each of the four nucleotides. If the nucleotide added is the next appropriate nucleotide and incorporated in the growing strand, then pyrophosphate—a natural by-product of nucleotide incorporation—is generated. The pyrophosphate is then converted into light by enzymatic reactions. The light generated is recorded by the instrument, and the sequence is thereby determined (Fig. 4-14).

The primary limitations of sequencing by synthesis are its ability to generate only relatively short sequences, problems generating a sequence when extensive secondary structure is present, and an inability to accurately characterize regions that contain more than four of the same nucleotide in tandem (i.e., homopolymers). The advantages are ease of use (i.e., the sequencing reaction occurs in a commonly used 96-well plate) and the sequence generated is present in context, which means surrounding, known sequences may be used as a sequencing control. The key to successfully using pyrosequencing for microorganism identification is knowledge of focal genetic targets that provide information sufficient for the differentiation of the microorganisms of interest.

Clinical Applications. Applications that use sequencing by synthesis have been introduced into some clinical microbiology laboratory. A major limitation has been the lack of FDA-approved products and an apparent lack of interest in supporting this technology by the company of ownership.

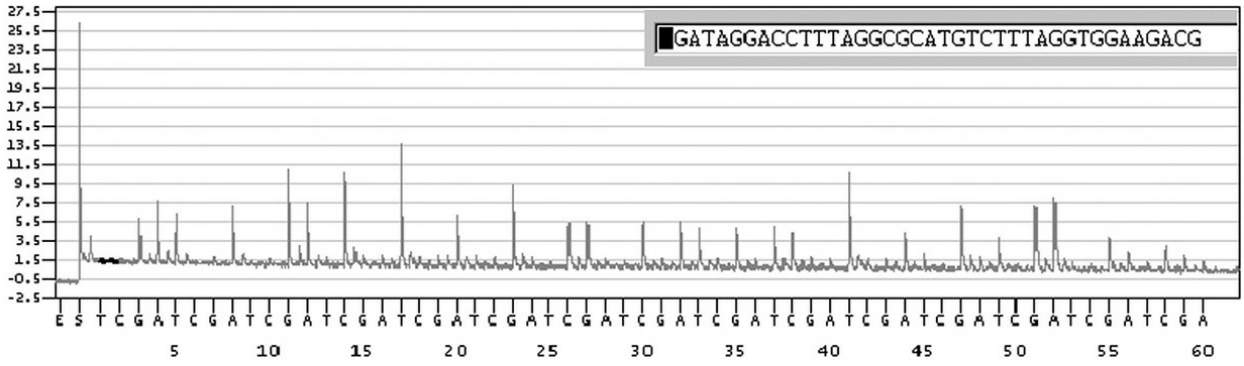
Research applications have included the identification and typing of bacteria, such as *H. pylori* and *L. monocytogenes*, and the differentiation of bacteria into clinically relevant groups.[118,239,359] It has also been used to study linezolid resistance in the enterococci.[327] Additional uses that would likely prove useful for the clinician may include the rapid identification of bacteria that cause infections in neonates and the rapid identification of the bacteria that cause meningitis.[172]

Pyrosequencing has also been used to identify clinically relevant fungi, nocardiae,

and mycobacteria.[112] We have used a broad-range PCR that targets the portion of the 16S rDNA that contains the hypervariable region A and have demonstrated excellent differentiation of the vast majority of the clinically relevant mycobacteria with pyrosequencing; this was achieved with the analysis of a region consisting of only 30 bp.[357] We chose this target because the hypervariable region A had been shown by several groups to be useful for the genetic differentiation of mycobacteria.[74,137,337] Similarly, we have targeted portions of the 16S rDNA gene that confer taxonomic information for *Nocardia* species and have achieved excellent results.[90,219]

The differentiation of viruses has also been demonstrated using this technology. An assay has been developed to determine the HCV genotype using pyrosequencing.[81] We have also used this method to differentiate the human polyomaviruses that may be recovered from human tissues and body fluids.[20]

Many of the pyrosequencing applications described for clinical microbiology laboratories use a broad-range PCR



■ **FIGURE 4-14** DNA sequence by pyrosequencing. This pyrogram was generated from a clinical specimen that contained acid-fast bacilli. Real-time PCR followed by pyrosequencing was performed because of the possibility of tuberculosis. The sequence generated, which is from the hypervariable region A of the 16S rDNA gene, identified the isolate as *M. intracellulare*.

for the microorganisms of interest, followed by the investigation of a compact variable region that confers the genetic information of interest. There are many additional potential applications for sequencing by synthesis for the diagnosis of infectious disease and for the characterization of the infecting microorganisms and the host. These applications may include the identification of the causative agents of infection and the detection of resistance-associated genetic mutations. Furthermore, the

characterization of SNPs or other genetic polymorphisms that may increase the risk of infection in the host could also be determined by this technology. To this end, we have used this technology to perform human toll receptor genotyping to study the effect of this mutation on human susceptibility to infection. Unfortunately, commercially available kits are not yet available; so users must rely on laboratory-developed assays, which has limited the widespread use of this technology.

Next-Generation Sequencing. Next-generation sequencing (NGS) may be performed using a variety of sequencing methods that have been developed to perform massive parallel sequencing (Image Plates 4-2E and F). These methods afford the opportunity to perform DNA sequencing of a large number of amplified products simultaneously and to sort this extensive amount of data using bioinformatics tools. Alternately, there are applications that afford long-read, single-molecule sequencing.

There are numerous potential applications in the area of microbiology. There is an entire subsection of our discipline that is utilizing this technology to study the microbiome. The human, microbiome, environmental interactions will prove to be a rich area of research and discovery for many years to come. Other groups have used a method termed *shotgun* NGS for pathogen discovery, which may have more immediate applications in clinical medicine. Finally, it is now possible to sequence entire bacterial genomes within a day using this technology. Such applications have been used in the ultimate strain typing assays to study epidemics. A detailed discussion of this technology, which is still in the early stages, is beyond the scope of this text, but select references are provided.

Microarray Analysis. A wide variety of microarrays have been used for many years in the basic sciences for many purposes, including infectious diseases.[14,26,33] These devices detect a multitude of signals simultaneously and may be used to detect genetic (DNA) targets, alterations in genetic targets, or differences in expression (mRNA).[33] They are useful in determining which genes are expressed and which genes are silenced in response to different stimuli. These discovery tools have proven useful when studying everything from infections to cancer. Briefly, products of nucleic acid amplification, which may be the result of broad-range PCR or RT-PCR, are applied to the microarray. The microarray consists of often thousands of hybridization sites, each of which provides information regarding the nucleotide composition of the amplicon. The signal generated from the microarray may be fluorescent or electrical. The major advantage of the microarray is its ability to examine thousands of hybridization reactions simultaneously. This feature also contributes to one of its major limitations, namely the amount of data generated from a single microarray may be overwhelming to the user and usually requires computers and advanced software packages for data analysis.

There have been modifications to this technology wherein only a very limited

number of hybridization sites are used. This approach affords the detection of a variety of different pathogens responsible for a particular type of disease (i.e., a syndromic approach to diagnostics). Some groups have employed microarray hybridization technology after organisms have grown in culture (i.e., blood culture). They have found that the growth (i.e., biologic amplification) is sufficient for the detection of the target organisms and a PCR step is not needed prior to amplification.

Clinical Applications. Microarray formats have been used extensively in discovery. They have been used to identify bacteria, mycobacteria, fungi, and viruses, to detect the genetic determinants of resistance, to study host response to infection, and to discover new drugs that may be useful to cure infections.[36,44,46,60,94,100,235,314] Regarding HIV, this technology has proven very useful for studying viral gene expression and the changes in the host cell expression profile after infection.[163,322,348,366,375]

There has been some realization to predictions made regarding the potential utility of microarrays in the clinical microbiology laboratory, with the introduction of two microarray platforms.[33] The GenMark eSensor system, which uses a bioelectric array following multiplex PCR/RT-PCR for the detection of respiratory viruses, was described earlier (Image Plates 4-2G and H). The Verigene system (Nanosphere, Northbrook, IL) is a limited microarray system that may be used with or without nucleic acid amplification (Image Plates 4-3A and B). One of the most exciting applications of this technology is the use of this system on blood culture bottles that signal positive. The Gram's stain is used to select the type of assay to be performed, a gram-positive panel, a gram-negative panel, and in the near future a yeast panel. These panels do not require PCR prior to hybridization with the microarray, since the growth or biologic amplification of the bacteria in the blood culture bottle provides sufficient target for detection. These panels contain an expanded group of organisms that are identified, as well as key genetic indicators of resistance.

The Verigene system may also be used to detect and differentiate the products of a multiplex PCR reaction, and thereby may be used to detect microbial pathogens directly in clinical specimens. The Verigene Enteric Pathogens Test, which is FDA approved, detects the common bacterial and viral pathogens that cause enterocolitis. This group also offers a *C. difficile* assay that detects both toxin genes as well as the ribotype 027 marker of hypervirulence. The Respiratory Virus Plus test detects influenza A and B viruses, identifies some viral subtypes, and detects the H275Y genetic marker of oseltamivir resistance. The Respiratory Pathogens Flex test is an expanded respiratory panel, which in addition to detecting influenza A and B and viruses RSV also detects adenovirus, human metapneumovirus, parainfluenza viruses 1–4, rhinovirus, and *Bordetella.* These and similar tests represent a major advance in the molecular syndromic approach to the diagnosis of infectious diseases.

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Real-Time Nucleic Acid Amplification

Real-Time PCR, which is also commonly called **rapid-cycle PCR**, has evolved from a "new" technique into the manner in which almost all PCR assays are performed in the clinical laboratories. It is incorrect to abbreviate this as RT-PCR, as that designation has traditionally been used for RT-PCR. This section reviews that basics and select applications of this technology.

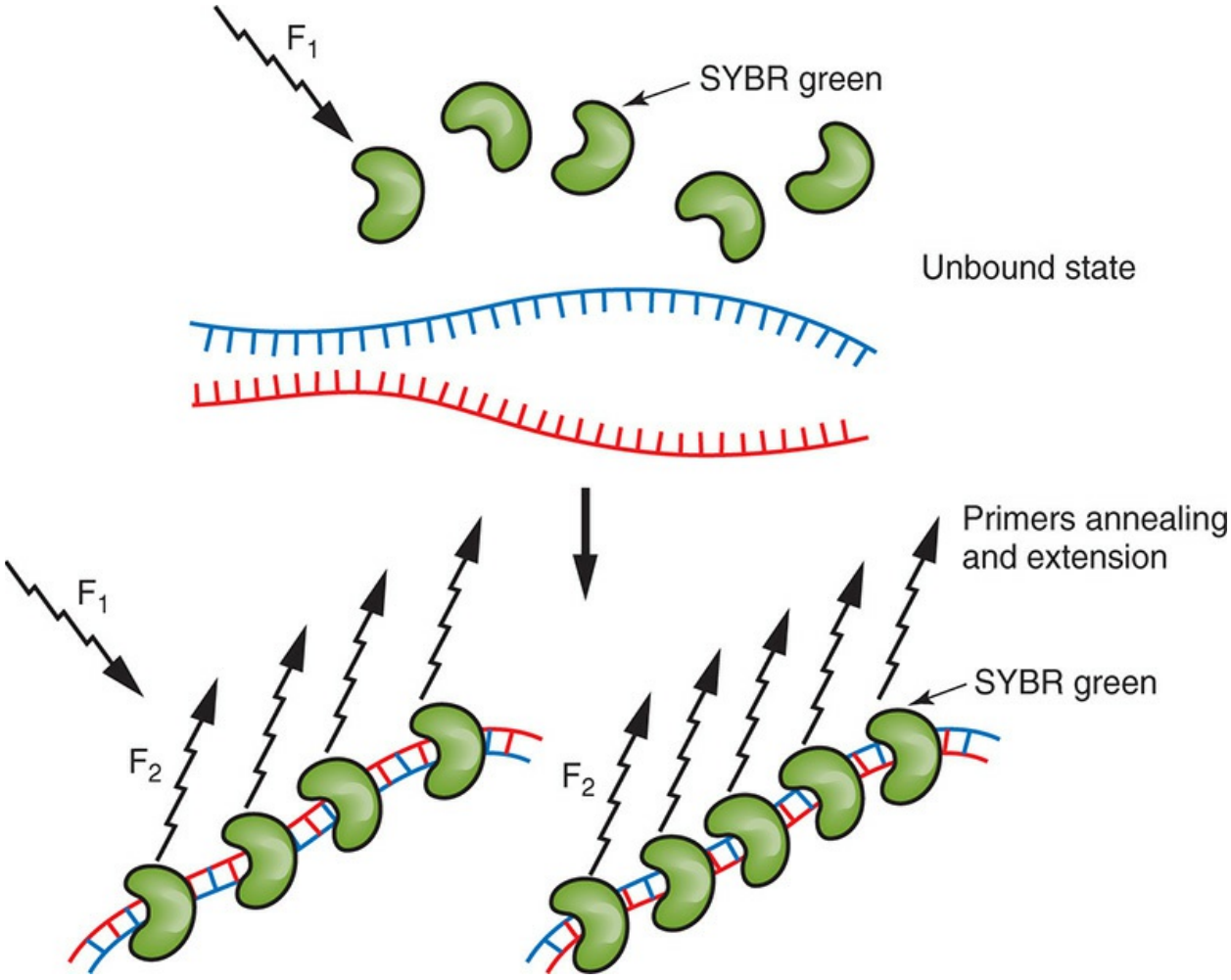
Two technologic advances resulted in the development of real-time nucleic acid amplification. The first was the development of methods of more rapid heat exchange (i.e., rapid heating and cooling) of the reaction chamber.[211] Traditional PCR, for example, occurs in a block cycler, in which the block consists of metal or some other solid material. The rate at which the solid block can be heated and cooled to the temperatures needed for PCR limits the rate at which the cycling may proceed. The rapid introduction of heated air and the removal of the heated air (cooling), which may be achieved through fans in real-time instruments, allows the cycling reaction to proceed more rapidly. The use of smaller reaction volumes also assists in the more rapid exchange of thermal energy. The second technical advance was in the development of a homogeneous reaction mixture.[211] This is a mixture in which the fluorogenic molecules that are used for the detection of the amplified products are present within the same reaction vessel in which amplification occurs. Furthermore, an assessment of the fluorescence from these molecules is usually obtained by the instrument once per amplification cycle. Evidence of amplification occurs during the PCR reaction in "real-time," which is why these assays are commonly referred to as real-time PCR. This type of amplicon detection represented a significant advance in nucleic acid chemistry, since it was more rapid than traditional methods of amplicon detection. In addition, this closed-tube system results in a significant reduction in the chance of amplicon contamination of the laboratory, since the reaction vessel does not have to be opened to analyze the amplicon. There are a variety of platforms available for real-time nucleic acid amplification[98] (Image Plates 4-1G and H). In addition to real-time PCR, the NASBA reaction, in the past, has been modified to a real-time format with detection using molecular beacons (see below). There is the possibility of modifying any nucleic acid amplification technology to a real-time format.

Methods of Detecting the Products of Real-Time Amplification

There are a variety of fluorogenic molecules that may be used for the detection of amplified nucleic acids in a homogeneous or real-time reaction. These may be

nonspecific, detecting any amplified nucleic acid, or specific, such as oligonucleotide probes, which hybridize with a specific sequence present in the amplicon. The nonspecific detection of amplicon using SYBR green and the use of third-generation binding dyes, as well as the three most commonly used types of oligonucleotide probes methods used for specific amplicon detection, will be covered. The specific fluorophore-labeled oligonucleotide probes that will be discussed are the hydrolysis probe (i.e., TaqMan) fluorescence resonance energy transfer (FRET) probes, and molecular beacons. Other types of detector molecules are also available, but a discussion of these is beyond the scope of this text.

SYBR Green. SYBR Green I is a dye that binds to the minor groove of double-stranded DNA. This dye emits very little fluorescence when in the unbound state, but generates considerably more fluorescence when bound to double-stranded DNA (Fig. 4-15). This property makes it useful for determining the presence of an amplified DNA product.



■ **FIGURE 4-15** SYBR green chemistry. SYBR Green I dye binds to the minor groove of double-stranded DNA. When DNA is single stranded, there is no minor groove; so SYBR green does not bind to DNA. However, after primer annealing and extension, SYBR green can bind and emit light (F_2) in response to the appropriate excitation wavelength (F_1). SYBR green is an excellent method of measuring nonspecific amplicon generation, since the more DNA is produced by PCR the more binding sites there are available for binding and the more fluorescence is generated.

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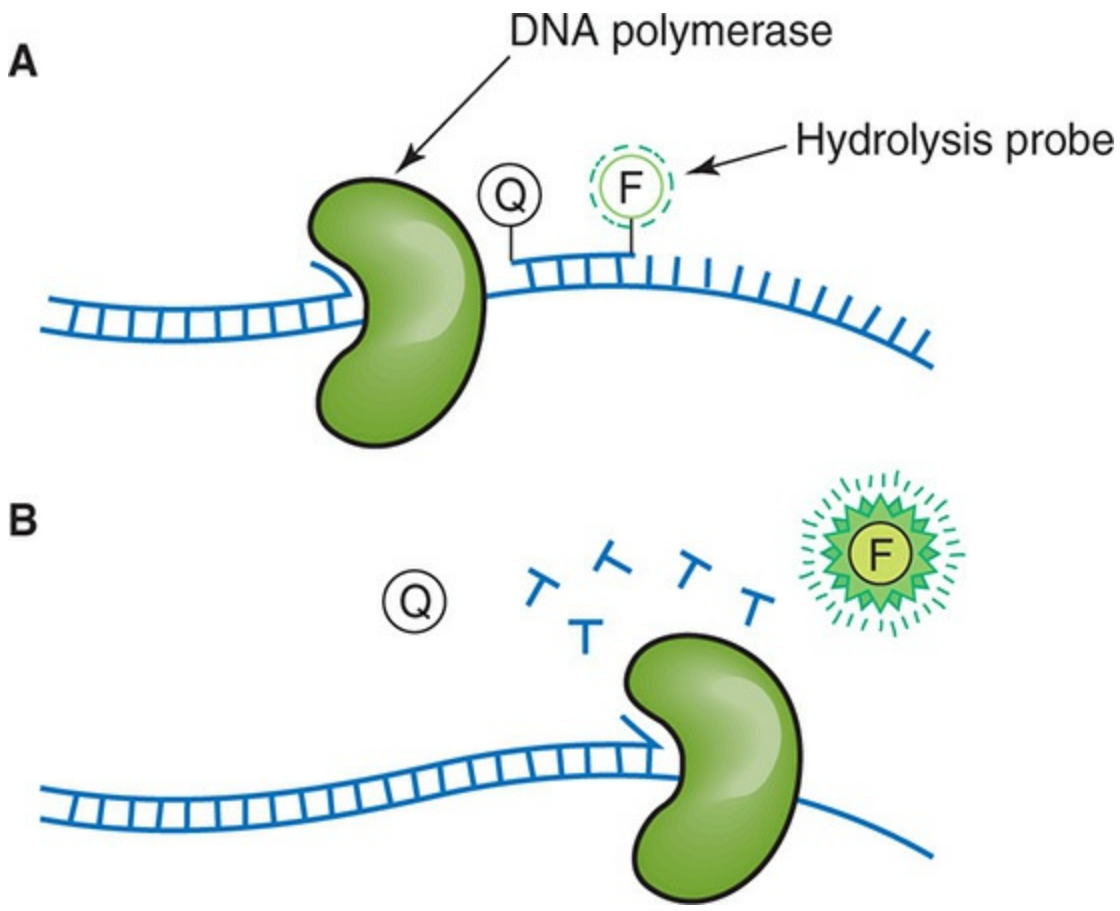
Multiple molecules of SYBR green may bind to a single amplicon, thereby making it a sensitive method of amplicon detection. Also, this dye is much less expensive than specific oligonucleotide probes that are labeled with fluorophores. The detection of amplicons using SYBR green is useful for optimizing the PCR reaction (i.e., determining the optimal concentrations of the primers and $MgCl_2$). It is also useful to determine if amplification has occurred prior to a definitive method of postamplification analysis, such as DNA sequencing or microarray analysis. The main disadvantage of SYBR green detection is that it is nonspecific and these detector molecules will bind to any double-stranded DNA, including the products of nonspecific amplification and primer dimers. This method of detection lacks the specificity achieved with the use of an oligonucleotide probe (described below). It is for this reason that SYBR green detection should never be used as the sole method of detection in a clinical assay.

Third-Generation DNA-Binding Dyes. Advances in double-stranded DNA-binding dyes have resulted in what has been termed *third-generation binging dyes*. These dyes more fully saturate the amplified DNA molecule, so much so that upon melt-curve analysis it is possible to differentiate even SNP. This is an inexpensive, sensitive means of assessing for a SNP, and when this SNP is located in an important location of deriving meaningful information. Although commercially available assays have not been developed, we commonly utilize this methodology to differentiate *M. abscessus* from *M. chelonae,* and use it to identify inducible clarithromycin susceptibility. We have also developed assays to detect the SNP responsible for a toll receptor mutation (discussed earlier) and to differentiate *Plasmodium falciparum* from other *Plasmodium* species.

Hybridization Probes. There are many different types of fluorescently labeled oligonucleotide probes that may be used for amplicon detection in a real-time PCR assay. The three that will be discussed are the most commonly used and cover the most important aspects of real-time detection chemistry. These are the hydrolysis

probes (i.e., Taqman probes), the FRET probes, and molecular beacons.

Hydrolysis or Taqman Probes. The hydrolysis probe detection chemistry relies on the 5′ to 3′ exonuclease activity of DNA polymerase. This function of DNA polymerase hydrolyzes any oligonucleotide that may bind to the single-stranded portion of the DNA molecule for which the complementary strand is being synthesized. In nature, this helps to ensure that only the appropriate nucleotides are incorporated into the newly synthesized strand of DNA. The hydrolysis probe chemistry exploits this property of DNA polymerase to generate a detectable signal. The hydrolysis oligonucleotide probe is labeled with both a fluorophore and a quencher molecule. In the absence of specific target, this molecule folds up to a certain degree, which places the quencher molecule in close enough proximity to the fluorophore so that the majority of any fluorescent signal emitted is immediately absorbed. When this probe binds to the complementary portion of DNA that has been generated by the previous cycles of PCR, it is hydrolyzed by DNA polymerase (Fig. 4-16). This hydrolysis affords the diffusion of the fluorophore away from the quencher molecule and the generation of light that is not quenched and, therefore, detectable.



■ **FIGURE 4-16** Hydrolysis probe chemistry. **A.** The close proximity of the quencher

molecule (*Q*) to the fluorescent molecule (*F*), both of which are attached to the intact hydrolysis probe, results in only minimal background fluorescence. **B.** The hydrolysis of this probe by the exonuclease activity of DNA polymerase as extension occurs results in diffusion of the fluorophore from the quencher molecule, so that fluorescence may occur when the fluorophore is appropriately excited.

FRET Probes. FRET probes function through the transfer of energy. In this system, there are two detector probes used instead of one. These probes hybridize in close proximity to one another on the amplicon, with a two- to five-nucleotide gap between the two probes. The probe on the upstream side of the pair has a fluorescein molecule attached to the 3′ end of the probe, and may be termed the *donor molecule*. The second probe in the FRET set is situated downstream in relation to the first probe, has an acceptor molecule, usually LC640 or LC705 when used with the LightCycler system, on the 5′ end of molecule; this has been termed the *acceptor molecule*. Other acceptor molecules may be used, but they must have the property of being excited by the wavelength of light that is emitted by the fluorescein or another donor molecule and, in turn, to emit light of another, detectable wavelength. In addition, the label on the 5′ end of the acceptor probe, this probe must be phosphorylated to prevent probe extension during PCR. Detection with the FRET probes works as follows: if the appropriate probe hybridization sequence is present in the amplicon, the FRET probes will hybridize. At this time, the donor molecule (the fluorescein) is excited by light energy introduced into the system by the instrument. When the excited donor molecule returns to its base energy state, it releases light of a particular wavelength. This released light excites the nearby acceptor molecule (LC640 or LC705), completing resonance energy transfer. The acceptor molecule is in turn excited to a heightened energy state. When the acceptor molecule returns to its base energy state, it releases light energy that is detected by the system (Fig. 4-17). Detection using FRET-based chemistry is by its nature highly specific, since the only way to receive the final energy signal is for four independent reactions occur. These independent reactions are the hybridization of the two individual primers that afford amplification and the hybridization of each of the probes that afford detection (Fig. 4-18).

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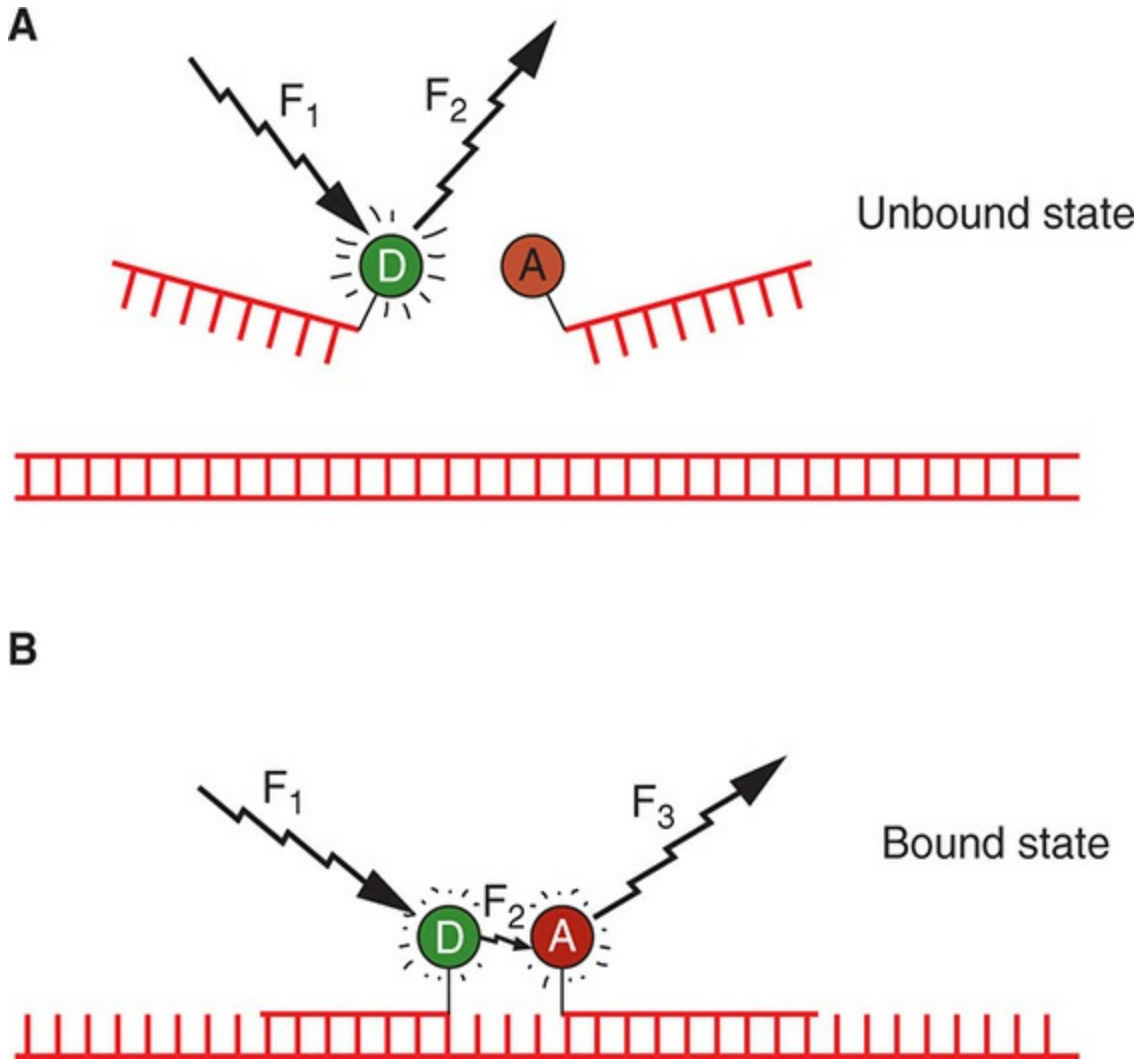


■ **FIGURE 4-17** FRET. Two probes, both of which are labeled with a fluorophore, are necessary to achieve FRET. **A.** The excitation wavelength (F_1) of the donor fluorophore (D) raises the energy state of the molecule and an emission occurs (F_2). This does not affect the acceptor fluorophore (A) when the probes are not hybridized, because they are not in close proximity to one another. **B.** When the two probes are hybridized and near to one another, then the emitted light from the excited donor fluorophore (F_2) acts as the excitation energy for the acceptor fluorophore, which in turn emits an particular wavelength of light of its own (F_3). The presence of the emission (F_3) from the acceptor molecule is evidence that probe hybridization has occurred.

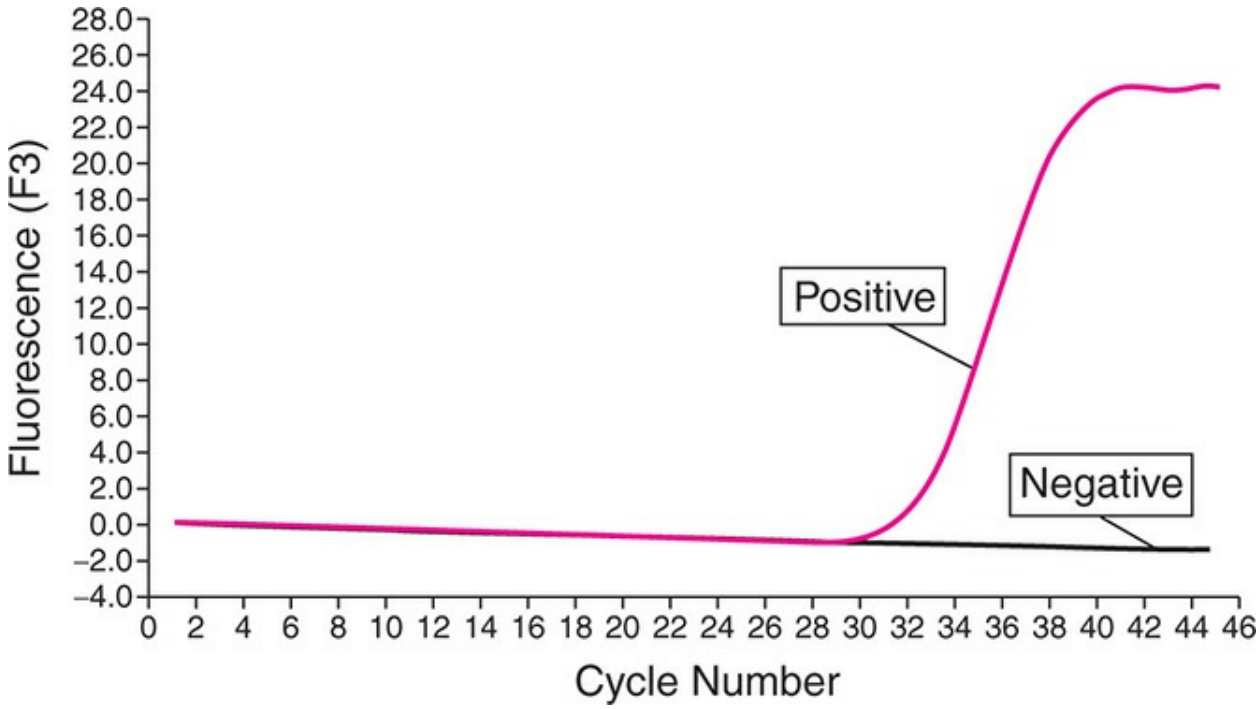
The use of FRET probes also allows the user to perform a postamplification melt-curve analysis. This is possible because of the nature of FRET probe interactions and the fact that unlike hydrolysis probes these probes are not hydrolyzed during detection. The reader will recall that the hydrogen bonds are responsible for the

hybridization between the oligonucleotide probes to the complementary DNA strand. When thermal energy is added to the system that exceeds the energy of these bonds, the bonds will be broken and the probe will "melt off" the complementary DNA target. The point at which half the probe molecules are bound and half are unbound is the melting point or T_M of the oligonucleotide.

Melting-curve analysis is performed after the PCR reaction is complete. Initially, the reaction mixture is cooled to a point well below the T_M of the individual FRET probes so that both will hybridize if the appropriate amplified DNA is present. At this point, an excitation wavelength of light is introduced into the instrument and a signal will be generated from the FRET reaction that occurs between the juxtaposed probes. Next, the reaction mixture is then slowly heated while continuous fluorescence measurements are taken. When the reaction mixture reaches and finally exceeds the T_M of the FRET probes, there will be a precipitous loss in the fluorescence signal. The first derivative of the slope of this curve results in a peak (Fig. 4-19). The perpendicular line that may be drawn through the center of this peak represents the experimentally derived T_M.

There are at least three important uses for melt-curve analysis. First, there should be a relatively predictable melting temperature for the expected amplicon, which may be confirmed using known standards. The demonstration of the appropriate melting temperature helps assure the user that the identification is correct, rather than a misidentification potentially obtained through the amplification and detection of a taxonomically related organism. Secondly, postamplification melt-curve analysis may be used to differentiate closely related organisms, where the probe hybridizes to an target area wherein there is a small degree of genetic variability that separates the species. Finally, the detection of nucleotide mismatches between the FRET probes and their corresponding probe hybridization site, just like that used for organism differentiation, may be used to detect other clinically important SNPs, such as those that may confer resistance to antimicrobials or those in the host that may denote relative susceptibility to infection.

Molecular Beacons. The next type of detector molecule to be discussed is the molecular beacon. This molecule, like the hydrolysis probe, contains both a fluorophore and a quencher molecule. The molecular beacon, however, is larger than typical hydrolysis probes and is not hydrolyzed by the DNA polymerase in order to achieve fluorescence in the presence of the appropriate amplicon. This molecule has a loop-and-stem configuration (Fig. 4-20). The loop portion of the molecule is the part of the molecule that is complementary to a specific part of the amplicon. The stem is a construct of complementary nucleic acids designed to keep the



■ **FIGURE 4-18** Positive and negative real-time PCR reactions. Positive real-time PCR reactions demonstrate an exponential increase in fluorescence, whereas the negative reactions retain only low, basal levels of fluorescence.

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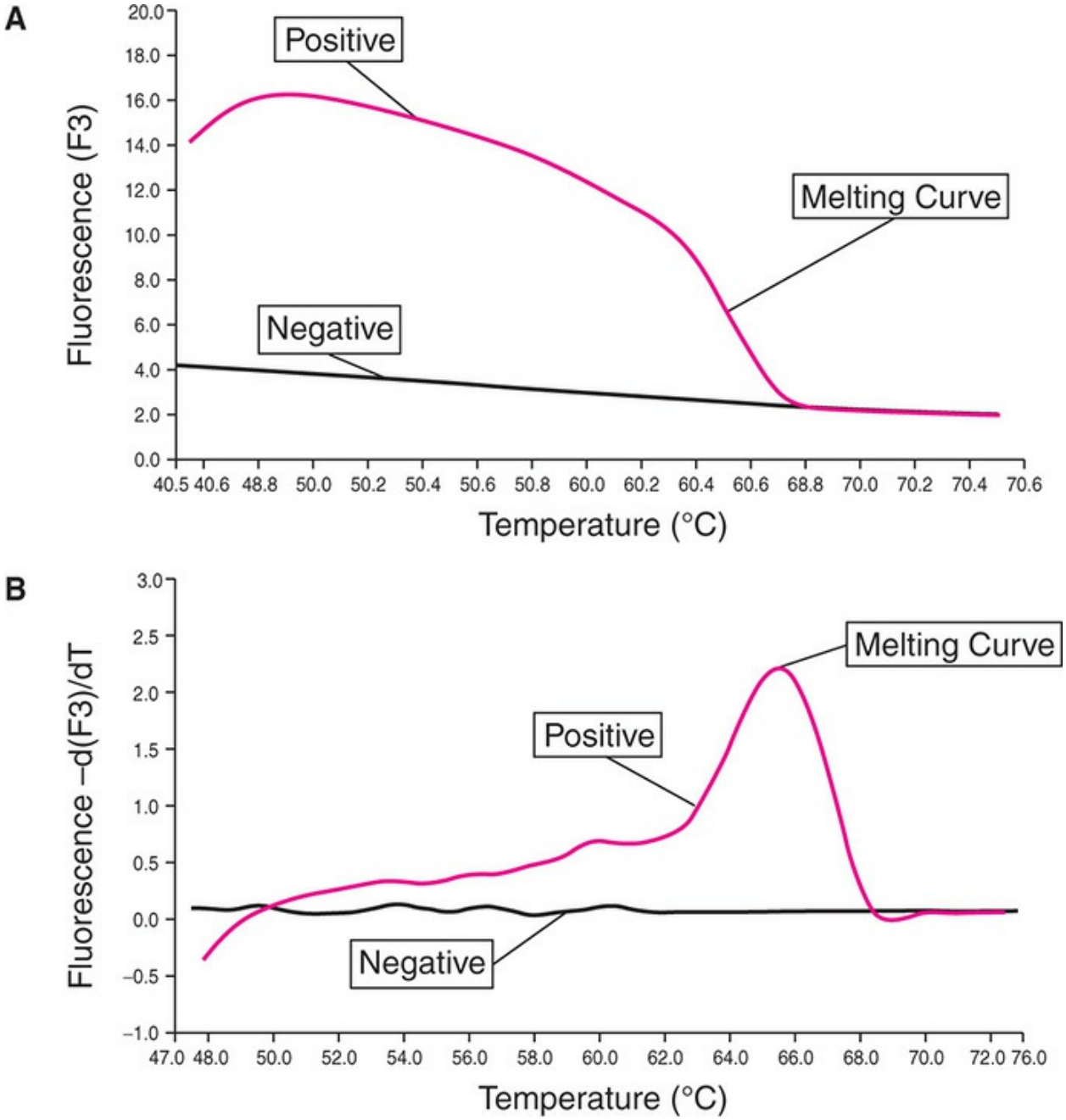
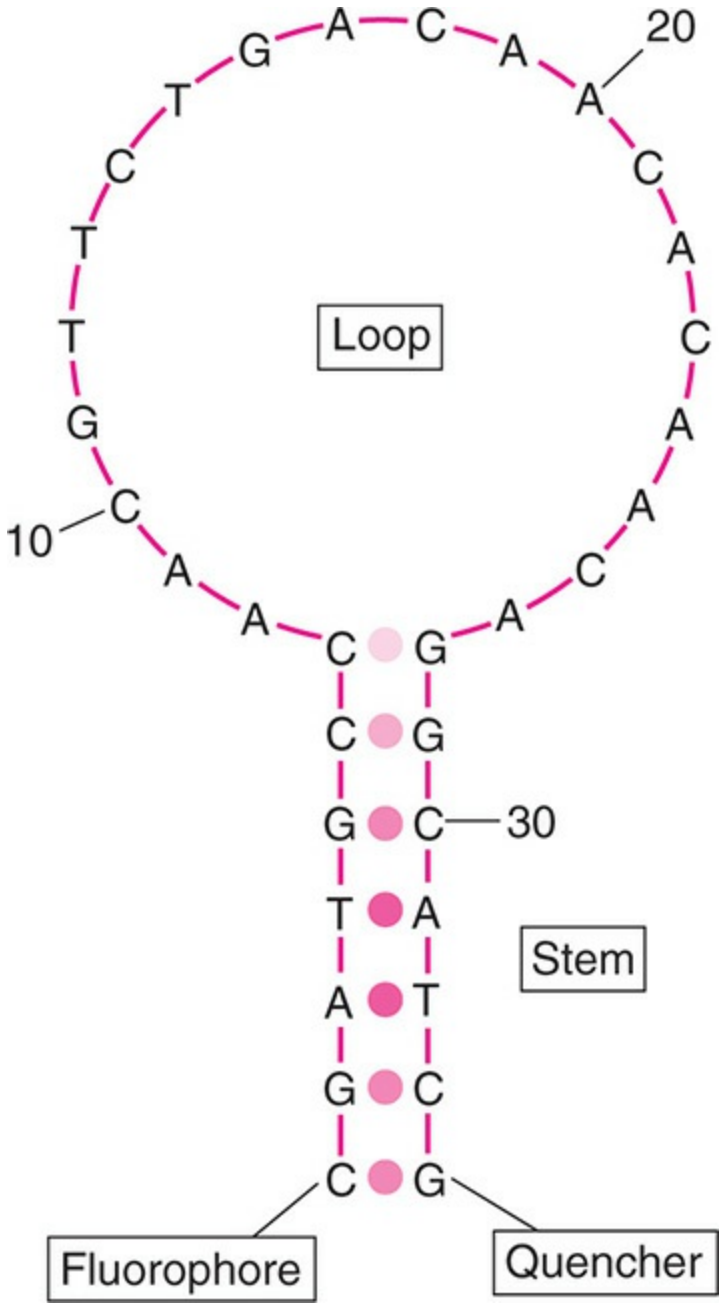


■ **FIGURE 4-19** The melting curves (**A**) and the first derivative of the curve the melting peaks (**B**) are demonstrated for one positive and one negative specimen in a real-time PCR that specifically detects *Staphylococcus aureus*.



■ **FIGURE 4-20** This molecular beacon was designed to detect the amplified product of a broad-range *Salmonella* PCR. It consists of 34 nucleotides and has a stem-loop configuration. This structure places the fluorophore in close proximity to the fluorescence-quenching molecule when the molecular beacon is not hybridized with its genetic target (i.e., the PCR is negative). When the appropriate amplicon is present, the thermodynamics favor hybridization over the stem-loop configuration, the molecule opens and hybridizes, and fluorescence is discernible, as the fluorophore is spatially separated from the quencher.

molecule closed, in the absence of a specific amplicon. The two respective ends of the

molecular beacon probe molecule are the sites of attachment for the fluorophore and quencher molecules. The fluorophore is juxtaposed with the quencher molecule when the beacon is closed (i.e., when it is not in the presence of the appropriate amplicon). When the beacon is in the presence of the appropriate amplicon, the thermodynamics favor hybridization with the amplicon over the closed, semi-self-hybridized state. When the molecular beacon is hybridized, the fluorophore is spatially separated from the quencher, and fluorescence can be detected by the instrument. Melting curves can be achieved with molecular beacons, but it is much more difficult to achieve the same quality of melt-curve results as those achieved with FRET probes.

It may seem difficult for the molecular microbiologist to choose which type of detector molecule to use for a real-time nucleic acid amplification assay. In part, this may be dictated by the type of instrumentation present in the laboratory. For example, if a SmartCycler is purchased, a molecular beacon or hydrolysis would be favored, whereas for the LightCycler, FRET-probe chemistry is favored. The ABI 7500 series (Applied Biosystems, Foster City, CA) and Roche (COBAS) TaqMan (Roche Diagnostics, Indianapolis, IN) platforms use hydrolysis probes. Some platforms, such as the RotorGene (Qiagen), are biochemically agnostic and support any type of probe chemistry. For the most part, the same ends (i.e., detection of the microorganism of interest) can be achieved by a variety of different means. The other major factor in determining the type of probe to use on a particular segment of DNA is the nucleotide composition of that segment. Factors that may influence probe selection include the G-C content of the region and the presence of secondary structure, among others.

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Clinical Applications. There are far too many applications to completely review here. In short, anything previously detected by traditional PCR has been migrated to a real-time PCR platform. The relative ease of this migration has contributed significantly to the implementation of real-time assays for a wide variety of clinically important microorganisms. The commercial availability of a wide variety of assays, the ease of use, the rapid turnaround time, the availability of assay design software for the development of laboratory-developed tests, and the closed-tube nature of these systems has facilitated the implementation of this testing in the clinical laboratory. These assays are not only used for the detection of the fastidious pathogens that have traditionally been approached using PCR, but also for common microorganisms that have been traditionally detected by culture (e.g., *S. aureus*) or EIA (e.g., *C. difficile*). Real-time assays for DNA viruses include assays for the herpesviruses, adenovirus, smallpox virus, and parvovirus B19, among others.[126,140,146,168,184,318,332,344] Postamplification melt-curve analysis has been used to differentiate HSV type 1 from HSV type 2, and such assays are commercially available and some are FDA

approved.[381] The quantitative nature of these assays has also been used to predict disease and monitor the viral load in response to therapy.[11,116,121,168,311,374] Quantitative PCR for the detection and monitoring of HIV, HCV, CMV, EBV, and the BK virus has become the standard of care. Real-time RT-PCR and similar technologies for RNA viruses include broad-range assays for enteroviruses that cause viral encephalitis and individual and multiplex assays for the detection of respiratory viruses.[27,55,129,176,284,330,369,370,376,378] RT-PCR for influenza A and B viruses and RSV has essentially replaced cell culture for the detection of these viruses. Real-time assays have also been used to rapidly more recently recognized and emerging viral pathogens, such as human metapneumovirus and the SARS coronavirus.[59,120,198,212,213,269,270] There are several FDA-approved respiratory viral panels that include essentially all respiratory viruses of medical importance.

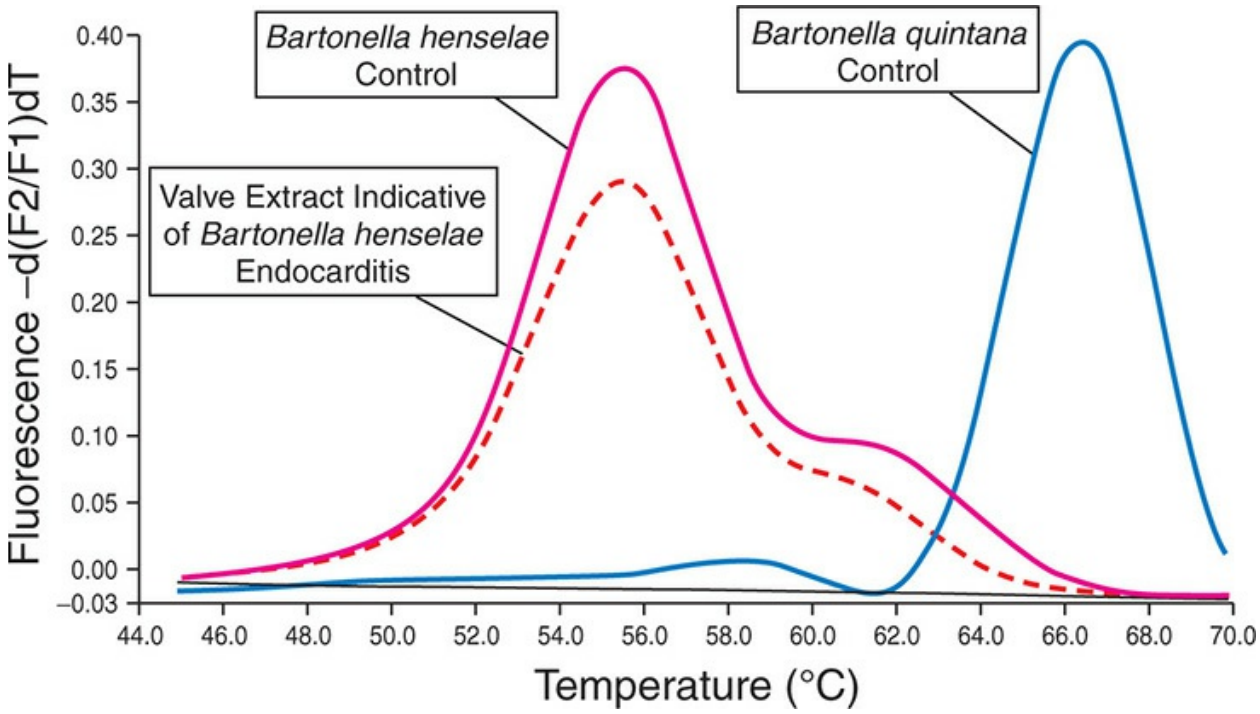
Numerous assays have been described that detect bacteria and the genes associated with resistance. There are several assays for the detection of group B *Streptococcus* for prenatal screening during pregnancy. There are also several platforms to choose from for the detection of methicillin-susceptible and methicillin-resistant *S. aureus*. These applications include the detection of nasal colonization, use on positive blood cultures that contain gram-positive cocci in clusters and wounds. There are assays for the detection of group A *Streptococcus,* which are primarily used as a more sensitive replacement for culture. A major change has been the virtual replacement of toxin-detecting immunoassays for *C. difficile* with rapid-cycle PCR. There is, however, some controversy surrounding this test, as it is so sensitive that it also likely detects patients with colonization; therefore, only detecting patients with the appropriate clinical presentation is very important.

In addition, the literature is replete with examples of real-time amplification assays for the detection and differentiation of a variety of microorganisms and resistance-associated genes. Many of these assays target the traditional microorganisms that do not grow on artificial media or are extremely difficult to culture, such as *Tropheryma whippelii*, *Rickettsia*, and *Bartonella* species.[92,170,389] Postamplification melt-curve analysis also has been used to differentiate the most common *Bartonella* species (Fig. 4-21). In addition to these applications, as PCR in the real-time formats has become widespread and more user-friendly, it is being applied to commonly encountered bacteria and their mechanisms of resistance. For example, real-time PCR and other molecular methods have been shown to be a reliable and more timely method for the differentiation of *S. aureus* from coagulase-negative staphylococci in blood culture bottles that signal positive and contain gram-positive cocci, and is a reliable method for determining oxacillin resistance in these *S. aureus* isolates.[326]

Real-time assays for fungi include assays for organisms that cannot be cultivated in the routine clinical microbiology laboratory, such as *P. jiroveci*, and for slow-growing

organisms, such as *H. capsulatum*.[158,174,197,218,227,255] We have demonstrated a greater than twofold increase in the sensitivity of PCR for *Pneumocystis* over a morphologic assessment of stained respiratory smears. Other applications have included the detection of invasive mold and yeast infection in immunocompromised patients and the detection of other systemic dimorphic fungal pathogens, such as *Coccidioides* and *Penicillium marneffei*.[24,158,267,272,276,288,336]

Another important application for these assays has been the rapid detection of mycobacteria, particularly *M. tuberculosis*. Mycobacteria are excellent candidates for detection



■ **FIGURE 4-21** Postamplification melt-curve analysis. Postamplification melt-curve analysis may be used to differentiate closely related species. In this example, the causative agent of the culture-negative endocarditis, *Bartonella henselae*, is distinguished from *Bartonella quintana* using this technique.

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and identification by molecular methods, since these microorganisms are slow-growing, some cause severe disease and death, and tuberculosis is a communicable disease for which transmission-control measures may be instituted. Several real-time assays have been developed for the rapid detection of *M. tuberculosis* directly from clinical specimens. Shrestha et al. describe a LightCycler assay that detects and

differentiates *M. tuberculosis* from nontuberculous mycobacteria using postamplification melt-curve analysis.[325] Excellent assays for the detection of *M. tuberculosis* are also available for SmartCycler and TaqMan users.[50,71,304] Assays have also been developed for the rapid detection of resistance to commonly used drugs, such as isoniazid and rifampin.[297,308,364] The Cepheid Xpert MTB/RIF is the only FDA-approved assay that detects *M. tuberculosis* and genetic targets of rifampin resistance.[38] This is performed in the standard Cepheid cartridge, which is easy to use and produces results in about an hour. This assay represents a significant advance in the detection of multidrug-resistant *M. tuberculosis.*

The detection and identification of parasites has also been approached using real-time nucleic acid amplification assays. A number of assays have been described for the rapid detection of otherwise difficult to detect parasite *Toxoplasma gondii*.[57,167,204] Other applications include the detection of stool parasites, such as *Cryptosporidium* and microsporidia, the identification of tissue parasites, such as *Leishmania* and *Trypanosoma*, and the detection and differentiation of the *Plasmodium* species, among many other applications.[22,31,43,61,86,96,150,229,319,377,383] Likely, the greatest advance in commercially available molecular parasitology was the release of the FDA-approved APTIMA *Trichomonas vaginalis* assay (Hologics, San Diego, CA). This assay, like many of the others from this company, uses TMA amplification. This assay is more sensitive than microscopy; in some studies the sensitivity was better than lateral flow EIA, whereas in other studies the sensitivities were equivalent.[157,245] The cost-effectiveness of screening all asymptomatic women has been called into question by Hathorn et al., because of the low prevalence of disease in this population; they offer an alternative suggestion of screening asymptomatic individuals in high-risk groups.[142] The precise way in which this assay fits into women's health programs remains to be determined.

Strain Typing

Hospital microbiologists and epidemiologists may need to determine relatedness among bacteria or fungi of the same species to determine if transmission of the organism of interest has taken place in a manner that may be addressed by infection control. For example, if *P. aeruginosa* has been isolated from four babies in a neonatal intensive care unit, the hospital epidemiologist may wonder if these are the same bacterial strain that has been transferred from a point source or if they are different strains and the infection has occurred merely by chance. The determination that the bacteria are all of the same strain strengthens the evidence that there is a point source of transmission of the bacteria and that there is a breakdown in techniques or personal hygiene, all of

which may be corrected.

Phenotypic methods of strain typing preceded genotypic methods, but many of these lack the high degree of discrimination that may be achieved by genotypic strain typing. Some others, such as bacteriophage typing, require a high degree of specialized expertise and the maintenance of considerable resources (i.e., libraries of bacteriophages). Antimicrobial susceptibility profiling, although not as definitive as some of the genotypic methods of profiling, is valuable, and uses information that is often already available because of susceptibility testing performed to guide therapy. Bacteria that have very different antimicrobial susceptibility profiles are likely not identical and therefore probably do not warrant more expensive genotypic strain typing.

The earliest molecular attempts at demonstrating relatedness among different groups of bacteria were accomplished with simple DNA–DNA hybridization studies. Groups of bacteria with high degrees of DNA homology, as determined through DNA–DNA hybridization studies, were considered to be more closely related, compared with groups that had poor DNA homology when compared with one another. For the most part, these studies confirmed what was considered to be true from phenotypic testing. For example, the DNA–DNA hybridization studies between *Staphylococcus* and *Micrococcus*, which are in the same family, would demonstrate greater homology than would a comparison between *Staphylococcus* and *Pseudomonas*.

Plasmids are small, circular DNA molecules that are present in many bacteria and exist separately from the chromosomal DNA. Plasmids are important because they may contain genes important for virulence, including genes that impart resistance to antimicrobial agents. Plasmids may also spread from one bacterium to another via conjugation and thereby disseminate these genes. Developments in electrophoresis that allowed for the separation of plasmids from the chromosomal DNA, and the differentiation of plasmids from one another, made possible plasmid profiling as an early means of strain typing. Plasmid profiling was one of the earliest genetic methods used to determine strain relatedness.

There have been many genetic methods described for analyzing the relationship of microorganisms. Methods that are not discussed here but that have proven useful include ribotyping, random amplified polymorphic DNA (RAPD) analysis, spoligotyping, and most recently applications of NGS.

Molecular typing methods may be categorized as those that require nucleic acid amplification for the typing process (amplification-based methods) and those that do not (non–amplification-based methods). This chapter will discuss one non–amplification-based method, pulsed-field gel electrophoresis (PFGE), which has been considered by many to be the standard for microbial typing. Two amplification-based typing methods will be discussed, PCR-RFLP, which combines the strengths of PCR with the typing of RFLP, and Rep-PCR, a typing method that targets repetitive genetic elements that occur naturally in bacteria.

Non–Amplification-Based Typing

Pulsed-Field Gel Electrophoresis. There are many strain typing methods that vary in the degree of separation that may be detected, cost and ease of use. Many of the

current methods of molecular strain typing rely on the use of a group of enzymes called **restriction endonucleases**. These enzymes are naturally occurring enzymes that cleave double-stranded DNA based on particular sequences termed *restriction sites*. Different enzymes cleave based on different sequences, and a variety of restriction endonucleases are commercially available. When the enzymes cleave the DNA, the two ends of the DNA produced may be either blunt or may have overhanging nucleotide (i.e., so-called sticky ends). When microbial chromosomal or plasmid DNA is extracted from the organisms and exposed to one or more restriction endonucleases, it will be cleaved depending on the restriction endonucleases used and the number of restriction sites present in the DNA. The fragments of DNA that result will vary in size, and may be separated from one another by gel electrophoresis and visualized by staining with ethidium bromide. This is a RFLP analysis. Microorganisms with the same RFLP pattern are likely to be closely related, whereas those with very different RFLP patterns are less likely to be closely related. Although RFLP patterns are highly reproducible and very accurate, problems may arise in assessing the hundreds of DNA fragments that may arise following restriction enzyme digestion. PFGE was devised as a means to simplify RFLP typing.

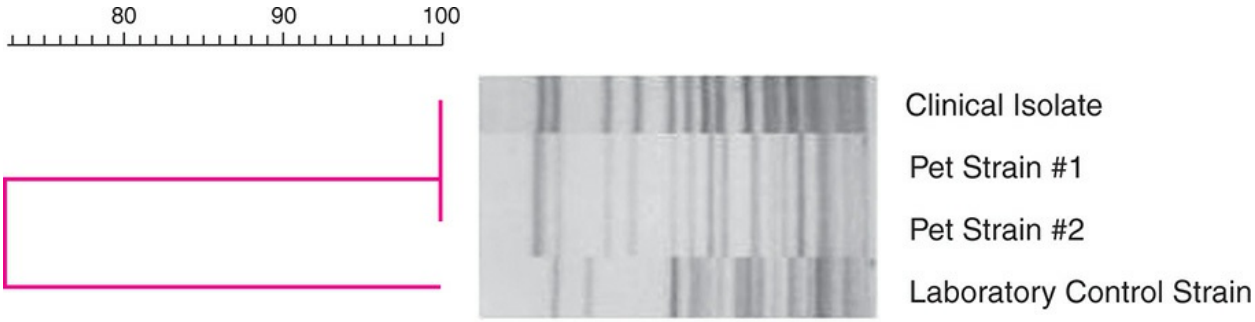
PFGE is considered by many to be the standard form of strain typing, to which newer methods should be compared. In short, this technique begins with the extraction of the chromosomal bacterial DNA of the strains of interest, which are exposed to a variety of restriction endonucleases. The type of restriction endonucleases used is usually determined by the type of bacteria under analysis, based on previous research that has demonstrated the efficacy of these enzymes. As above, the restriction endonucleases cut the chromosomal DNA into a variety of pieces, depending on the number of restriction sites present in that particular strain. The fragments of chromosomal DNA are then separated using a specialized type of electrophoresis. The chromosomal restriction profile resulting from PFGE usually results in 5 to 20 fragments that range in size from 10 to 800 kb[350,352] (Fig. 4-22). These are visualized, photographed, and submitted for image analysis. Computer programs are then used to determine if the strains under analysis are indistinguishable or distinguishable and the degree to which these may be distinguished. The process has been termed *DNA fingerprinting* when the process of RFLP is combined with Southern blot transfer and probe hybridization.[350]

Amplification-Based Typing

There are a number of postamplification methods of analysis that may be used for microbial typing. The newest and most powerful are being developed using NGS. The standard amplification-based typing methods include single-stranded conformation polymorphism analysis, amplified fragment length polymorphisms, arbitrarily primed PCR, RFLP analysis of the PCR product (PCR-RFLP), and analysis of PCR products of repetitive elements present in bacterial genomes (rep-PCR). The discussion of all these methods is beyond the scope of this text. We will briefly describe the latter two types of analysis, PCR-RFLP and rep-PCR, as examples of amplification-based methods of microbial typing

PCR-RFLP. Just as restriction endonucleases may be used to ligate the chromosomal DNA of bacteria, these enzymes may also be used to cleave an amplicon. The selection of the endonuclease(s) used with PCR-RFLP must be chosen with care, since the amplicon is orders of magnitude smaller than the bacterial chromosome and is less likely to randomly contain restriction sites. The enzymes are often chosen based on previous knowledge of restriction sites that are variably present in the amplicons and, therefore, may be useful for the comparison of strains. The size of the amplicon and the number of potential restriction sites that it may contain imposes limitations on the differentiating capabilities of this technology.

RFLP analysis of the PCR product is commonly performed on assays directed against the rDNA. When the PCR product of an assay that targets the rDNA is analyzed by RFLP, it may be referred to as PCR ribotyping. Even when the rDNA is analyzed, it is important to target particular areas, to achieve type-specific information.[169,185] PCR-RFLP has been used to type all different kinds of microorganisms, including bacteria such as *Campylobacter* and *Borrelia* species and atypical fungi such as *P. jiroveci*.[58,160,199] In addition to strain comparisons, PCR-RFLP patterns may be used for organism identification. The use of this technology in this manner relies on the use of highly specific PCR primers. This technique, for example, has been used to identify fastidious bacteria, such as *Bartonella* species, and difficult-to-identify parasites, such as hookworms and filarial worms.[104,105,171,294] This technology has also proven very useful for the differentiation of *Nocardia* species, which is clinically important given the differences in antimicrobial susceptibilities within this group.[208,338] The nonribosomal genes that are used for



■ **FIGURE 4-22** Pathogenic isolate of *Pasteurella multocida*. The analysis of a pathogenic isolate of *Pasteurella multocida* (clinical isolate) was genetically indistinguishable from two phenotypically different *P. multocida* isolates obtained from the patient's cat, but is clearly distinguishable from the laboratory control strain.

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PCR-RFLP include either housekeeping genes, such as *rpoB*, or any gene that may contain strain-specific information for a particular group of organisms (i.e., the genes that encode for the enzymes responsible for hippurate hydrolysis for *Campylobacter jejuni*).[166,294,339]

REP-PCR. Repetitive DNA elements in prokaryotic genomes form the basis for rep-PCR. These repetitive elements are the hybridization sites for the primers used in rep-PCR. These primers, which are complementary to the interspersed repetitive sequences, generate through PCR amplification variably sized DNA fragments, which may be separated by gel electrophoresis (Fig. 4-23). The electrophoretic patterns may be used to determine the relatedness of bacterial strains that are being compared. A single primer set may be used for a variety of gram-positive and gram-negative bacteria, or more than one primer set may be used to target different repetitive elements. Rep-PCR has been used to type enterohemorrhagic *E. coli* isolates to investigate animal-to-human transmission.[95,102,321] It, like other typing techniques, has been used to track both gram-positive and gram-negative bacteria that are resistant to antimicrobial agents and those of importance to the hospital epidemiologist.[25,67,323,356] The information generated through rep-PCR may also be used for microorganism identification. This technology, for example, has been used to identify organisms and examine diversity with the *Streptomyces* genus and to identify *Bartonella* species.[196,300]

Clinical Applications of Microbial Typing. Genetic comparisons of microorganisms have been used to investigate outbreaks in hospitals, in cities, and even across the nation.[35,117,190,210,232,273,390] It is common for potential nosocomial outbreaks to be

investigated using genetic typing methods. A possibility of an outbreak is often suspected when the frequency of isolation of a pathogenic microorganism exceeds its usual baseline recovery rate. This may be in a particular place, such as the number of methicillin-resistant *S. aureus* blood isolates in the hospital in general or a ward in particular, or it may be the number of *Salmonella* isolates reported to a county health department.

Given the fragility of premature babies, these techniques are commonly used to investigate outbreaks in neonatal intensive care units.[188,234,309] Molecular typing methods are also commonly used to investigate infections caused by bacteria that are resistant to antimicrobial agents.[88,148,159,309] In some instances, genetic typing methods have been used to successfully determine the point source of an outbreak. Outbreaks and the transmission of *M. tuberculosis* are often studied using molecular typing methods.[79,189,307,329,351,372]

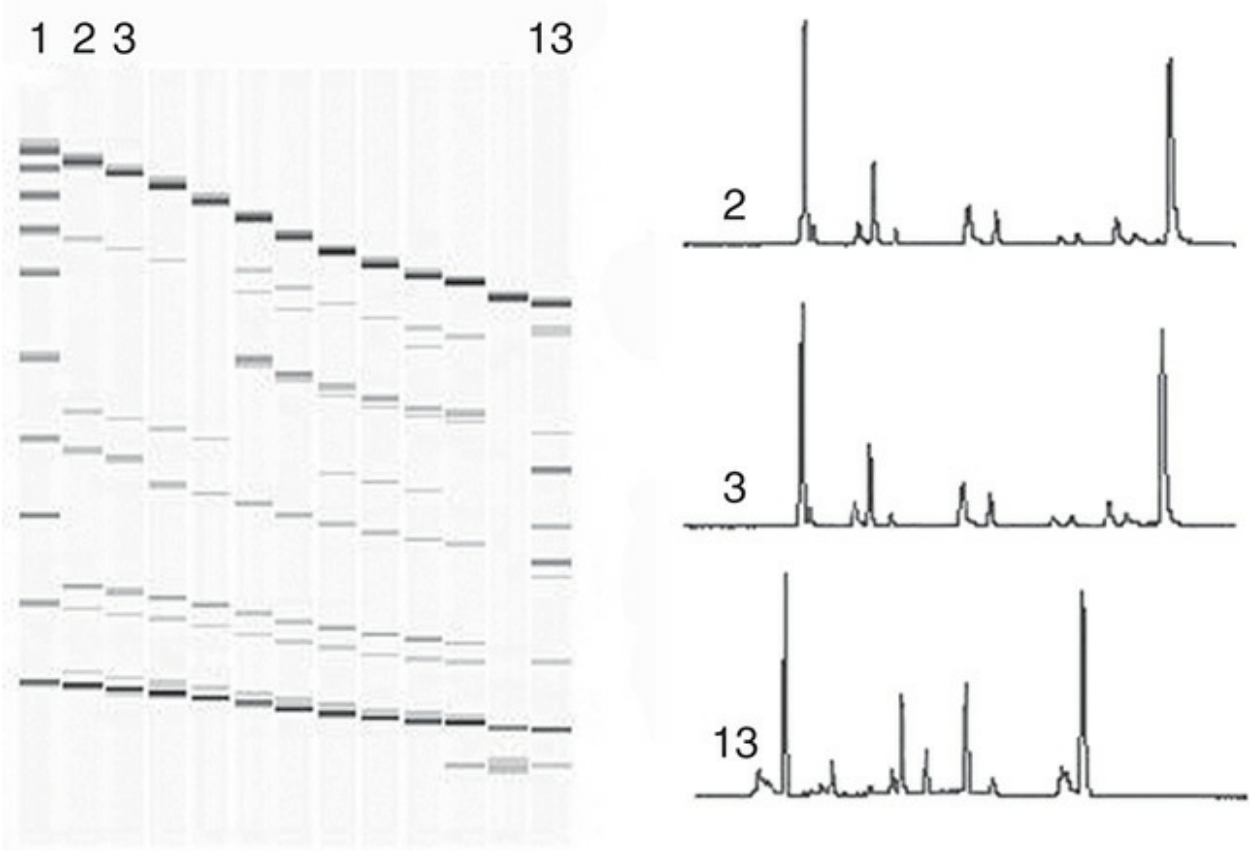
Public health epidemiologists from across the country have for many years now been collaborating by submitting standardized typing information obtained from strains of bacteria that cause foodborne disease into a national public database in a program called PulseNet. The PFGE fingerprint patterns are submitted from participants in all 50 states. PulseNet is designed as an early warning system for foodborne diseases. More information about this national effort to track and rapidly curtail bacterial foodborne illnesses may be obtained at www.cdc.gov/pulsenet.

Mass Spectrometry

A variety of different types of mass spectrometry have been used by clinical and environmental chemists for the detection and characterization of molecules. It has been demonstrated that this type of technology is useful for the identification of microorganisms by a mass spectrometric profile of the proteins, largely rRNA proteins, of the organism.

MALDI-TOF mass spectrometry is the type of mass spectrometry used in clinical microbiology.[153,187] Currently, two FDA-approved, commercially available mass spectrometers are available for use in clinical microbiology laboratories. These include the MALDI Biotyper (Bruker, Billerica, MA) and the Vitek MS (bioMérieux, Durham, NC) (Image Plates 4-3C and D).

In brief, bacteria or yeast from a colony is mixed with matrix supplied by the manufacturer on a position on the test slide. This is placed into the mass spectrometer, wherein this spot will be activated by a laser. The matrix absorbs much of the energy from the laser and converts it into heat. The heat vaporizes the outer portion of the specimen. The molecules move through a vacuum space at a different rate based on the mass-to-charge (m/z) ratio, and this “time of flight” is



■ **FIGURE 4-23** Rep-PCR of *Staphylococcus* isolates. The fragment analysis *(right)* reflects the patterns of the bands in the gel that is performed following rep-PCR. The ladder (Lane *1*) is used as a comparative standard. The fragments from the isolates represented in Lanes *2* and *3* are indistinguishable, whereas these are clearly different from those produced by the isolate represented in Lane *13*.

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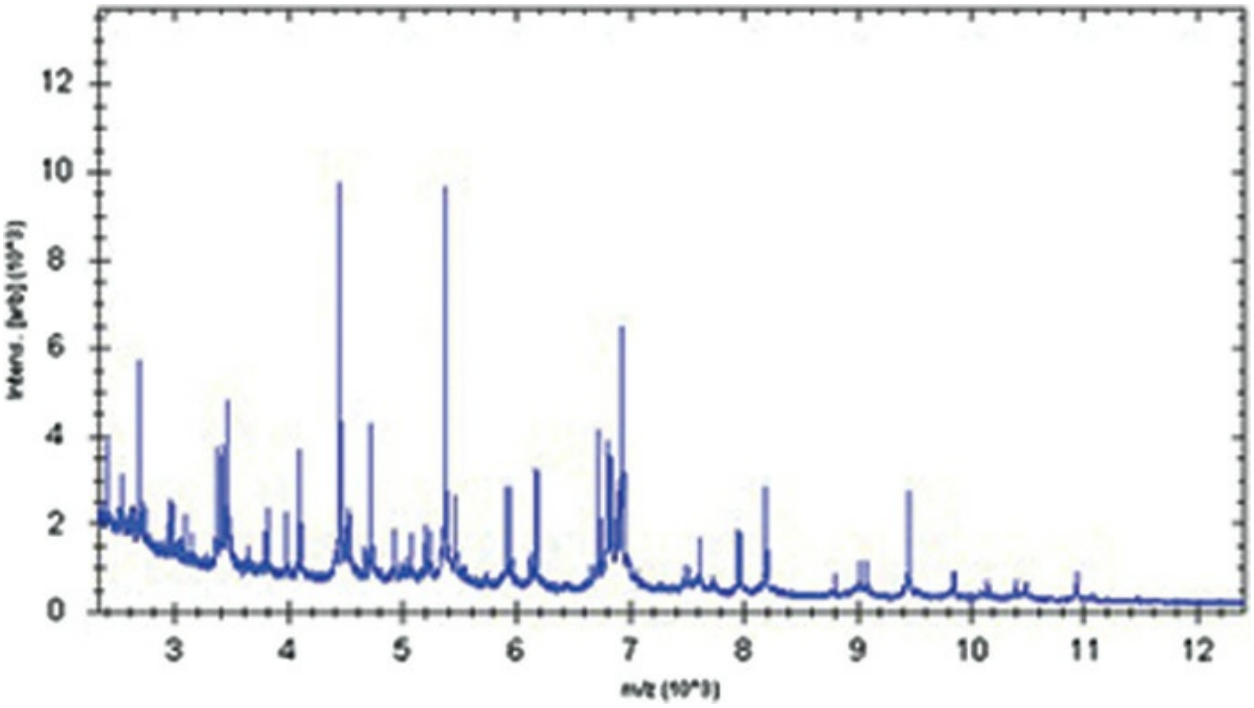
determined by the arrival of the different molecules at the detector. A summation of the time of flight for all molecules present will produce a spectrum (Fig. 4-24). This spectrum is then electronically compared with all the spectra in the library to determine the best match (Fig. 4-25) and subsequently the identification of the microbe (Fig. 4-26).

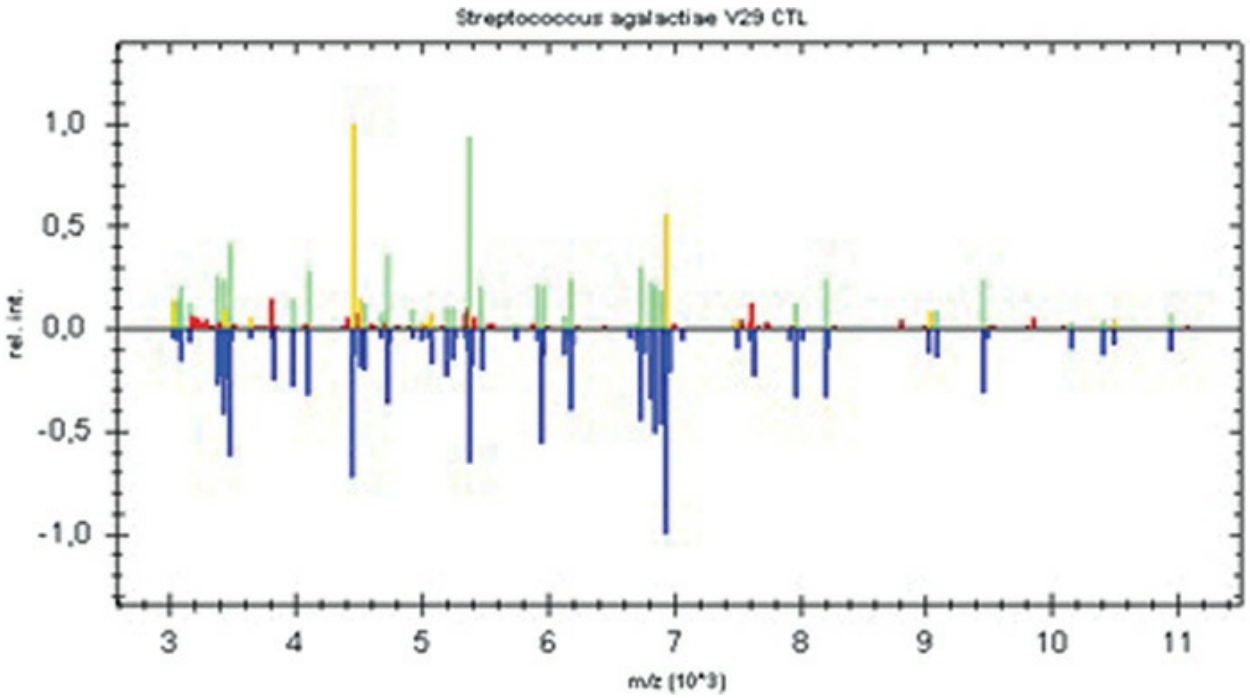
The literature is replete with manuscripts demonstrating the overall excellent ability of these mass spectrometers to identify a wide variety of microorganisms. Upon initial assessment, acquisition of these instruments may appear prohibitive because of a high capital cost. However, when one assesses the materials used, labor, and time to identification, then the return on investment for these instruments is excellent. Although these factors are important to justify the purchase of these instruments, the

greatest advantages are in the clinical impact. These instruments identify a wide variety of organisms within minutes with a high degree of accuracy. The delivery of accurate organism identification information early to treating physicians affords the opportunity for providers to assure appropriate therapeutic coverage. In addition to the direct identification of cultivated microorganisms, others have processed the sediment of blood culture bottles that signal positive as a means to more rapidly identify the causes of bloodstream infections.

There are, of course, some limitations, but these are few. For example, this technology cannot differentiate *Shigella* from *E. coli*. As with any assay, it is important to appropriately validate every assay and determine means to handle



■ **FIGURE 4-24** The MALDI-TOF spectrum produced by a microorganism.



■ **FIGURE 4-25** The comparison of the MALDI-TOF spectrum produced by that test microorganism with the library.

Mb	Detected Species	Log(Score)
	Streptococcus agalactiae V29 CTL	2,361
	Streptococcus agalactiae DSM 6784 DSM	2,352
	Streptococcus agalactiae 03 198 CTL	2,275
	Streptococcus agalactiae 04 158 CTL	2,250
	Streptococcus agalactiae CNR 10 CTL	2,230
	Streptococcus agalactiae 03 145 CTL	2,230
	Streptococcus agalactiae 03 102 CTL	2,215
	Streptococcus agalactiae DSM 16828 DSM	1,984
	Streptococcus agalactiae DSM 2134T DSM	1,797
	Streptococcus equi ssp zooepidemicus ATCC ...	1,731

■ **FIGURE 4-26** The computer-generated match that is determined by electronically comparing the spectrum of the unknown, test organism with all the spectra in the library.

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any shortcomings. Overall, MALDI-TOF is a cost-effective significant advance in the identification of a wide variety of microorganisms in clinical microbiology.

Conclusion

The molecular revolution continues and is stronger than ever. Industrial microbiologists and engineers are delivering innovative solutions to improve the laboratory-based diagnosis of infectious diseases. The number of molecular applications for the detection and characterization of all types of microorganisms, many of which are easy-to-use, FDA-approved assays. Many of these are taking a syndromic approach to testing, with assays targeting a broader ranges of organisms that cause a particular type of disease (e.g., gastroenteritis), even if these organisms are taxonomically unrelated (e.g., bacteria, viruses, and/or parasites or fungi targeted by the same assay). Simultaneously, clinical microbiologists for the first time are utilizing mass spectrometry to more quickly identify common microorganisms and, in many instances, more accurately identify less commonly encountered microorganisms. Finally, the use of genetic typing of microbes remains needed to demonstrate relatedness among microorganisms continues to provide useful information regarding the transmission of infectious diseases. Although a variety of molecular methods are available, the use of NGS promises yet another powerful tool for the molecular epidemiologist.

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CHAPTER 5

Medical Bacteriology: Taxonomy, Morphology, Physiology, and Virulence

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Taxonomy: Classification, Nomenclature, and Identification of Bacteria

- The Naming of Bacteria
- Phenotypic Identification of Bacteria
- Phylogenetic Criteria for Classification of Bacteria

Basic Bacterial Anatomy and Physiology

- Bacterial Size and Shape
- Nuclear Structure, DNA Replication, Transcription, and Translation
- Cytoplasm
- Cytoplasmic Membrane
- Bacterial Cell Wall Structure
  - Gram-Positive Bacterial Cell Walls
  - Gram-Negative Bacterial Cell Walls
  - "Acid-Fast" Bacterial Cell Walls

Bacterial Endospores
Bacterial Surface Structures
Capsules
Flagella
Other Locomotor Organelles
Fimbriae (Pili)
Genetic Exchange and Recombination in Bacteria
Requirements for Bacterial Growth and Metabolism
Carbon
Carbon Dioxide
Oxygen
Nitrogen
Growth Factors
Kinetics of Bacterial Cell Growth
General Bacterial Metabolism and Energy Generation
Fermentation
Utilization of Pyruvate
Bacterial Virulence Factors and Pathogenicity
Definitions and Concepts
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Exotoxins and Endotoxins
Bacterial Superantigens

Over the three centuries that have passed since Leeuwenhoek first observed bacteria and protozoa with his primitive microscope, a vast amount of knowledge has been accumulated about the microbial world. Microorganisms are found in all environments, including the soil, water, and air. They are able to participate in all vital life functions that are observed in higher, more complex life forms and are found in association with the nonliving environment and with all other living things. By their activities in these environments, microorganisms are an integral part of the balance of life. Using microorganisms, biochemical and genetic processes that occur in all forms of life have been elucidated, and the innumerable roles of microorganisms in environmental cycles are only now beginning to be fully appreciated. By necessity, subsequent chapters in this textbook deal with a relatively small number of microorganisms that are capable of causing disease in humans.

By the end of the 19th century, Louis Pasteur had experimentally dispelled the myth of spontaneous generation, and Robert Koch, among others, had shown that microorganisms were able to cause infectious diseases, such as anthrax and tuberculosis. Although current techniques allow more direct assessments of microbial virulence and pathogenicity, making Koch's original postulates somewhat inapplicable, these fundamental principles still serve as the basis for the unequivocal link between microorganisms and infectious diseases. **Koch's postulates** are outlined in Box 5-1.

Taxonomy: Classification, Nomenclature, and Identification of Bacteria

The taxonomy of bacteria specifically refers to three basic concepts: classification, nomenclature, and identification. **Classification** is the process of systematically dividing organisms into groups, with the species being the smallest and most definitive level of division. Classification also refers to the grouping of described species into genera, and on through the levels of families, orders, classes, and phyla. Historically, new species have evolved from the recognition of isolates that have phenotypic characteristics that differ from similar organisms (e.g., other members of the family *Enterobacteriaceae*). These isolates are then compared with other organisms using a large battery of phenotypic tests. Present-day applications of molecular techniques (e.g., DNA hybridization, DNA sequencing, and polymerase chain reaction [PCR]-based amplification) have enabled taxonomists to identify microbial "signature sequences" and other genetic elements that establish genetic relatedness between and among different microorganisms. Based on systematic comparisons of genetic, chemotaxonomic, and phenotypic traits, new taxa would be proposed and named via publication. The process of naming organisms is delineated by rules of nomenclature.

BOX 5-1

Koch's Postulates

1. A given organism must be present in every case of a given disease;
2. The microorganism can be isolated from (or demonstrated in) specimens associated with that disease state;
3. Inoculation of the isolate into susceptible animals produces a similar disease; and
4. The same organism that is associated with the initial disease state can be

recovered from representative specimens from the experimentally infected animal.

The Naming of Bacteria

Efforts to codify the taxonomy and nomenclature of bacteria started in earnest at the beginning of the 20th century with the work of Chester, Buchanan, and others.[12,13,20,64] During the early 20th century, committees of interested microbiologists were convened to organize bacterial taxonomy, to promulgate rules regarding the validation of new bacterial names, and to establish procedures for changes in nomenclature (Box 5-2). These discussions resulted in the publication of the International Code of Nomenclature of Bacteria in 1948. Sequential revisions of this document were published by the International Committee of Systematic Bacteriology in 1958, 1973, and 1992.[83,84] Priorities and rules for the naming of bacteria were established beginning on May 1, 1953, and on January 1, 1980, the first "Approved List of Bacterial Names" was published in the *International Journal of Systematic Bacteriology* (IJSB).[80] This list was subsequently amended and republished in 1989.[81] Quarterly issues of IJSB (now called the *International Journal of Systematic and Evolutionary Microbiology*, IJSEM) routinely list the names of new species (i.e., Validation Lists). Descriptions of new species can be published either in IJSEM or in a variety of other American and foreign journals. If the description is published in a journal other than IJSEM, the name and the literature reference containing its description must subsequently appear in an IJSEM Validation List. As of the beginning of the new millennium, 4,314 validly named prokaryotic species names have appeared either in the "Approved List of Bacterial Names," in IJSEM, or in IJSEM Validation Lists.[80,81] More recently, provisions for proposed species names that are currently nonculturable but that have been characterized by molecular methods (see below) have also been established.[59,60] New or revised species designations can be proposed by any investigator; the criteria for publication, including the naming of the new species are cited in Box 5-2. A detailed description of the morphologic, biochemical, and genetic characteristics of the organism is given, and there is designation of a living culture of the organism as the "type strain" of the species. This type strain is deposited in reference type culture collections (e.g., the **American Type Culture Collection [ATCC]** and the **National Type Culture Collection [NTCC]**); so the new species will be available to other investigators. Challenges to the validity of new species names are made by publishing a "request for an opinion" from the Judicial Commission of the International Union of Microbiological Societies. This commission usually refers the request to smaller

committees that deal with specific groups of organisms, such as the *Enterobacteriaceae* or the *Pasteurellaceae*.

BOX 5-2

Rules for the Nomenclature of Bacteria

1. There is only one correct name for an organism. When more than one name exists for the same specimen, the oldest legitimate name for that organism has precedence. Occasionally, proposed and accepted names may be changed to reflect proper latinized endings (e.g., the name *Alloiococcus otitis* being changed to *Alloiococcus otitidis*).
2. Names that cause errors or confusion should be rejected.
3. All names that are in Latin or are "Latinized" (i.e., given endings that agree in terms of proper usage and gender [masculine, feminine, neuter]), regardless of origin.
4. The first word of the name (genus name) is always capitalized.
5. The second word (species or specific epithet) is not capitalized.
6. Both the genus and the species name, together referred to as the species, are either underlined or italicized when appearing in print.
7. The correct name or a species or higher taxonomic designations is determined by valid publication, legitimacy of the name with regard to the rules of nomenclature, and priority of publication.
8. Genus and species names appearing in the IJSEM can be changed according to the following rules:
   a. In transferring a species from one genus to another, the species epithet is retained (e.g., *Campylobacter pylori* became *Helicobacter pylori*);
   b. If a type strain is found to actually belong to another genus, the type strain genus is considered invalid; and
   c. If an organism is included in two or more genera or has two or more species designations, the name of the genus/species containing the correct type strain is considered the valid name.

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Phenotypic Identification of Bacteria

Phenotypic identification procedures remain the principal method for identification of most isolates in the clinical microbiology laboratory (Box 5-3). The focus of identification efforts is at the genus and species level, with no attempt to describe the

hierarchy to which a given organism belongs. At present, taxonomic schemes, such as those published in the four-volume 1984-to-1989 edition of *Bergey's Manual of Systematic Bacteriology*, the 1994 edition of *Bergey's Manual of Determinative Bacteriology*, and various laboratory manuals and journals, reflect groupings of bacteria in nonhierarchical schemes based strictly on phenotypic characteristics. In the preface to the 1994 edition of *Bergey's Manual of Determinative Bacteriology*, J.G. Holt, the editor in chief, states that "the arrangement of the book is strictly phenotypic; no attempt has been made to offer a natural classification. The arrangement chosen is utilitarian and

BOX 5-3

Methods for Characterization of Microorganisms in the Clinical Microbiology Laboratory

1. **Cellular morphology**—This includes cell size, cell shape, and the arrangement of cells with respect to one another.
2. **Staining characteristics**—This usually refers to the Gram's and acid-fast stains, but may include the description of other microscopic characteristics, such as the presence of spores, metachromatic granules, vacuoles, etc.
3. **Motility**—Motility may be observed microscopically with a wet preparation, or by inoculation of semi-solid motility medium.
4. **Presence/absence of spores**—The presence of spores is particularly useful for identifying *Clostridium*, *Bacillus,* and related species. Spores can generally be discerned on Gram's stains, but occasionally specific spore stains may be helpful.
5. **Growth characteristics:**
   a. **Rapidity of growth**—Most organisms isolated in the clinical laboratory grow within 1 to 2 days. However, certain bacteria (e.g., actinomycetes, mycobacteria) may take considerably longer.
   b. **Morphology of colonies on growth media**—Bacteria vary in their appearance on solid culture media. With experience, colony morphology on various media coupled with a few rapid tests (e.g., oxidase, catalase) is often sufficient for genus-level characterization of organisms growing from clinical specimens.
   c. **Optimal atmospheric conditions for growth**—Microorganisms can be characterized by their requirements for oxygen and, depending on this requirement, can be classified as **aerobic** (requires O_2), **facultative** (can grow in the presence or absence of O_2), or **anaerobic** (optimal growth/any growth in the absence of O_2). The growth of most organisms is also

stimulated by CO_2, and, for **capnophilic** bacteria, $CO_2$is required for growth (e.g., *Neisseria gonorrhoeae*). Other organisms, such as *Campylobacter jejuni*, may require atmospheres with slightly decreased O_2tension; these organisms are termed *microaerophilic.*

d. Optimal temperature for growth—Most organisms isolated in clinical laboratories grow optimally at 35°C to 37°C. Ability to grow at various temperatures may be helpful in characterizing some isolates (e.g., campylobacters, certain nontuberculous mycobacteria).

e. Colony morphology on nonselective, selective, and differential media—Clinical laboratories routinely inoculate specimens onto both nonselective and selective media in order to provide optimal conditions for recovery of organisms in a specimen. Selective media use antimicrobial agents (e.g., colistin and nalidixic acid in colistin–nalidixic acid [CNA] agar) or dyes and bile salts (e.g., MacConkey agar; eosin–methylene blue [EMB] agar) to inhibit the growth of some organisms and allow the growth of others. In addition, some selective media are also differential, which means that the colonies growing on a given medium have an appearance that discloses information about a phenotypic characteristic or characteristics. These features may suggest the possible identity of the organism (e.g., the colorless colonies of lactose-nonfermenting *Shigella* species versus the pink/red colonies of lactose-fermenting *E. coli* on MacConkey agar).

6. **Biochemical characteristics**—Along with cellular and colonial morphology and the appearance of colonies on selective and differential media, biochemical and enzymatic tests form the basis for most identification procedures performed in clinical microbiology laboratories. In general, biochemical (phenotypic) characteristics refer to the formation of distinct biochemical end products from defined substrates, the production of acid from various carbohydrates, and the presence of certain bacterial enzymes as determined by chromogenic substrates or other methods.

7. **Serologic tests**—Serologic tests for bacterial identification usually involve the detection of antigens by enzyme or fluorescence immunoassays, or agglutination assays (see Chapter 3). Serologic identification methods may also be used to confirm identifications

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obtained by other methods. For example, *Salmonella* species can be identified by phenotypic methods, but serotyping of *Salmonella* isolates

for somatic and flagellar antigens is usually performed by slide agglutination with group- or type-specific antisera.

8. **Analysis of metabolic end products or structural components of organisms**—Metabolic end product analysis is performed indirectly with virtually all microbial identification systems, in which a metabolic end product produces a visually detectable change in an indicator of some kind. However, these analyses may also be performed by other methods, such as gas chromatographic analysis of a sampling of broth culture after extraction with various organic solvents. This technique is especially helpful for genus and species identification of anaerobic bacteria. Chemotaxonomic approaches (e.g., cell wall outer membrane and peptidoglycan analysis, cell wall composition, detection of electron transport components such as cytochromes and menaquinones by gas–liquid or high-pressure liquid chromatography) are also helpful, but are not used in most laboratories. Cell wall analysis has enjoyed success in some hospital laboratories as a method for differentiation and identification of mycobacteria and related organisms.
9. **Molecular genetic analysis**—Molecular methods include G+C% content, DNA–DNA hybridization, PCR, and DNA base sequencing. Although these methods are not used routinely in many hospital laboratories, they enabled the development of molecular assays that are now used in many laboratories for detection and identification of mycobacteria, chlamydiae, systemic fungi, and some bacteria. Amplification techniques (e.g., PCR, ligase chain reaction, strand-displacement amplification, nucleic acid sequence-based identification, and transcription-mediated amplification) are also being used in many clinical laboratories for direct detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urogenital specimens, the detection and differentiation of respiratory viruses, the detection of *C. difficile* and for viral load determinations in patients with HIV-1, transplant-associated viral infections (e.g., CMV, BK, and EBV), and hepatitis C infections.
10. **Proteomic analysis—** Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) is a type of mass spectrometry that is commercially available and has become commonly used in the clinical microbiology laboratory, in many instances replacing traditional biochemical identification. Microorganisms, most commonly bacteria and yeasts, are placed on a test slide and mixed with matrix material. The specimen is submitted for laser desorption/ionization and the molecules of the microorganisms are separated largely by mass and their "time of flight." The spectrum formed based largely on the protein content of the organisms is compared with a library of spectra of known microorganisms and thereby identification can be achieved in minutes at a very low cost per test.

is intended to aid in the identification of bacteria."[13,46] In this context, an operational definition of a **species** is a collection of strains that share many common phenotypic characteristics. A bacterial **strain** is derived from a single culture isolate, and a species **type strain** represents the permanent example of the species that carries the reference name and is part of a culture collection (e.g., ATCC). Species are assigned to morphologically and biochemically defined **genera**, which, in turn, are assigned to **families**, each of which also has certain morphologic, physiologic, and biochemical features. Since taxonomic decisions and controversies are arbitrated by committee, individual knowledge and bias introduced at this level make phenotypic classification above the species level somewhat arbitrary.[87] Nomenclature rules that imply a hierarchical classification have been accepted for years, however, as taxonomic convention for these nonhierarchical classification schemes. Consequently, family names have the latinized ending ***-aceae*** (e.g., the family *Enterobacteriaceae*, the family *Neisseriaceae*), order names have the ending ***-ales*** (i.e., Order *Eubacteriales*), and tribe names end in ***-eae*** (e.g., the Tribe *Proteae*). The incorporation of phenotypic data into computer-assisted databases of commercially available identification systems has provided systematic organization for the vast amount of phenotypic data that has accumulated. These data will no doubt continue to serve the clinical laboratory as the principal method for bacterial identification. The development of elegant and powerful genetic techniques, however, has made hierarchical bacterial classification not just a possibility but an inevitability.

Phylogenetic Criteria for Classification of Bacteria

Because the nucleotide sequence of DNA is unique to individual species of microorganisms, analysis of nucleic acid relatedness between and among microbes was recognized as a potential tool for unraveling bacterial taxonomy and nomenclature. Initially DNA was analyzed for its **G + C% (guanine plus cytosine percent)** content. This analysis is limited, however, by the fact that two organisms with divergent DNA sequences can have similar G+C% contents. Subsequently, it was found that **DNA–DNA hybridization** yielded more useful information. Double-stranded DNA can be separated into its two component strands by heat or high salt concentrations. On cooling or on lowering the salt concentration, the two single strands will reanneal or hybridize into the double-stranded forms by specific base-pairing (see below). The extent to which two single strands of DNA reanneal with one another is an indirect assessment of their relatedness.[86] Using this technique, it was found that organisms with about 15% or more sequence mismatches do not reanneal under ideal conditions; so DNA from distantly related organisms will not hybridize. Consequently, DNA–DNA hybridization

has been most useful for comparing organisms at or below the species level. In this context, a **bacterial species** can be defined as a group of bacteria that exhibit greater than 70% to 75% DNA relatedness, with 5% or less divergence in related nucleotide sequences.[86,96]

In the 1970s, geneticists began to examine ribosomal RNA sequences as a method for demonstrating microbial relatedness. This work centered on the **16S rRNA molecule**, which is a part of the 30S subunit of the bacterial ribosome (see below). This molecule is 1,500 to 1,800 nucleotides in length, is easily isolated from bacterial organisms, and has the same structural and functional roles in all bacterial cells. Small-subunit ribosomal RNAs have remained highly conserved during evolution; mutations in these highly conserved base sequences are usually lethal, and organisms with such mutations do not survive and propagate. In addition, since these molecules are an integral part of a complex, protein-containing structure (i.e., the bacterial ribosome), horizontal transfer of rRNAs to other organisms is rare. By examining the nucleotide sequences of the 16S rRNA, phylogenetic relationships and evolution of organisms from common ancestral types can be ascertained. Ribosomal RNA sequence analysis is most useful for determining the relatedness of microorganisms above the genus level, although this technique has been used to validate existing phenotypic differences between closely related genera and species. Strains of the same species usually show greater than 97% similarity in the rRNA sequences.[86]

Since its inception, sequencing studies of small-subunit RNA has had a tremendous impact on bacterial systematics. In the late 1970s, analysis of 16S rRNA from diverse bacterial groups (i.e., methanogenic [methane-producing] bacteria, halophilic bacteria, thermoacidophilic bacteria, etc.) revealed that these organisms were completely divergent from other bacteria and from eukaryotic organisms and heralded the division of the microbial world into two domains—the *Archaea* and the *Bacteria*—and reinforced the phylogenetic separation of eukaryotic organisms into a third domain, the *Eukaryota*. The development of DNA sequencing in the late 1970s and reverse transcription PCR (RT-PCR) technology in the early to mid-1980s enabled the amplification and sequencing of small-subunit rDNA. Extensive study and cataloging of these sequences in the 1980s and 1990s form the basis of the proposed phylogenetic classification scheme. The second edition of *Bergey's Manual of Systematic Bacteriology* reflects a complete departure from the first edition in its phylogenetic classification scheme. The second edition of *Bergey's Manual of Systematic Bacteriology* began publication in 2001 with volume 1, covering the *Archea* and the phototrophic bacteria. Subsequent years have seen the publication of volume 2 (the *Proteobacteria*) in 2005, volume 3 (the *Firmicutes*) in 2009, and volume 4 (*Bacteroidetes*, *Spirochaetes*, *Tenericutes* [*Mollicutes*], *Acidobacteria*,

Fibrobacteres, *Fusobacteria*, *Dictyoglomi*, *Gemmatimonadetes*, *Lentisphaerae*, *Verrucomicrobia*, *Chlamydiae*, *Planctomycetes*) in 2011.

The prokaryotic world is divided into two domains—the ***Archaea*** and the ***Eubacteria***.[99] Eukaryotic organisms are placed in Domain ***Eukaryota***. *Archaea* and *Bacteria* probably evolved from an ancestral organism, as evidenced by similarities in amino acid sequences of large structural and regulatory molecules and enzymes, including adenosine triphosphatases and elongation factors that function in protein synthesis. The *Archaea* include two major branches—the ***Euryarchaeota*** and the ***Crenarchaeota***. The *Euryarchaeota* include the methanogenic bacteria, the sulfate-reducing bacteria, aerobic **halophilic** (salt-requiring) and **thermoacidophilic** (optimal growth at 50°C to 60°C at pH less than 5) bacteria, and anaerobic **hyperthermophilic** bacteria. The *Crenarchaeota* are all **thermophilic** (optimal growth at 50°C to 60°C), organisms that are either sulfur-reducing heterotrophs or aerobic organisms capable of oxidizing sulfur or other inorganic and organic substrates. Although *Archaea* are similar to the *Eubacteria* in size, nucleoid structure, cell growth, and division, they also differ in several respects. The genome size of the *Eubacteria* ($0.6–12 \times 10^6$ base-pairs) can be up to three times larger than that of the *Archaea* ($1–4 \times 10^6$ base-pairs). The *Archaea* have cell walls composed primarily of protein and lack the murein peptidoglycan found in the *Eubacteria*. Although both groups have 70S ribosomes, those of the *Archaea* are insensitive to inhibition of protein synthesis by streptomycin or chloramphenicol, but, like the *Eukaryota*, they are sensitive to inhibition by diphtheria toxin. The opposite characteristics are present in the *Eubacteria*. In short, the *Archaea* encompasses bacteria that are environmental "extremophiles"; this group contains no human pathogens.

Organisms in the Domain *Eubacteria* (or Domain *Bacteria*, as addressed in *Bergey's Manual*) are divided into divisions or phyla. Most of these phyla are composed of environmental species, such as the thermophilic and/or photosynthetic bacteria. Human pathogens are distributed among eight different phyla. Most of the clinically significant gram-negative bacteria belong to the **Phylum *Proteobacteria***, which is divided into five classes, designated **α-, β-, γ-, δ-,** and **ε- *Proteobacteria***. All of the enteric bacteria, most of the nonfermentative species, fastidious gram-negative coccobacilli and cocci, and the campylobacters belong to the various classes within the Phylum *Proteobacteria.* **Phylum *Firmicutes*** includes the clostridia, eubacteria, peptococci, aerobic spore-forming gram-positive bacilli (e.g., *Bacillus* and related genera), the staphylococci, streptococci, and enterococci. **Phylum *Actinobacteria*** includes the aerobic and anaerobic actinomycetes and related organisms, the aerobic gram-positive cocci, and several other genera of facultative gram-positive bacilli. **Phylum *Bacteroidetes*** contains *Bacteroides* group organisms (*Bacteroides*, *Porphyromonas*, and *Prevotella*), flavobacteria, sphingobacteria, and related nonfermentative gram-negative bacilli. **Phylum *Fusobacteria*** is comprised of the

fusobacteria and the genus *Streptobacillus*. **Phylum *Spirochaetes*** and **Phylum *Chlamydiae*** include the spiral-shaped bacteria (e.g., *Borrelia, Treponema*, and *Leptospira*) and the *Chlamydia* and *Chlamydophila* genera, respectively. **Phylum *Tenericutes*** includes the mycoplasmas and ureaplasmas. Box 5-4 provides a complete outline of the classification scheme for organisms associated with humans and human disease that appears in the new edition of *Bergey's Manual of Systematic Bacteriology*.

Despite the current emphasis on molecular methods for classification, phenotypic criteria continue to be the mainstay of bacterial identification in the clinical microbiology laboratory. With the methods outlined in Box 5-3, along

BOX 5-4

Hierarchical Classification of Clinically Significant Bacteria in *Bergey's Manual of Systematic Bacteriology, Second Edition*

Hierarchical Classification of Clinically Significant Bacteria in *Bergey's Manual of Systematic Bacteriology, Second Edition*

				Domain Bacteria Phylum *Proteobacteria*	
Class	**Subclass**	**Order**	**Suborder**	**Family**	**Genera**
β-*Proteobacteria*		*Caulobacteriales*		*Caulobacteraceae*	*Brevundimonas, Caulobacter*
		Rhizobiales		*Bartonellaceae*	*Bartonella*
				Bradyrhizobiaceae	*Afipia, Agromonas* *Bosea, Bradyrhizobium*
				Brucellaceae	*Brucella, Ochrobactrum Pseudochrobactrum*
				Methylobacteriaceae	*Methylobacterium, Protomonas*
				Phyllobacteriaceae	*Mesorhizobium*
				Rhizobiaceae	*Agrobacterium, Rhizobium*
		Rickettsiales		*Anaplasmataceae*	*Aegyptianella, Anaplasma, Cowdria, Ehrlichia, Neorickettsia, Wolbachia*
		Sphingomonadales		*Sphingomonadaceae*	*Sphingomonas*
β-*Proteobacteria*		*Burkholderiales*		*Alcaligenaceae*	*Achromobacter, Alcaligenes, Bordetella, Oligella, Sutterella, Taylorella*
				Burkholderiaceae	*Burkholderia, Cupriavidus, Lautropia, Pandoraea, Paucimonas, Ralstonia, Wautersia*
				Comamonadaceae	*Acidovorax, Comamonas, Delftia*
		Neisseriales		*Neisseriaceae*	*Allysiella, Aquaspirillum, Chromobacterium, Eikenella, Kingella, Simonsiella, Vitreoscilla*
		Nitrosomonadales		*Spirillaceae*	*Spirillum*
γ-*Proteobacteria*		*Aeromonadales*		*Aeromonadaceae*	*Aeromonas*
				Succinivibrionaceae	*Anaerobiospirillum, Succinomonas, Succinovibrio*
				Shewanellaceae	*Shewanella*
		Cardiobacteriales		*Cardiobacteriaceae*	*Cardiobacterium, Dichelobacter, Suttonella*
		Enterobacteriales		*Enterobacteriaceae*	*Arenophonus, Biostraticola, Brenneria, Buchnera, Budvicia, Buttiauxella, Calymmatobacterium, Cedacea, Citrobacter, Chronobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Levinea, Mangrovibacter, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Photorhabdus, Pleisiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Saccharobacter, Salmonella, Samsonia, Serratia, Shigella, Sodalis, Tatumella, Thorselia, Trabulsiella, Wigglesworthia, Xenorhabdus, Yersinia, Yokenella*
		Legionellales		*Coxsiellaceae*	*Aquacilla, Coxiella*
				Legionellaceae	*Fluoribacter, Legionella, Sarcobium, Tatlockia*
		Unclassified Legionellales			*Rickettsiella*
		Oceanospirillales		*Oceanospirillaceae*	*Balneatrix*

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		Pasteurellales		*Pasteurellaceae*	*Actinobacillus, Aggegatibacter, Avibacterium, Basfia, Bibersteinia, Chelonobacter, Gallibacterium, Haemophilus, Histophilus, Lonepinella, Mannheimia, Nicoletella, Pasteurella, Phocoenobacter, Volucribacter*
		Pseudomonadales		*Moraxellaceae*	*Acinetobacter, Moraxella, Psychrobacter*
				Pseudomonadaceae	*Chryseomonas, Flavimonas, Pseudomonas*
		Thiotrichales		*Francisellaceae*	*Francisella*
		Vibrionales		*Vibrionaceae*	*Listonella, Photobacterium, Vibrio*
		Xanthomonadales		*Xanthomonadaceae*	*Stenotrophomonas*
δ-*Proteobacteria*		*Desulfovibrionales*		*Desulfovibrionaceae*	*Bilophila, Disulfovibrio, Lawsonia*
ε-*Proteobacteria*		*Campylobacteriales*		*Campylobacteraceae*	*Arcobacter, Campylobacter*
				Phylum *Firmicutes*	
Class	**Subclass**	**Order**	**Suborder**	**Family**	**Genera**
Bacilli		*Bacillales*		*Alicyclobacillaceae*	*Alicyclobacillus, Sulfobacillus*
				Bacillaceae	*Alkalibacillus, Amphibacillus, Anoxybacillus, Aquisalibacillus, Bacillus, Caldalkalibacillus, Cerasibacillus, Exiguobacterium, Falsibacillus, Filobacillus, Geobacillus, Gracilibacillus, Halalkalibacillus, Halobacillus, Halolactibacillus, Jeotgalibacillus, Lentibacillus, Lysinibacillus, Marinibacillus, Natronobacillus, Oceanobacillus, Ornithinibacillus, Paraliobacillus, Paucisalibacillus, Pelagibacillus, Piscibacillus, Pontibacillus, Saccharococcus, Salibacillus, Salimicrobium, Salinibacillus, Salirhabdus, Salsuginibacillus, Sediminibacillus, Tenuibacillus, Terribacillus, Thalassobacillus, Tumebacillus, Ureibacillus, Virgibacillus, Viridibacillus, Vulcanibacillus*
				Listeriaceae	*Brochothrix, Listeria*
				Paenibacillaceae	*Ammoniphilus, Aneurinibacillus, Brevibacillus, Cohnella, Fontibacillus, Oxalophagus, Paenibacillus, Saccharibacillus, Thermicanus, Thermobacillus*
				Pastueriaceae	*Pasteuria*
				Planococcaceae	*Filibacter, Kurthia, Paenisporosarcina, Planococcus, Planomicrobium, Sporosarcina*
				Sporolactobacillaceae	*Marinococcus, Sporolactobacillus, Tuberibacillus*
				Staphylococcaceae	*Gemella, Jeotgalicoccus, Macrococcus, Nosocomiicoccus, Salinicoccus, Staphylococcus*
		Lactobacillales		*Aerococcaceae*	*Abiotrophia, Aerococcus, Dolosicoccus, Eremococcus, Facklamia, Globicatella, Ignavigranum*
				Carnobacteriaceae	*Agitococcus, Alkalibacterium, Allofustus, Alloiococcus, Atopococcus, Atopostipes, Carnobacterium, Desemzia, Dolosigranulum, Granulicatella, Isobaculum, Lacticigenium, Lactosphaera, Marinilactibacillus, Trichococcus*
				Enterococcaceae	*Atopobacter, Bavariicoccus, Catellicoccus, Enterococcus, Melissococcus, Pilibacter, Tetragenococcus, Vagococcus*
				Lactobacillaceae	*Lactobacillus, Paralactobacillus, Pediococcus*
				Leuconostocaceae	*Fructobacillus, Leuconostoc, Oenococcus, Weissella*
				Streptococcaceae	*Lactococcus, Lactovum, Streptococcus*

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Phylum *Firmicutes*

Class	Subclass	Order	Suborder	Family	Genera
Clostridia		*Clostridiales*		*Clostridiaceae*	*Clostridium, Sarcina*
				Eubacteriaceae	*Acetobacterium, Alkalibacter, Eubacterium, Mogibacterium*
				Gracilibacteriaceae	*Gracilibacter*
				Lachnospiraceae	*Lachnospira, Butyrivibrio*
				Peptococcaceae	*Peptococcus*
				Peptostreptococcaceae	*Anaerococcus, Anaerosphaera, Filifactor, Finegoldia, Helcococcus, Peptoniphilus, Peptostreptococcus*
				Veillonellaceae	*Acidaminococcus, Megasphaera, Selenomonas, Veillonella*

Phylum *Actinobacteria*

Class	Subclass	Order	Suborder	Family	Genera
Actinobacteria	*Actinobacteridae*	*Actinomycetales*	*Actinomycineae*	*Actinomycetaceae*	*Actinomyces, Actinobaculum, Arcanobacterium, Mobiluncus*
			Corynebacterineae	*Corynebacteriaceae*	*Bacterionema, Corynebacterium, Caseobacter, Turicella*
				Dietziaceae	*Dietzia*
				Gordoniaceae	2009 Proposal to combine family *Gordoniaceae* and family *Nocardiaceae* in an emended family *Nocardiaceae* (see below)
				Mycobacteriaceae	*Mycobacterium*
				Nocardiaceae	*Gordonia*, Micropolyspora, *Nocardia, Rhodococcus, Skermania, Williamsia*
				Tsukamurellaceae	*Tsukamurella*
			Micrococcineae	*Brevibacteriaceae*	*Brevibacterium*
				Cellulomonadaceae	*Cellulomonas, Oerskovia, Paraoerskovia, Tropheryma*
				Dermabacteriaceae	*Brachybacterium, Dermabacter, Devriesea, Helcobacillus*
				Dermacoccaceae	*Demetria, Dermacoccus, Kytococcus*
				Dermatophilaceae	*Dermatophilus*
				Microbacteriaceae	*Agreia, Agrococcus, Agromyces, Aureobacterium, Clavibacter, Leifsonia, Microbacterium*
				Micrococcaceae	*Arthrobacter, Kocuria, Micrococcus, Nesterenkonia, Renibacterium, Rothia, Stomatococcus*
				Promicromonosporaceae	*Cellulosimicrobium, Promicromonospora*
				Sanguibacteraceae	*Sanguibacter*
			Micromonosporineae	*Micromonosporaceae*	*Micromonospora*
			Propionibacterineae	*Nocardioidaceae*	*Nocardioides*
				Propionibacteriaceae	*Arachnia, Propionibacterium, Propioniferax*
		Bifidobacteriales		*Bifidobacteriaceae*	*Bifidobacterium, Gardnerella*

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Phylum *Bacteroidetes*					
Class	**Subclass**	**Order**	**Suborder**	**Family**	**Genera**
	Coriobacteridae	*Coriobacteriales*	*Micromonosporineae*	*Coriobacteriaceae*	*Atopobium, Collinsella, Cryptobacterium, Eggerthella, Paraeggerthella, Slackia*
Bacteroidia		*Bacteroidales*		*Bacteroidaceae*	*Bacteroides, Acetofilamentum, Acetomicrobium, Acetothernus, Anaerorhabdus*
				Porphyromonadaceae	*Narnesiella, Dysgonomonas, Porphyromonas, Tannerella*
				Prevotellaceae	*Prevotella, Xylanibacter*
Flavobacteria		*Flavobacteriales*		*Flavobacteriaceae*	*Bergeyella, Capnocytophaga, Chryseobacterium, Elizabethkingia, Empedobacter, Flavobacterium, Myroides, Riemererella, Wautersiella, Weeksella*
Sphingobacteria		*Sphingobacteriales*		*Sphingobacteriaceae*	*Pedobacter, Sphingobacterium*
Phylum *Fusobacteria*					
Class	**Subclass**	**Order**	**Suborder**	**Family**	**Genera**
Fusobacteria		*Fusobacteriales*		*Fusobacteriaceae*	*Fusobacterium*
				Leptotrichiaceae	*Leptotrichia, Sneathia, Streptobacillus,*
Phylum *Spirochaetes*					
Class	**Subclass**	**Order**	**Suborder**	**Family**	**Genera**
Spirochaetes		*Spirochaetales*		*Leptospiraceae*	*Leptonema, Leptospira, Turneriella*
				Spirochaetaceae	*Borrelia, Spirochaeta, Treponema, Cristispira*
Phylum *Chlamydiae*					
Class	**Subclass**	**Order**	**Suborder**	**Family**	**Genera**
Chlamydiae		*Chlamydiales*		*Chlamydiaceae*	*Chlamydia, Chlamydophila*
				Parachlamydiaceae	*Neochlamydia, Parachlamydia*
				Simkaniaceae	*Simkania,* Candidatus Rhabdochlamydia
				Waddliaceae	*Waddlia*
Phylum *Tenericutes*					
Class	**Subclass**	**Order**	**Suborder**	**Family**	**Genera**
Mollicutes		*Acholeplasmatales*		*Acholeplasmataceae*	*Acholeplasma*
		Anaeroplasmatales		*Anaeroplasmataceae*	*Anaeroplasma, Asteroleplasma*
		Entomoplasmatales		*Entomoplasmataceae*	*Entomoplasma, Mesoplasma*
				Spiroplasmataceae	*Spiroplasma*
		Mycoplasmatales		*Mycoplasmataceae*	*Eperythrozoon, Haemobartonella, Mycoplasma, Ureaplasma*

with various identification schemes and computer-assisted databases, most bacteria isolated in the clinical laboratory can be placed in a taxonomic framework that allows a genus and species name to be determined. This approach fulfills the role of the clinical microbiology laboratory in providing physicians with accurate and timely organism identifications and determining the susceptibility of these organisms to antimicrobial agents. Physicians, in turn, use this information to guide their selection of appropriate therapeutic interventions, to monitor the clinical response of the patient, and to evaluate the patient's clinical course.

In order to provide the reader with sufficient background information for understanding subsequent chapters regarding specific bacterial pathogens and the diseases that they cause, the next section of this chapter deals with the general morphology, physiology, and virulence mechanisms of bacteria. This discussion will provide the information necessary for understanding the nuances of morphology, staining properties, growth, metabolic features, and biochemical characteristics of the organisms discussed in subsequent chapters. The basic morphology and physiology of fungi, parasites, and viruses are presented in Chapters 21, 22, and 23, respectively.

Basic Bacterial Anatomy and Physiology

Bacteria are prokaryotic, while fungi, protozoa, and other organisms are eukaryotic. Eukaryotic cells contain a nucleus with a nuclear membrane enclosing multiple chromosomes, while prokaryotic cells have a single chromosome (nucleoid) that is not enclosed in a nuclear membrane. Eukaryotic cells also possess a variety of subcellular organelles with specialized functions, such as mitochondria (sites of aerobic respiration) and chloroplasts (sites of photosynthesis in green plants). In fact, these subcellular organelles probably evolved from prokaryotic organisms that entered the eukaryotic cells and developed symbiotic relationships with them over time by losing metabolic functions associated with a free-living existence and developing features or attributes that benefitted the "host" organism. Prokaryotic and eukaryotic cells differ substantially in many other characteristics; these are briefly described in Table 5-1.

Bacterial Size and Shape

Bacterial cells have a wide variety of sizes and shapes. Most bacteria are generally 0.2 to 2 µm in diameter and 1 to 6 µm in length, although many environmental organisms may be as long as 100 µm. Bacteria exist in four basic morphologies: spherical cells, or **cocci**; rod-shaped cells, or **bacilli**; spiral-shaped cells, or **spirilla**; and comma-shaped cells, or **vibrios**. Arrangements of coccal cells in pairs, chains, or clusters define groups of organisms called **diplococci**, **streptococci**, and **staphylococci**, respectively (Fig. 5-1). Rod-shaped organisms may be regular in morphology, may be somewhat shorter (i.e., "**coccobacillary**"), or may appear club- or dumbbell-shaped ("**coryneform**"). Comma-shaped cells generally define a basic characteristic of certain species (e.g.,

TABLE 5-1 Properties of Prokaryotic and Eukaryotic Cells

Characteristic	Prokaryotic (Eubacterial) Cells	Eukaryotic Cells
Major groups	Bacteria, blue-green algae	Algae, fungi, protozoa, plants, animals
Cell wall	Contains peptidoglycan, lipid, protein	Variable; when present, contains chitin (fungi) or cellulose (green plants)
Nuclear structure		
Nuclear membrane	Absent	Present

Chromosomes	Single, closed, circular double-stranded DNA	Multiple, linear chromosomes
Ploidy	Haploid	Most are diploid; haploid (fungi)
Transcription/translation	Continuous, with short-lived mRNA and polyribosome (polysome) formation	Discontinuous; long-lived mRNA transcribed in nucleus and translated in cytoplasm
Histones	Absent	Present
Cytoplasm		
Ribosomes	Present: 70S (50S + 30S)	Present: 80S (60S + 40S)
Mitochondria	Absent	Present
Golgi complex	Absent	Present
Endoplasmic reticulum	Absent	Present
Cytoplasmic membrane	Present: phospholipids, no sterols (except for *Mycoplasma* species)	Present: phospholipids and sterols (cholesterol, ergosterol)
Triglycerides	Absent	Present
Motility	Flagella (simple)	Flagella (complex); pseudopodia; cilia and other complex locomotor organelles)

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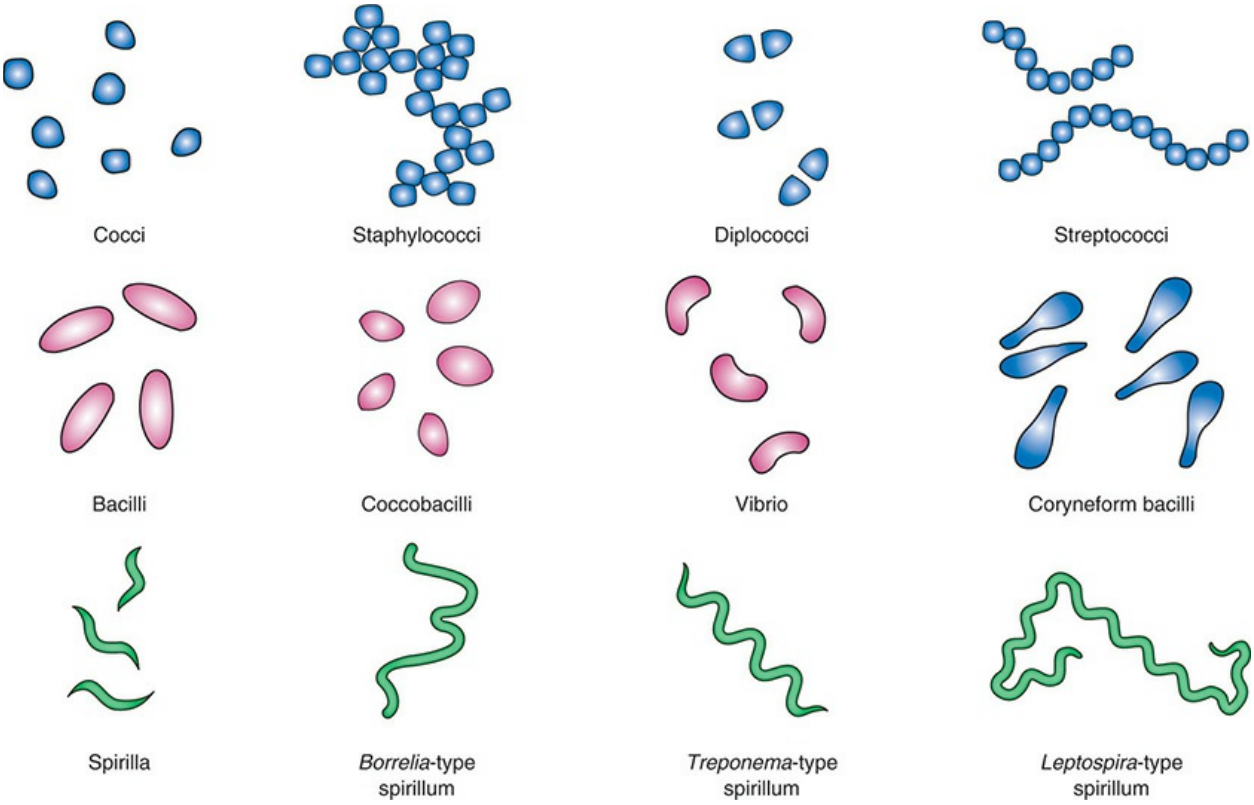
Energy generation	Cytoplasmic membrane-associated	Mitochondria
Sexual reproduction	Absent (unnecessary)	Present in most; may be evolutionarily lost in some fungi and absent in some protozoa
Recombination/gene exchange	Chromosomal or plasmid gene exchange via transformation, transduction, or conjugation)	Diploid zygote formed from haploid germ cells; meiosis results in genetic recombination

Vibrio species). The same is true for certain other spiral-shaped bacteria (e.g., *Campylobacter*, *Helicobacter*, *Borrelia*, and *Treponema* species), in which spiral formation may be loose (about 4 coils per organism) or tight (14 to 20 coils per organism). In addition to their size, shape, and cellular arrangement, bacteria can be further differentiated on the basis of their staining characteristics with the **Gram's stain**. Using this staining technique, most bacteria can be classified as gram-positive or gram-negative. The Gram's stain helps to differentiate bacteria based on their cell wall structure, which is discussed later in this chapter. The structure of a generalized bacterial cell (both gram-positive and gram-negative) is depicted in Figure 5-2.



■ **FIGURE 5-1** Basic morphologies of various bacteria.

Nuclear Structure, DNA Replication, Transcription, and Translation

The inheritable characteristics of all living organisms are determined by the structure of the genetic material. The genetic material of an individual cell is composed of **DNA** organized into single or multiple chromosomes. Collectively, the genetic material is referred to as the genome of the organism. In the vast majority of prokaryotic

organisms, the **genome** is composed of a single, covalently closed, circular chromosome of double-stranded DNA (dsDNA); in some organisms (e.g., *Borrelia burgdorferi*, the streptomycetes) linear DNA is present. In the *Archaea* and the *Bacteria*, this circular chromosome is not bounded by a membrane, but is free in the cytoplasm in a discrete central portion of the bacterial cell called the **nucleoid**. Bacterial cellular DNA has a molecular weight of about 106 kDa, measures 300 to 1,400 μm in length, and is present in the cell in a **supercoiled** state (i.e., the double-stranded molecule is twisted up on itself like a "twisted" rubber band). Individual genes are arranged linearly on the chromosome. The nucleoid represents about 10% of the cell volume, although DNA is only 2% to 3% of the dry weight of the cell. In *Escherichia coli*, the chromosome contains about 5×10^6 base-pairs, and its length is

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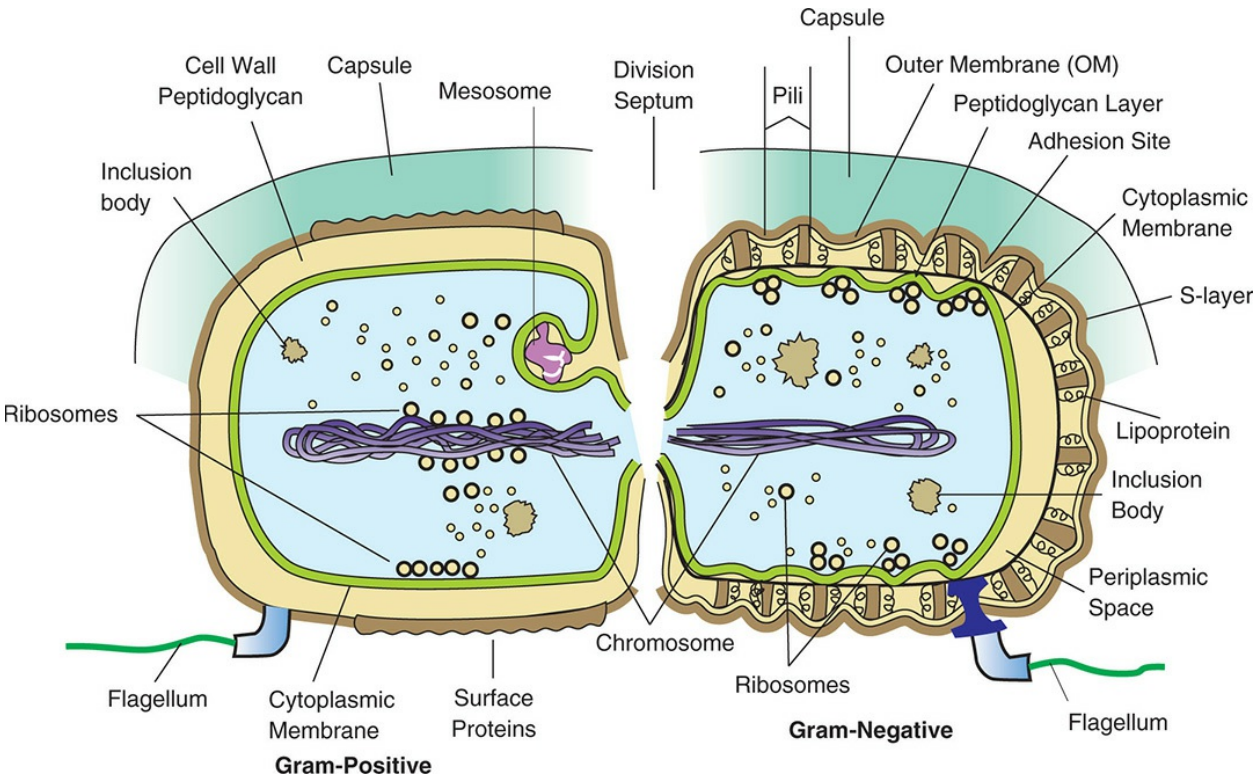

■ **FIGURE 5-2** Cross-section through a generalized bacterial cell. The left half of this figure depicts the structure of a gram-positive bacterium; the right half shows the structure of a gram-negative bacterium.

about 1,000 times the length of the bacterial cell in which it is contained. Unlike similar processes in eukaryotic cells, DNA replication and transcription of the DNA into

messenger ribonucleic acid (mRNA) occur continually. The chromosome also appears to be attached to the inner aspect of the cell membrane at certain points. In **eukaryotic** organisms, the genetic material is organized into several **chromosomes** within the nucleus. The chromosomes are, in turn, associated with several basic proteins called histones, which help to stabilize chromosomal structure. The chromosomes of eukaryotic organisms are separated from the rest of the cellular material by a **nuclear membrane**, which is composed of a lipid bilayer that is similar in composition to the cell membrane. The nuclear membrane also contains pores that allow passage of small molecules into and out of the nucleus.

Nucleic acids of all bacteria, like other organisms, are composed of **polynucleotides** (a polymer consisting of nucleotides) that are comprised of the following three components (Fig. 5-3): (1) a cyclic, 5-carbon sugar (ribose in RNA; deoxyribose in DNA), (2) a purine (adenine, guanine) or pyrimidine (cytosine, thymine, uracil) base attached to the 1′ carbon atom of the pentose by an *N*-glycosidic bond, and (3) a phosphate (PO_3) attached to the 5′ carbon of the pentose by a phosphodiester linkage. Deoxyribose moieties are linked together via alternating phosphate groups to form a chain that has a characteristic helical coil, and the bases are directed toward the central axis of the coil. Such a structure composes a single strand of nucleic acid (ssDNA).

The double-helix structure of DNA, specifically, results from the interaction between two complementary single strands of nucleic acid. Complementarity is associated with the sequence of bases on a single strand and the hydrogen bonding that occurs between specific bases on the complementary strand (Fig. 5-4). The **purine** bases are **adenine** (**A**) and **guanine** (**G**), and the **pyrimidine** bases are **cytosine** (**C**), **thymine** (**T**), and **uracil** (**U**) (Fig. 5-3). The purine adenine will specifically base-pair only with the pyrimidine thymine, while the purine guanosine will specifically base-pair only with the pyrimidine cytosine. Antiparallel chains of ssDNA are held together by three hydrogen bonds between C and G and two hydrogen bonds between A and T (Fig. 5-4). Native DNA exists as a double-stranded helix, while RNA exists primarily in a single-stranded form as **messenger RNA** (**mRNA**) and in partially double-stranded forms in **ribosomal RNA** (**rRNA**) and **transfer RNA** (**tRNA**) molecules. In all RNA species, uracil is present in place of thymine (see Fig. 5-3). The sequence of purine and pyrimidine bases in DNA constitutes the genetic code, with specific codons (three base-pair sequences) coding for specific amino acids. Single-stranded mRNA is synthesized from double-stranded DNA during the process of **transcription** by a **DNA-dependent RNA polymerase**, in which a complementary strand of mRNA is synthesized with the "sense" strand of dsDNA as the template. The mRNA then becomes associated with **ribosomes**, which are the sites of protein synthesis.

Both prokaryotic and eukaryotic organisms harbor large numbers of ribosomes. In prokaryotic organisms, the ribosomes are 70S; in eukaryotic organisms, the ribosomes

are 80S. The "S" refers to a Svedberg unit, which is an indirect measure of the size of the ribosome as determined by its rate of sedimentation when subjected to ultracentrifugal force. The 70S bacterial ribosome has a molecular weight of about 80 kDa and exists in a dissociated state as two subunits termed the *30S* and the *50S subunits*. The 30S subunit contains the 16S RNA species, while the 50S subunit contains both 23S and 5S RNA; together, the subunits also contain

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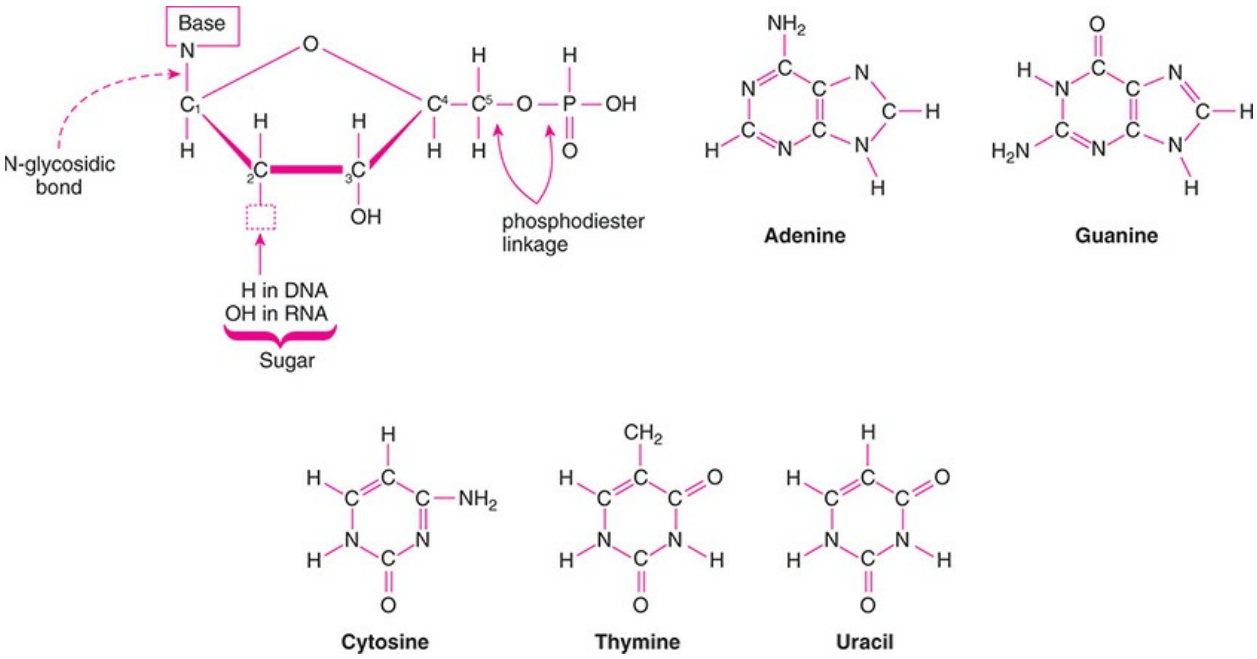
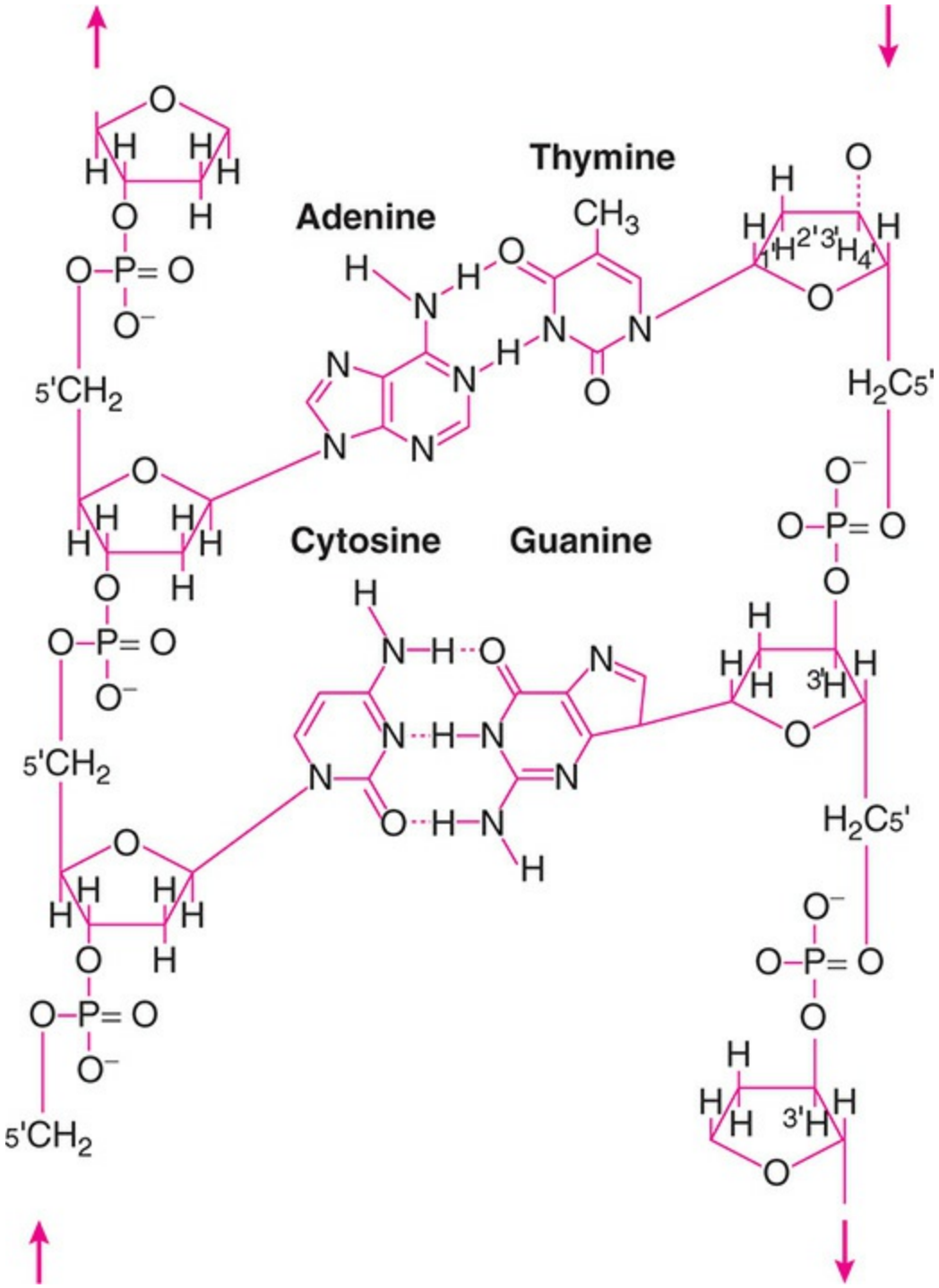


■ **FIGURE 5-3** Molecular structure of polynucleotides and nucleic acid bases. Polynucleotides consist of a cyclic, 5-carbon sugar (ribose or deoxyribose), a purine or pyrimidine base attached to the 1′ carbon atom of the sugar by an *N*-glycosidic bond, and a phosphate group linked to the 5′ carbon of the sugar by a phosphodiester linkage. The structures of the two purine (adenine and guanine) and the three pyrimidine (cytosine, thymine, and uracil) bases are also shown.



■ **FIGURE 5-4** In the DNA molecule, the two polynucleotide strands of the DNA double helix are "antiparallel," that is, the 3′-OH terminus of one strand is adjacent to the 5′-P terminus of the complementary strand. The bases, which are directed toward the central axis of the helix, hold the two polynucleotide strands together by relatively weak hydrogen bonds. Adenine pairs with thymine via two hydrogen bonds, while cytosine pairs with guanine via three hydrogen bonds. These interactive forces between the polynucleotide

strands can be overcome by thermal energy (heat) or by strong alkali in the process of denaturation.

about 50 ribosomal proteins. rRNA comprises 70% of the total cellular RNA. The remaining cellular RNA is found as tRNA (16%) and mRNA (14%).

When complexed with an mRNA transcript from the DNA, the 50S and 30S ribosomal subunits form the intact 70S ribosome found in bacterial cells. Ribosome–mRNA aggregates, termed *polyribosomes* or *polysomes*, contain all components of the protein-synthesizing system; polysomes are essentially chains of 70S ribosomes (monomers) attached to mRNA. Histone or histone-like proteins that serve to stabilize the nascent polypeptides synthesized by the polysomes have only recently been found in small amounts in association with *E. coli* DNA, whereas the occurrence of polyamine proteins (e.g., putrescine and spermidine) associated with bacterial DNA is well known. The mRNA is "decoded" in association with several ribosomes in a polysome–mRNA complex, where the **codon–anticodon base-pairing** occurs through molecules of tRNA during the process of **translation**. tRNA molecules bear the **specific anticodons** corresponding to the codons of the mRNA and also carry the corresponding, covalently linked amino acid. The tRNA–amino acid complex interacts with the ribosome such that a peptide bond is formed between the amino acid on the tRNA and the nascent peptidyl–tRNA. Interaction of tRNA–amino acid, ribosomes, adenosine triphosphate (ATP) and several cofactors result in the formation of a specific polypeptide chain. Thus, the genetic code present in the DNA is translated into structural protein molecules (i.e., "building blocks") or enzymes that, in turn, catalyze the synthesis and degradation of all the other cellular components. The synthesis of new DNA molecules, called **replication**, occurs by the uncoiling and "unzipping" of the dsDNA molecule by a **DNA gyrase** enzyme, and the synthesis of complementary strands of DNA by a DNA-dependent DNA polymerase.

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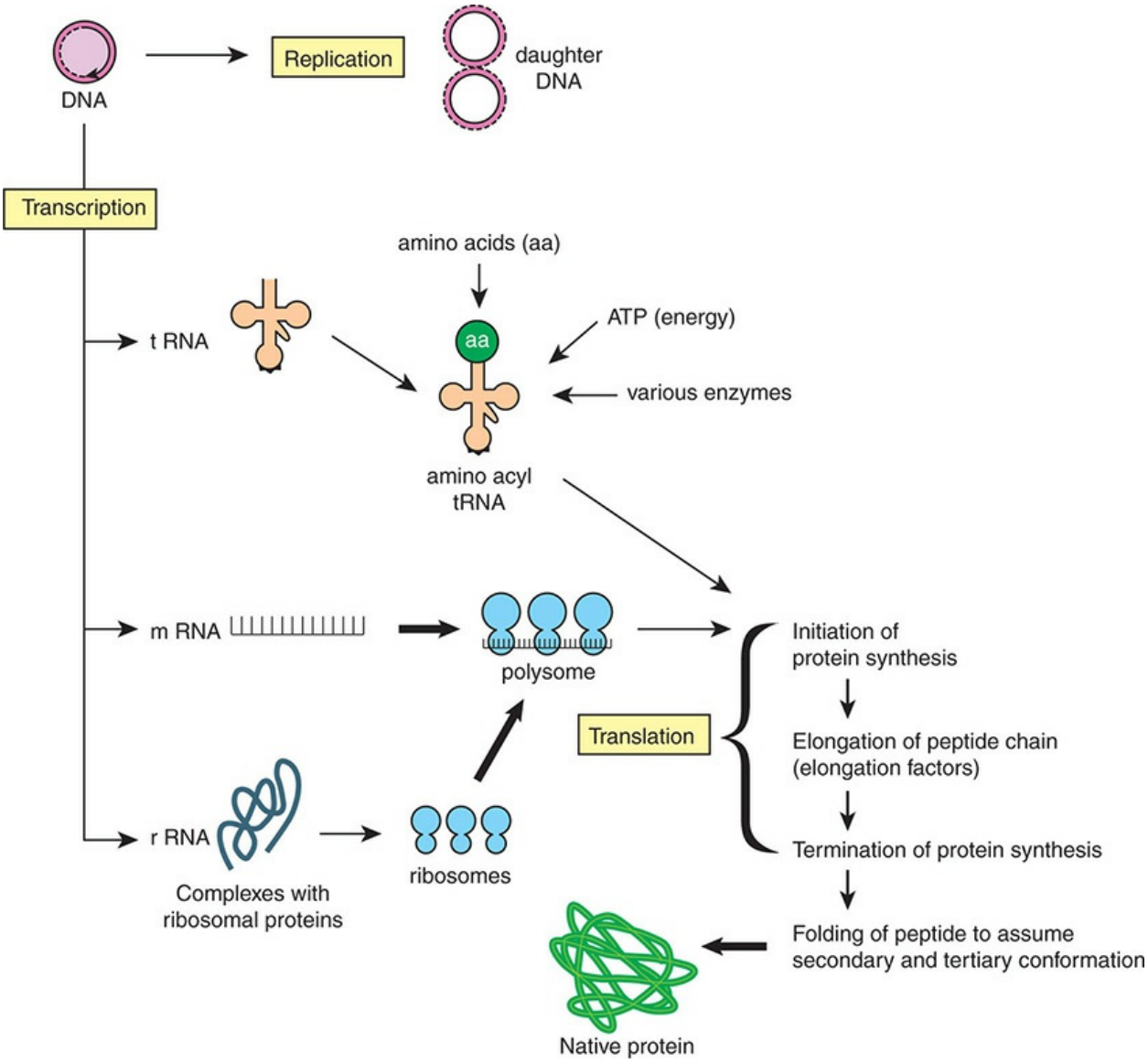
Each new dsDNA molecule contains a single strand of the parent DNA. The relationship among DNA replication, RNA transcription, and translation of the genetic code into proteins is summarized in Figure 5-5.

In addition to its utility for determining genetic relatedness among bacteria (see above), sequencing of rRNA molecules has revealed nucleotide sequences that are unique to individual species. Since these unique RNA sequences are also highly conserved and exist in multiple copies within the ribosomes of a bacterial cell, synthetic oligonucleotides that can hybridize with these unique sequences can be used to both detect and identify bacteria. Such an approach forms the basis for nucleic acid probe technology for direct detection of microorganisms in clinical specimens or for

identification of microorganisms recovered in culture.

Cytoplasm

The cytoplasm is an amorphous gel containing enzymes, ions, and a variety of granules, many of which represent food and energy reserves. The cytoplasmic enzymes of prokaryotic cells function in both anabolic and catabolic processes, and many of these enzymes are associated with the inner aspect of the cell membrane (see below). Prokaryotic cells lack separate, membrane-bounded subcellular organelles, while eukaryotic cells contain a variety of subcellular structures (e.g., mitochondria, endoplasmic reticula, etc.) composed of or bounded by phospholipid bilayer membranes. Intracellular, cytoplasmic inclusions or granules represent accumulations of food reserves (polysaccharides, lipids, or polyphosphates). The numbers and types of storage granules



■ **FIGURE 5-5** Replication, transcription, and translation of the genetic code in prokaryotic organisms. DNA is replicated by DNA-dependent DNA polymerase to produce two double-stranded DNA (dsDNA) molecules. The genetic code in the dsDNA is copied to produce a single-stranded RNA called messenger RNA (mRNA) during the process of transcription. Transfer RNA (tRNA) and ribosomal RNA (rRNA) are also transcribed. rRNA becomes complexed with specific proteins to form part of the structure of the ribosome. The mRNA becomes complexed with ribosomes to form polysomes, which are the site of protein synthesis. On the polysome, codons specific for individual amino acids are recognized by anticodons on tRNA molecules by specific base-pairing. Specific codons correspond to different amino acids attached to aminoacyl tRNA molecules. During the stages of translation, protein synthesis is initiated, polypeptide chains are elongated, and synthesis is eventually terminated with the release of a protein molecule.

vary with the medium and the functional state of the cells. Starch is the principal storage product among the *Neisseria* and *Clostridium* species, while glycogen is the major storage material of enteric bacteria. *Bacillus* and *Pseudomonas* species accumulate 30% or more of their dry weight as a high-molecular-weight lipid called **poly- β-hydroxybutyrate**. High-molecular-weight polymers of polyphosphate known as **metachromatic granules** or **volutin** occur in *Corynebacterium* species, *Yersinia pestis*, and *Mycobacterium* species. These volutin granules appear reddish-pink when stained with methylene blue. Intracytoplasmic inclusions that are found mostly among environmental bacteria include magnetosomes and gas vesicles. **Magnetosomes** are composed of various forms of iron and impart a magnetic axis to the organism that allows it to orient itself in relation to the earth's magnetic field. **Gas vesicles** permit the organism to maintain buoyancy at a certain level in an aqueous environment. Both of these inclusions enable the organisms to adjust locations in response to growth conditions and nutrient concentrations.

Extrachromosomal DNA is frequently present in the cytoplasm of prokaryotic organisms in the form of **plasmids**. Plasmids exist as covalently closed circles of dsDNA that range in size from about 1 kilobase (kb) to greater than 400 kb, which is equivalent to about 10% of the size of the *E. coli* chromosome. They are generally not found in eukaryotic organisms, although some subcellular organelles in eukaryotic organisms (e.g., mitochondria) do contain DNA molecules that resemble bacterial plasmids. Plasmids are capable of autonomous replication, are inherited by progeny bacterial cells, and may contain the genetic information for a variety of structures or functions related to bacterial virulence, including genes for antimicrobial resistance, virulence-related adhesins, toxin production, and resistance to heavy metal ions. Some plasmids, termed *conjugative plasmids*, encode for enzymes that facilitate transmission of plasmids to other bacterial cells. Some bacteria may also possess **transposons** and **insertion sequences**, which are sequences of DNA that are able to insert into different, unrelated sites on either the chromosome or a plasmid. Insertion of these elements into DNA does not require that the transposon base sequence be homologous with its insertion site (see below).

Cytoplasmic Membrane

The cytoplasm of all bacterial cells is surrounded by a cytoplasmic membrane. The bacterial cytoplasmic membrane lies immediately within the cell wall peptidoglycan layer in gram-positive bacteria, and adjacent to the periplasmic space in gram-negative bacteria (discussed below). The basic structure of the cytoplasmic membrane is a phospholipid bilayer in which various constituent proteins are embedded. The membrane is comprised of 30% to 60% phospholipid and 50% to 70% protein by

weight. Most bacterial cell membranes contain phosphatidyl glycerol, phosphatidyl ethanolamine, and diphosphatidyl glycerol; they do not contain sterols (e.g., cholesterol or ergosterol). The only prokaryotic exceptions to this are the mycoplasmas and ureaplasmas, which incorporate sterols from the growth medium into their cell membranes. The fatty acids that compose the lipid portion of the phospholipid bilayer generally contain 15 to 18 carbon backbones and are usually saturated or monounsaturated.

The cell membrane of prokaryotes possesses several functions that are relegated to specialized intracytoplasmic organelles in eukaryotic organisms. The bacterial cell membrane contains enzymes that are active in cellular respiration and oxidative phosphorylation, peptidoglycan and complex lipid biosynthesis, DNA replication, and outer membrane biosynthesis in gram-negative bacteria. In phototrophic bacteria, the early events associated with light harvesting also occur at the cell membrane level. The cell membrane contains the machinery for the synthesis and secretion of enzymes and bacterial toxins and provides an insulating barrier across which there may form an ion gradient or membrane potential that is used for energy production; such energy may be used for flagellar movement, chromosomal mobilization, etc. The membrane structure allows the retention of metabolites and exclusion of many external compounds. Some membrane proteins are involved in the active transport of materials (e.g., certain monosaccharides and disaccharides) into the cytoplasm. Such specific membrane-associated carrier proteins are termed *permeases*. **Mesosomes**, which are invaginations of the cytoplasmic membrane that extend into the cytoplasm, may function to increase the available membrane surface area for catabolic and anabolic cellular enzymes. They may also function in DNA replication and DNA duplex separation in actively growing cells. However, there is also evidence suggesting that mesosomes may actually be artifacts that result from the fixation techniques used for electron microscopy.

Bacterial Cell Wall Structure

The bacterial cell wall provides structural rigidity, confers shape to the cell, and forms a physical barrier against the outside environment. The rigid component of the cell wall of all bacteria is composed of **peptidoglycan**. Peptidoglycan is found in all bacterial species except for the cell wall–deficient mycoplasmas and ureaplasmas. This structure is composed of a backbone of alternating carbohydrate moieties of ***N*-acetylglucosamine and *N*-acetylmuramic acid** in β-1,4 linkage (Fig. 5-6). Short tetrapeptides, generally composed of identical short chains of D- and L-amino acids, are attached to the *N*-acetylmuramic acid residues via a peptide bond to the lactyl group on C3. These short chains contain unusual amino acids not generally found in proteins, including D-isomers of alanine and D-glutamic acid (gram-positive bacteria), *meso*-diaminopimelic acid (*meso*-DAP) or lysine (gram-negative bacteria). Some of

these tetrapeptides are, in turn, linked to one another by short peptides forming cross-bridges between adjacent peptidoglycan strands (Fig. 5-7). The types of amino acids found and the degrees of cross-linkage are variable components of the peptidoglycan structure. For example, in *Staphylococcus aureus*, most of the *N*-acetylmuramic acid residues are cross-linked to adjacent peptidoglycan strands by five glycine residues, thereby providing a rather tight, rigid cell wall structure (Fig. 5-7A). In gram-negative bacteria such as *E. coli*, the cross-linkage is directly between the *meso*-DAP of one peptidoglycan "chain" and the terminal D-alanyl residue on an adjacent strand (Fig. 5-7B). The degree of such cross-linking determines whether a cell wall structure is termed *tight* (highly cross-linked) or *loose*. Among the gram-positive bacteria, there are

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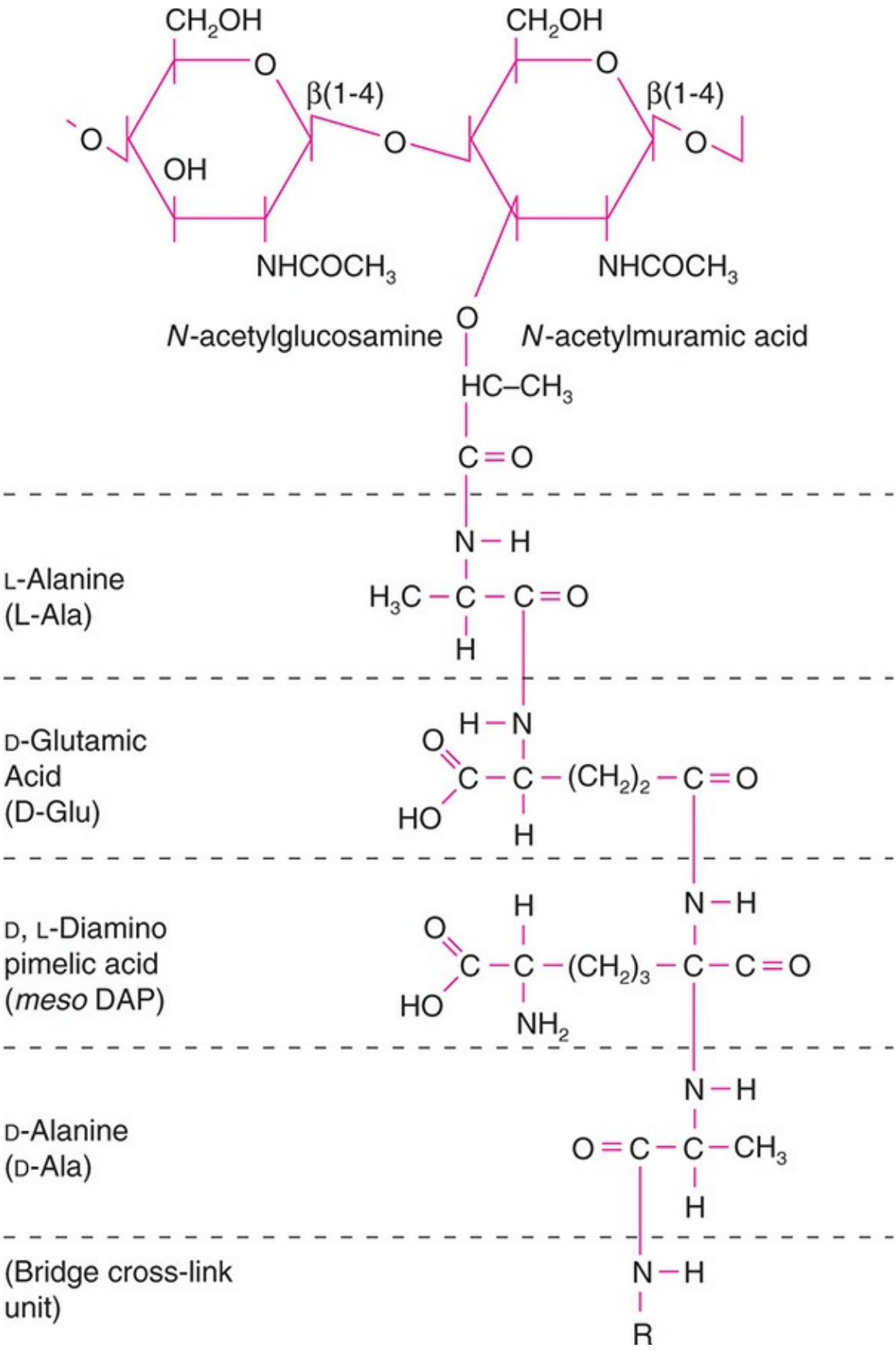
CH2OH
O
β(1-4)
O
OH
NHCOCH3
CH2OH
O
β(1-4)
O
NHCOCH3
N-acetylglucosamine
N-acetylmuramic acid
O
HC–CH3
C=O
N–H
L-Alanine
(L-Ala)
H3C–C–C=O
H
H–N
D-Glutamic
Acid
(D-Glu)
O
C–C–(CH2)2–C=O
HO
H
H
D, L-Diamino
pimelic acid
(meso DAP)
O
C–C–(CH2)3–C–C=O
HO
NH2
N–H
N–H
D-Alanine
(D-Ala)
O=C–C–CH3
H
N–H
(Bridge cross-link
unit)
R

■ **FIGURE 5-6** Structure of the repeating peptidoglycan unit of *Escherichia coli*.

over 100 different peptidoglycan chemotypes that differ by having various amino acid substituents attached to the lactyl group of *N*-acetylmuramic acid or that have different linkage units comprising the interpeptide bridges. Slight changes in the cell wall chemotype may occur on exposure to high salt concentrations or cell wall–active antimicrobial agents such as methicillin.

Bacterial cell wall biosynthesis is a continual process. New peptidoglycan polymers are exported from the cell and linked to preexisting cell wall polymers at the inner aspect of the cell wall by **penicillin-binding proteins**. At the same time, older peptidoglycan material overlying the newly synthesized structures is continually being removed by cell wall autolysins. Antimicrobial agents such as penicillins and cephalosporins inhibit bacterial cell growth by binding to the penicillin-binding proteins, thereby inhibiting polymer renewal at the inner aspect of the wall. Continuous autolytic hydrolysis of older peptidoglycan materials causes a thinning of the wall, eventually resulting in cell lysis.

Gram-Positive Bacterial Cell Walls. The gram-positive bacterial cell wall (Fig. 5-8A) is almost 80 nm thick and is composed mostly of several layers of peptidoglycan; in fact, anywhere from 40% to greater than 80% of the dry weight of some gram-positive cell walls may be peptidoglycan. Trapped within this peptidoglycan matrix are a variety of proteins, polysaccharides, and unique molecules called teichoic acids. Teichoic acids are polymers of either ribitol (5-carbon) or glycerol (3-carbon) units joined together by phosphodiester linkages (Fig. 5-9). Ribitol teichoic acids are associated with the cell wall, while glycerol teichoic acids are associated with the inner aspect of the bacterial cell membrane. Ribitol teichoic acids are covalently linked to the peptidoglycan via the C6 hydroxyl group of *N*-acetylmuramic acid, while the glycerol teichoic acids are linked to glycolipids of the cytoplasmic membrane. The latter molecules are termed *lipoteichoic acids*. They are linked to the outer lipid layer of the cell membrane and extend into the cell wall. Teichoic acids of different bacteria are further modified by addition of "R" groups including ester-linked D-alanine or D-lysine residues or *O*-glycoside-linked glucose, galactose, or *N*-acetylglucosamine. Teichoic acids stabilize the cell wall, maintain the association of the wall with the cell membrane, chelate small ions necessary for cell function and cell wall integrity, and participate in cellular interaction and adherence to mucosal or other surfaces. Teichoic acids may also function in peptidoglycan synthesis and septum formation during growth and reproduction and may also play a role in the competence of some gram-positive bacteria to undergo transformation. In some organisms, the teichoic acids are antigenic and form the basis for antigenic grouping (e.g., the group D antigen in group D streptococci and members of the genus *Enterococcus*).

In the various groups of pathogenic gram-positive bacteria, other cell wall structures that are important virulence determinants may also be present. For example, M protein, a well-recognized virulence factor of group A β-hemolytic streptococci, is associated with lipoteichoic acids in the streptococcal cell wall and extends out of the wall as a fimbrial protein (see Chapter 13).[45] The group antigens of the group A, B, C, F, and G β-hemolytic streptococci are also nonteichoic acid polysaccharides that are found in the cell wall. The C polysaccharide found in the cell walls of *Streptococcus pneumoniae* is a complex lipoteichoic acid composed of ribitol and phosphate substituted at various points with *N*-acetyl-D-galactosamine, D-glucose, *N*-acetyl-2,4-diamino-2,4,6-trideoxyhexose in *O*-glycosidic linkage and choline in diester linkage.[49]

Gram-positive (and some gram-negative) bacterial cell walls also possess a cell wall component called the **S-layer**. S-layers consist of protein or glycoprotein molecules of 50 to 120 kDa molecular weight that self-assemble on the outer surface of the organism in oblique, square or hexagonal, packed lattice-like structures.[7,82] S-layer material may constitute up to 20% of the total cell protein. On electron microscopy, the S-layer appears as an extra "layer" on top of the cell wall. S-layer proteins are poorly conserved and can differ markedly even between related species. Depending on the species, the S-layers have a thickness between 5 and 25 nm and possess identical pores 2 to 8 nm in diameter. S-layers exhibit either an oblique, square, or hexagonal lattice symmetry. Depending on the lattice symmetry, the S-layer is composed of one (P1), two (P2), three (P3), four (P4), or six (P6) identical protein subunits, respectively. For many bacteria, the S-layer represents the outermost interaction zone with the environment and its functions are diverse and vary from species to species. In *Archaea,* the S-layer is the only cell

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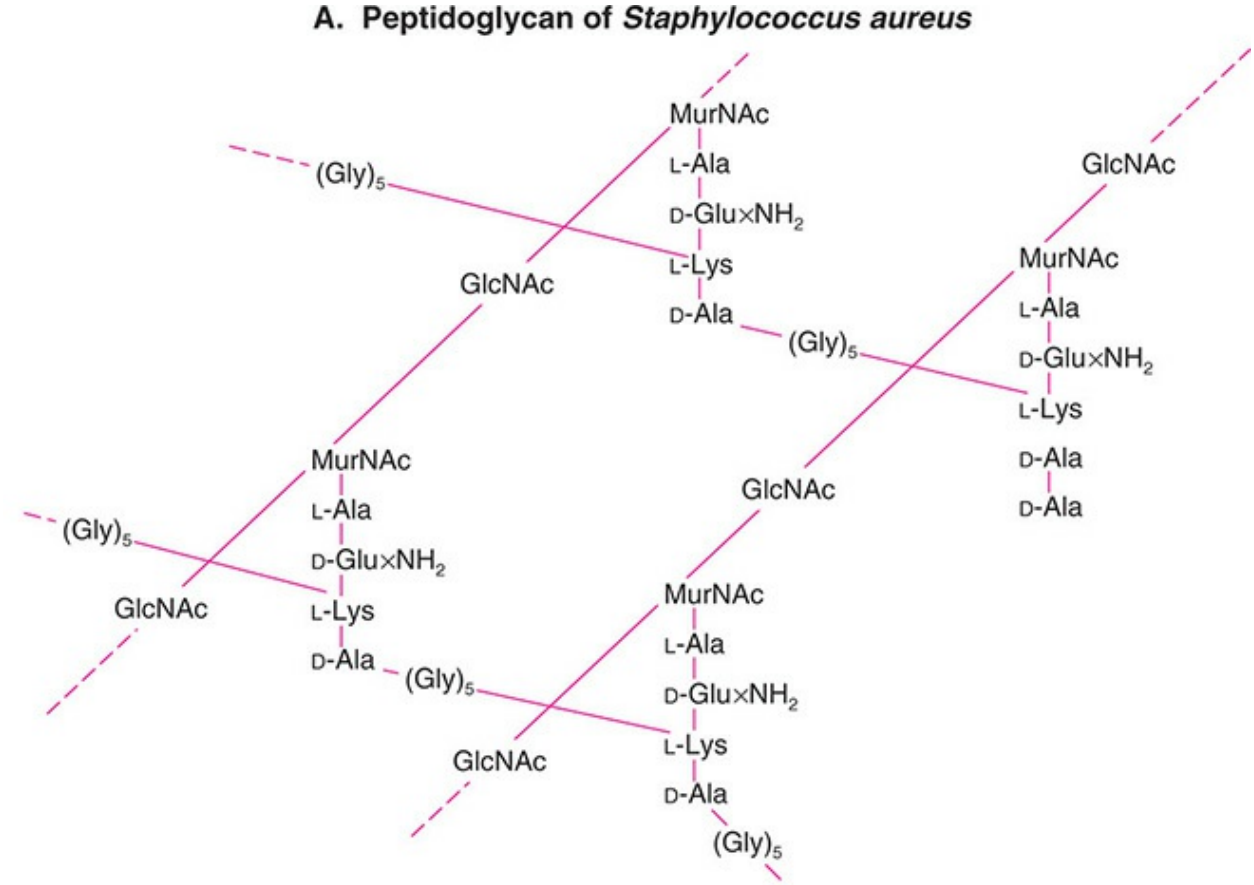

■ **FIGURE 5-7** Structure of the peptidoglycan in *Staphylococcus aureus* and *Escherichia coli*.

wall component and, therefore, is important for mechanical stabilization. Additional functions associated with S-layers include protection against bacteriophages and resistance against low pH. The S-layer may act as a barrier against high-molecular-weight substances (e.g., lytic enzymes) and may function as an adhesin in the case of glycosylated S-layers. S-layers may act to stabilize the cell membrane and to provide attachment sites for periplasmic proteins. In pathogenic microorganisms, the S-layer may inhibit phagocytosis and/or prevent the binding of immunoglobulin and complement. Freeze-etch electron microscopic techniques have demonstrated S-layers in *Bacillus*, *Lactobacillus*, *Clostridium*, *Campylobacter*, and *Aeromonas* species. In *Bacillus anthracis*, it is now thought that the S-layer is this organism's major cell wall antigen and, along with the capsule and the two toxins, may contribute to virulence.[57]

Gram-Negative Bacterial Cell Walls. The cell wall of gram-negative bacteria (Fig. 5-8B) is thinner than that of gram-positive bacteria but is structurally more complex.

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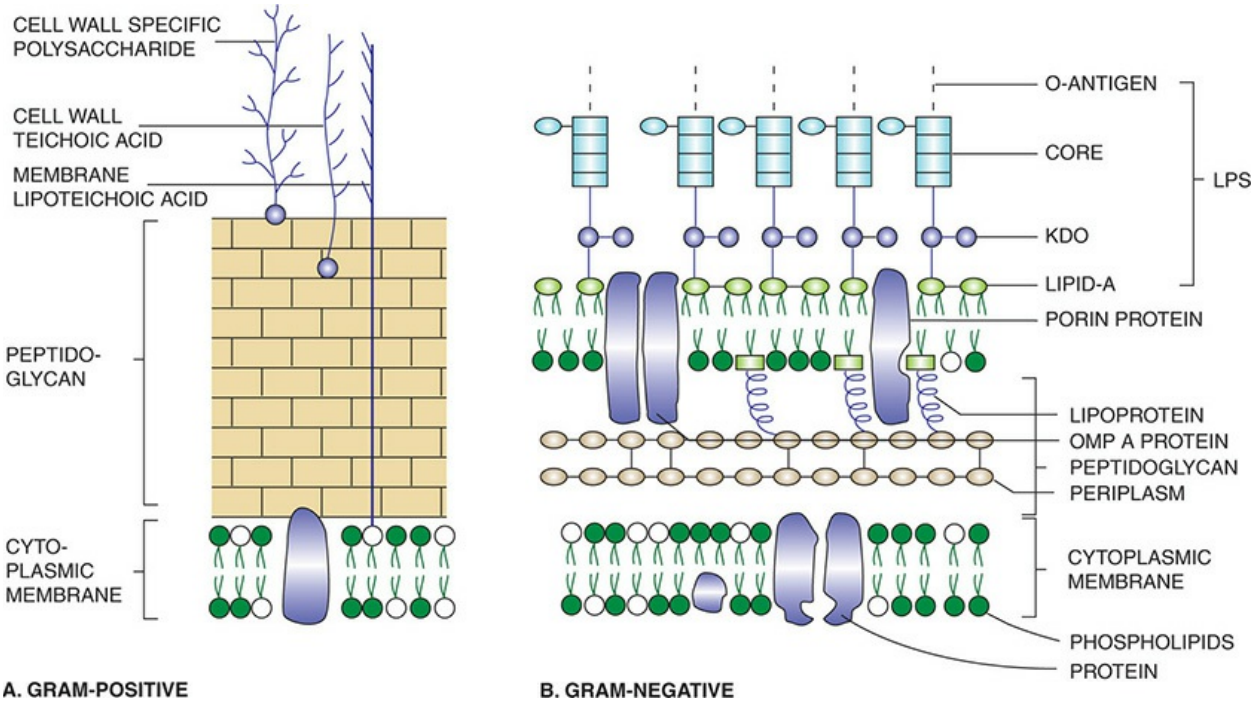
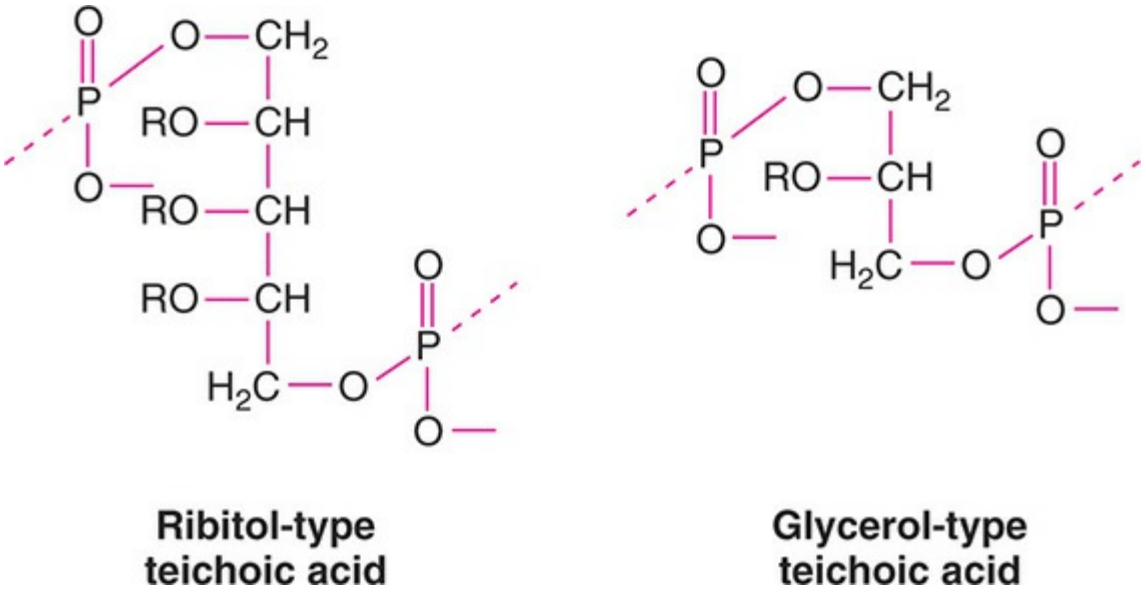


■ **FIGURE 5-8** Structure of the cell wall of gram-positive (**A**) and gram-negative (**B**) bacteria. KDO, keto-deoxy-octulonate; LPS, lipopolysaccharide.

Immediately outside of the cytoplasmic membrane is the **periplasmic space**. The periplasmic space contains degradative enzymes (alkaline phosphatase, proteases,

nucleosidases, β-lactamases, and aminoglycoside phosphorylases) and specific binding and transport proteins for vitamins, amino acids, and ions. A **single-unit-thick peptidoglycan layer** forms the outer border of the periplasmic space. Because the peptidoglycan layer is only one layer thick, cross-linking occurs only to adjacent peptidoglycan strands rather than to layers of peptidoglycan either closer to or more external to the individual cell surface. Cross-links are formed from the carboxyl group of the terminal D-alanine residue on one chain to the free amino group of a *meso*-DAP residue on an adjacent chain (Fig. 5-7B). The peptidoglycan layer of gram-negative bacteria is fairly "loose"; that is only about half of the peptide chains attached to the *N*-acetylmuramic acid residues are actually involved in cross-linking. Moreover, cross-linking within the peptidoglycan occurs directly from the muramyl tetrapeptide on one peptidoglycan chain to the muramyl tetrapeptide of an adjacent peptidoglycan chain (i.e., no interpeptide bridges as in Fig. 5-7A).

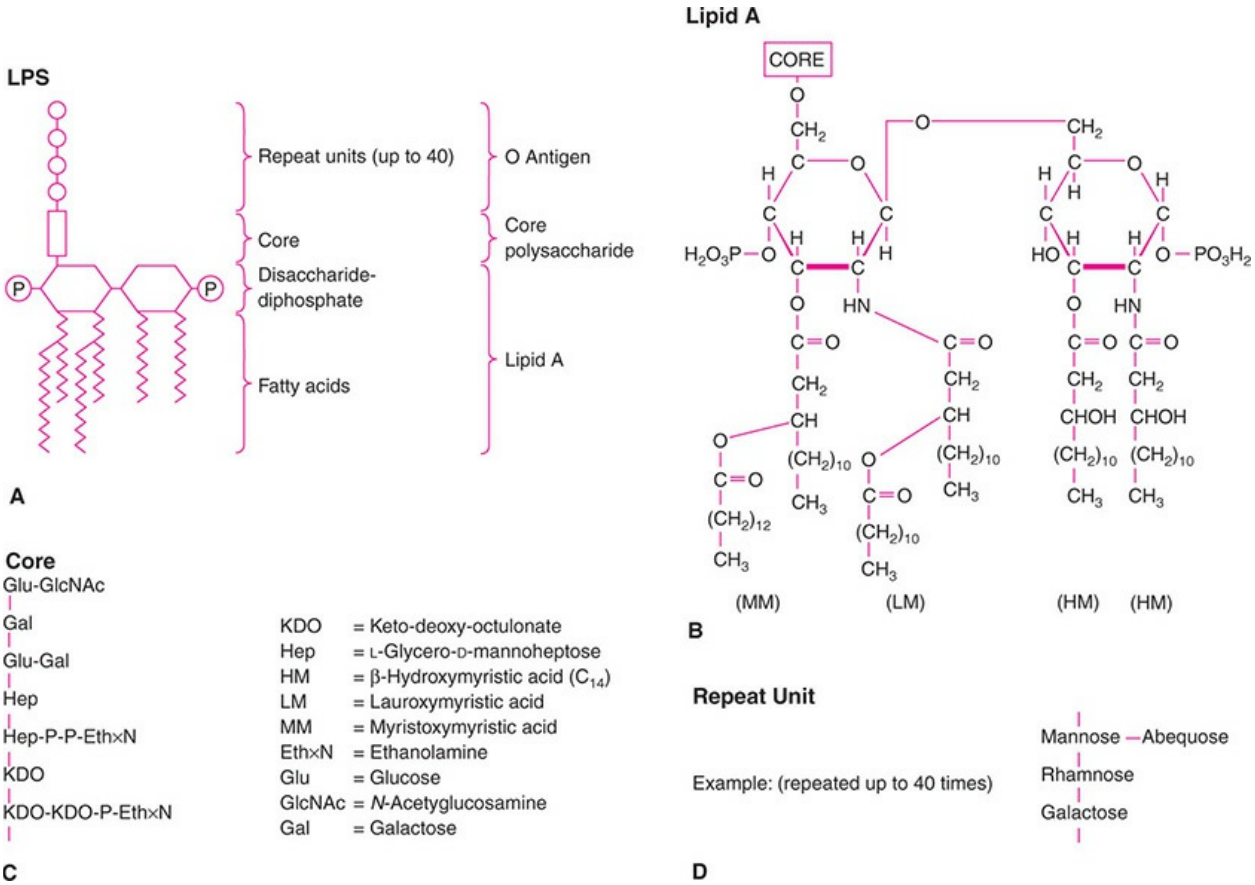


■ **FIGURE 5-9** Structure of teichoic acids of gram-positive bacteria. Ribotol teichoic acid is shown on the left; glycerol teichoic acid is shown on the right. "R" group substitutions may include ester-linked D-alanine or D-lysine or *O*-glycosidic links to glucose, galactose, or *N*-acetylglucosamine.

Outside of the thin peptidoglycan layer is the **outer membrane**. This outer membrane has a basic structure similar to the cytoplasmic membrane; that is, a phospholipid bilayer in which various other large molecules are embedded. The outer membrane is anchored to the peptidoglycan layer by small, strongly lipophilic **murein lipoproteins** that are attached covalently to the amino group of diaminopimelic acid in the peptidoglycan and extend across the periplasmic space as an α-helical structure.

The other end of this lipoprotein is noncovalently embedded in the lipid structure of the outer membrane.

A structural component that is unique to the gram-negative outer membrane is **lipopolysaccharide (LPS)** (Fig. 5-10A). LPS molecules are the major surface antigenic determinants (called **somatic** or **O antigens**) in gram-negative bacteria and are responsible for the endotoxin activity of gram-negative cells. LPS molecules are high-molecular-weight, complex glycolipids consisting of three components: a complex, hydrophobic, lipid portion called **lipid A**, a **core polysaccharide region** that links lipid A to the more external structures of the molecule and that is generally similar in structure within a given bacterial genus or species, and **O-specific (somatic antigen) polysaccharide side chains**, which are regions of variable biochemical structure that impart unique



■ **FIGURE 5-10** The lipopolysaccharide (LPS) of the gram-negative cell envelope. **A.** Segment of the polymer showing the arrangements of the major constituents. **B.** Structure of lipid A of *Salmonella typhimurium*. **C.** Polysaccharide core. **D.** Typical repeat unit (*Salmonella typhimurium*). (Redrawn from Brooks GF, Butel JS, Ornston E, et al. Jawetz,

Melnick, and Adelberg's Medical Microbiology. 19th Ed. Norwalk, CT: Appleton & Lange, 1991.)

serologic identity to gram-negative species. The lipid A moiety of the LPS is embedded in the outer leaflet of the outer membrane, with the core polysaccharide and the O-specific side chains projecting from the outer membrane surface like whiskers. Each *Salmonella* serotype, for example, has characteristic O-specific side chains that allow serologic confirmation of biochemical identifications and afford strain typing when possible foodborne *Salmonella* outbreaks are being investigated. The structure of LPS has been studied most extensively in *Salmonella* species and *E. coli*.

Lipid A is composed of a glucosamine disaccharide in which the hydroxyl groups are esterified to uncommon β-hydroxy fatty acids like β-hydroxymyristic acid (C14), myristomyristic acid, and lauromyristic acid (Fig. 5-10B). Additional fatty acids may be attached via hydroxyl groups to other unsubstituted locations on the myristic acid molecule; these additional substitutions differ among the various genera of gram-negative bacteria. Attached to the lipid A portion of the LPS is the core polysaccharide. The core polysaccharide contains two unique carbohydrates: **3-deoxy-D-mannooctulosonate** (formerly called **2-keto-3-deoxyoctonoic acid [KDO]**), an 8-carbon sugar, and **heptose**, a 7-carbon sugar. Core KDO forms covalent connections between lipid A and heptose moieties in the core polysaccharide. Additional sugars (e.g., *N*-acetylglucosamine, glucose, and galactose) may also be found in the core polysaccharide. The core polysaccharide structure is fairly conserved within a given genus, but may vary from species to species. The O-specific side chains are attached to the core polysaccharide and are responsible for the antigenic specificity of individual isolates. These side chains contain a variable number (up to about 40) of repeating oligosaccharide units comprised of three to five monosaccharides each. These antigenically specific side chains often contain unusual or uncommon carbohydrate residues, including aminohexuronic acid, 6-deoxyhexoses, and 2,6-dideoxyhexoses. The lipid A moiety appears to be the principal component responsible for the manifestations of endotoxin activity in patients with gram-negative bacterial sepsis (e.g., fever, shock, vascular collapse, and hemorrhage). Endotoxin can also activate complement and can cause disseminated intravascular coagulation. The generalized structure of the LPS of *Salmonella* species is shown in Figure 5-10A, while the structure of lipid A, the core polysaccharides, and somatic antigens are shown in Figure 5-10B, C, and D, respectively.

Dissociation of the outer membrane LPS can be partially accomplished by treatment of cell suspensions with ethylenediamine tetraacetic acid (EDTA), which chelates the divalent cations of the outer membrane. Subsequent treatment with lysozyme hydrolyzes the peptidoglycan layer of gram-negative bacteria, and the cells may be lysed. The dependence of the integrity of the outer membrane on calcium

and magnesium ions is one of the principal reasons for the inclusion of these ions in media that is used for antimicrobial susceptibility testing.

Some gram-negative bacteria (e.g., *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Bordetella pertussis*) have **lipo-oligosaccharides (LOS)** rather than LPS in their cell walls.[11,42,102] LOS contains lipid A and an oligosaccharide core containing KDO, but it does not have a long-chain polysaccharide O antigen as is found in the LPS of enteric bacteria. These molecules, like endotoxin, possess a range of biologic activity, including general toxicity, pyrogenicity, and the unique ability to induce B-cell mitogenicity and polyclonal B-cell activation. Antigenic variation seen in *N. gonorrhoeae* is partially due to high-frequency structural modulation of the LOS, which has also been shown to affect gonococcal adherence to mucosal surfaces and susceptibility to the bactericidal action of normal human serum.[77,78]

The gram-negative outer membrane also contains phospholipids and proteins. The phospholipids are similar to those found in the cytoplasmic membrane and include phosphatidyl ethanolamine and phosphatidyl glycerol. Proteins also constitute a significant portion of the outer membrane. Those that are present in greatest concentrations are called **principal or major outer membrane proteins** (**OMPs**). These proteins fall into three major groups. **Porin proteins** are proteins that form trans-outer membrane channels through which lower-molecular-weight materials (e.g., amino acids, sugars, ions) are allowed into the periplasmic space. Many of these porin proteins are composed of three identical proteins that form a "doughnut-shaped" pore. Porins also help to limit passage of many antimicrobial agents into the cell. **Transmembrane proteins** are nonporin proteins that span the outer membrane, extend through the periplasm, and are associated with the peptidoglycan layer of the cell wall. They may function in exoenzyme production and secretion, transport of specific proteins, attachment to surfaces, or in binding of antimicrobial agents to their cell-surface targets (e.g., penicillin-binding proteins). **Peripheral proteins** are responsible for transmembrane transport of molecules that are also too large for porin entry. Most of these proteins are components of substrate-specific permease systems (e.g., siderophore binding and transport of iron into the cell). Lipoproteins (see above) are the smallest of the OMPs and serve to stabilize the cell wall via covalent linkage with the peptidoglycan.

The structure of the bacterial cell wall has direct, practical importance to the microbiologist because the type of cell wall structure is largely responsible for the **Gram's stain** reaction. This differential stain divides the majority of bacteria into two groups—**the gram-positive** and the **gram-negative bacteria**. In the Gram's stain procedure, cells are (1) stained with **crystal violet**; (2) treated with i**odine** to form a crystal violet–iodine complex within the cell; (3) washed with an **organic solvent**

(acetone–alcohol); and (4) stained again with the red counterstain **safranin**. In gram-positive bacteria, the purple crystal violet–iodine complex is retained within the cell after washing with acid–alcohol because the thick peptidoglycan layer does not allow the crystal violet–iodine complex to be washed out of the cell. In gram-negative bacteria, the crystal violet–iodine complex is leached from the cell (i.e., the cells become colorless) because of disruption of the lipid-rich outer membrane by the acetone–alcohol organic solvent. These colorless cells must be counterstained in order to be seen under the light microscope; this counterstain is provided by safranin. Gram-positive bacteria appear blue-purple under the microscope, while gram-negative bacteria are stained red by the safranin counterstain.

Some gram-negative bacteria do not have the complex cell wall structure described above. Organisms in the *Cytophaga–Flexibacter–Flavobacterium* group have outer membranes containing ornithine-aminolipids and sulfonolipids as their principal components, along with large amounts of branched-chain fatty acids with odd-numbered carbon side chains. Organisms in *Archaea* lack a peptidoglycan structure and have instead a "pseudopeptidoglycan" composed of proteins, glycerol esters, and *N*-acetyltalosaminuronic acid (i.e., *meso*-DAP and *N*-acetylmuramic acid are absent). Some *Archaea* lack even this rudimentary peptidoglycan, having instead a thin cell wall consisting of proteins or sulfated polysaccharides.

"Acid-Fast" Bacterial Cell Walls. A modification of the gram-positive cell wall is seen in organisms belonging to the genera *Mycobacterium*, *Nocardia,* and *Corynebacterium*. In these organisms, lipids account for as much as 60% of the dry weight of the cell wall. These organisms contain molecules called **mycolic acids** in their cell walls. Mycolic acids are large, α-substituted, β-hydroxy fatty acids that occur as esters attached to cell wall polysaccharides. Mycolic acids vary in the number of carbon atoms; those with 30 carbons (C30) are found among the corynebacteria (corynemycolenic acids), those with C50 are found in *Nocardia* species (nocardic acids), and those with C90 or more constitute the mycolic acids found in the genus *Mycobacterium*. In *Mycobacterium tuberculosis*, the unique mycolic acid **6,6 ′-dimycolyltrehalose** is known as **cord factor** (Fig. 5-11A). This molecule is associated with virulence of *M. tuberculosis* and has a wide range of biologic activities, including cell membrane cytotoxicity, inhibition of polymorphonuclear cell migration, induction of granuloma formation, adjuvanticity, antitumor activity, and ability to activate the alternative complement pathway.

The cell membrane of mycobacteria is similar to other bacterial unit membranes except that it contains **phosphatidylinositol mannosides** and **lipoarabinomannan**. External to the membrane is the peptidoglycan layer which is composed of *N*-acetylglucosamine in β-1,4 linkage to *N*-glycolylmuramic acid. The murein layer is cross-linked by tetrapeptide bridges containing L-alanine, D-glutamate, and *meso*-DAP residues. Some of the *N*-glycolylmuramic acid residues are linked by phosphodiester

bonds to an overlying layer of branched-chain polysaccharide macromolecules called **arabinogalactans** (containing arabinose and galactose moieties). The distal arabinose residues of this layer are linked to the overlying mycolic acids. The hydrocarbon chains of the mycolic acids are intercalated with those of numerous other wall-associated lipids and glycolipids. These cell wall-associated lipids include those with medium (C24 to C36) and short (C12 to C20) fatty acyl groups. These wall-associated lipids include trehalose sulfolipids (Fig. 5-11B). The trehalose sulfolipids, typified by the principal sulfolipid of *M. tuberculosis* **2,3,6,6′-tetraacyltrehalose-2′-sulfate**, are associated with mycobacterial virulence

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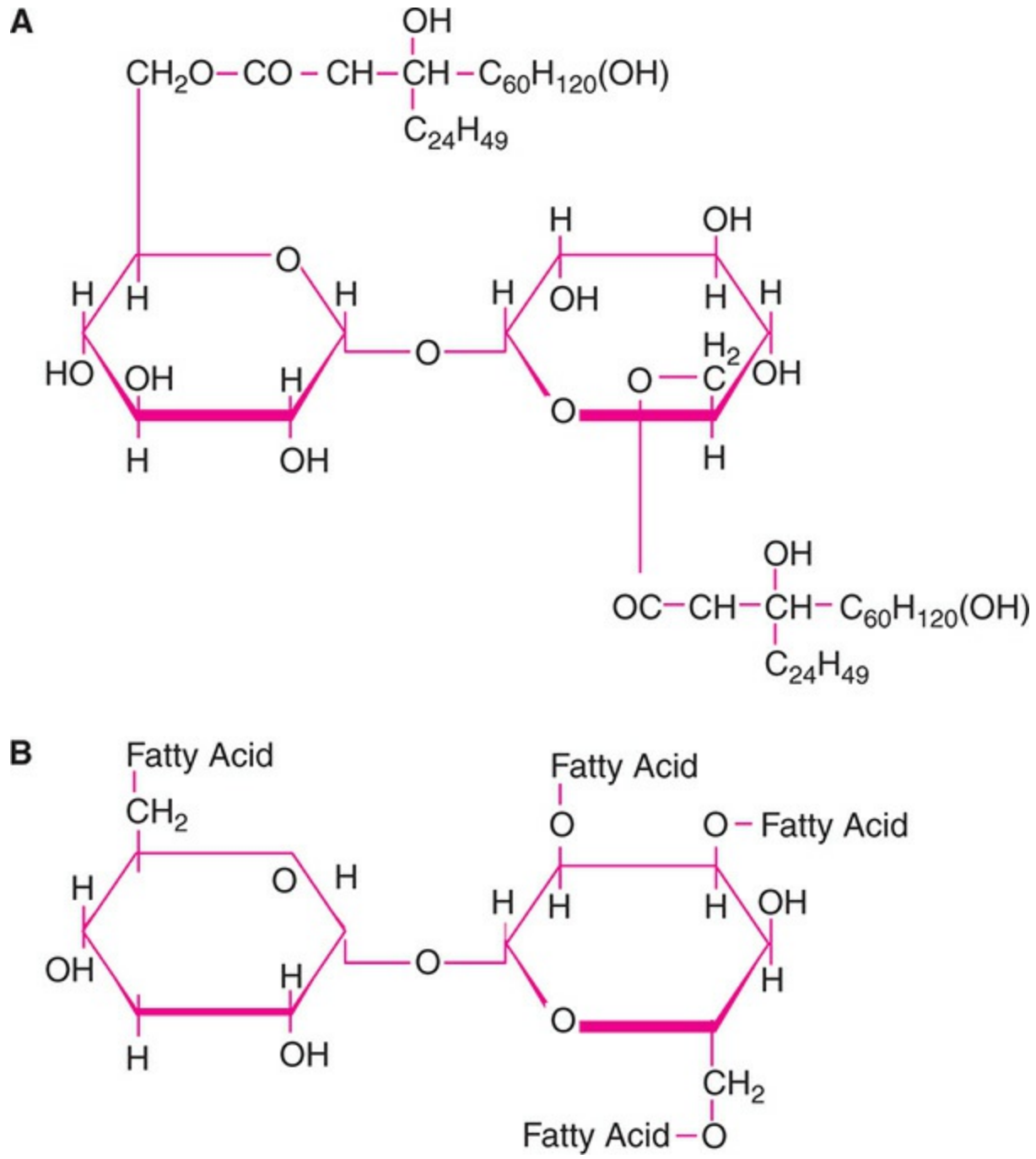

■ **FIGURE 5-11** Molecular structure of specialized lipids found in the cell wall of *Mycobacterium tuberculosis*. **A.** Molecular structure of cord factor (6,6′-dimycolyltrehalose) produced by *Mycobacterium tuberculosis*. **B.** Molecular structure of the principal sulfolipid (2,3,6,6′-tetraacyltrehalose-2′-sulfate) of *M. tuberculosis*.

in that these molecules may act to prevent phagosome–lysozyme fusion following phagocytosis of the mycobacterial cells, thereby allowing the organisms to survive as

facultative intracellular parasites. Protruding from the plasma membrane through the peptidoglycan, arabinogalactan, and mycolic acid layers of the wall are substituted phospholipids (phosphatidylinositol mannosides) and lipopolysaccharides (lipoarabinomannans) that are attached to the outer leaflet of the mycobacterial cell membrane. These molecules provide a noncovalent link between the cell membrane and the cell wall. Proteins that are embedded in the mycobacterial cell wall are involved in biosynthesis and construction of the cell wall polymers and some apparently also function as porins.

Organisms that are acid-fast are stained red with the basic dye carbol fuchsin and are resistant to decolorization with acid–alcohol. Because of the hydrophobicity of the mycobacterial cell wall, penetration of the dye into the cell is enhanced by heat treatment (i.e., the Ziehl–Neelsen method) or incorporation of detergent into the dye (i.e., the Kinyoun method). Resistance to decolorization by acid–alcohol (i.e., "acid-fastness") is associated with the mycolic acid–arabinogalactan moieties that constitute the bulk of cell wall materials external to the peptidoglycan layer. The soluble lipids contribute to but do not determine the acid-fast properties of mycobacterial cells, since extraction of these lipids diminishes but does not destroy the ability of the cells to retain carbol fuchsin (i.e., their acid-fastness). Mechanical disruption of the cell wall and extraction of the cell wall lipids with ethanolic alkalis that remove both free and esterified lipids destroys the acid-fast properties of these organisms, indicating that the total lipid content of the cell wall is responsible for the acid-fast staining property.

Bacterial Endospores

Endospores are spherical or oval structures formed within certain bacterial species that represent a dormant or "resting" stage in the growth cycle of the organism. Among clinically significant bacteria, endospores are formed by aerobic gram-positive bacilli belonging to genus *Bacillus* and other related genera, and by the anaerobic gram-positive bacilli in the genus *Clostridium.* In these genera, endospores are formed in response to nutritional deprivation within the vegetative bacterial cell. They are highly resistant to the injurious effects of heat, drying, pressure, and many chemical disinfectants. Sterilization temperatures (i.e., 120°C for 15 to 20 minutes) are required to kill spores. The heat resistance of bacterial endospores is believed to be due to reduced amounts of water in the core of the spore itself. The size, shape, and location of incipient endospores in stationary-phase cells of *Clostridium*, *Bacillus,* and related species are helpful for characterization and identification of certain species within these two genera (see Chapters 14 and 16). The endospores may be spherical, subspherical or oval in shape, they may differ in their location within the cell (i.e., central, terminal, or subterminal), and they may or may not swell the cell. Endospores generally do not stain with routine staining methods like the Gram's stain, and appear as refractile, nonstaining bodies in smears.

Under the stimulus of certain environmental conditions such as the exhaustion of nutrients (i.e., glucose, nitrogen, or phosphate) or exposure to suboptimal temperatures or redox potentials, the nuclear material divides into two nucleoids, and one becomes separated from the other by a membranous septum. The septum then grows together and the spore core becomes engulfed in a double membrane. Between the two

membranes, a cortex layer is deposited by the membranes. This cortex consists primarily of peptidoglycan material. The cortex layer thickens and accumulates calcium ions because of the chelating activity of a unique molecule called **dipicolinic acid**. The core becomes protected by the high concentration of calcium ions tightly cross-linking the peptidoglycan material and all available water in the spore is expelled. Several layers of the spore coat (a keratin-like substance that is rich in disulfide bonds) are laid down, and the endospore is liberated on the death and lysis of the mother vegetative cell. Endospores may remain viable for prolonged periods. When the spore is placed in a favorable environment in the presence of particular stimuli (e.g., the presence of particular amino acids or carbohydrates and water), spore outgrowth occurs. Upon this stimulus, enzymes are activated which degrade the spore cortex and release the peptidoglycan material, calcium ions, and dipicolinic acid. RNA synthesis begins, followed by protein synthesis and, eventually, DNA synthesis. A new vegetative cell results.

Bacterial Surface Structures

Capsules. Some bacteria possess a capsule external to the outer layer of the cell wall. The capsule may be thick or thin, and may be closely or loosely associated with the external aspect of the cell wall. Loosely associated capsular material may also be referred to as a **slime layer** or **glycocalyx**. Capsular material is usually polysaccharide in nature; these polysaccharides may be polymers of single monosaccharides (glucans, dextrans, levans) or heteropolysaccharides containing both hexose and pentose sugars, plus ribitol, glycerol, or other sugar alcohols. Phosphates are also frequently present. In most cases, the capsule is synthesized at the level of the cell membrane; components are synthesized and exported out of the cell by an isoprenoid lipid "carrier" system, in which the components become attached to "primer" capsular material already present on the surface of the cell. In some cases, such as the glucan capsule of *Streptococcus mutans*, the capsule is synthesized by a class of extracellular and cell wall-associated enzymes called **glucosyltransferases**.[9] The action of these enzymes on dietary sucrose creates a branched, **insoluble glucan** matrix that specifically interacts with the tooth surface and with receptors on the *S. mutans* cell. Subsequent formation of acids from dietary sucrose and from intracellular glycogen

stores in *S. mutans* and other organisms leads to formation of dental caries.

The bacterial capsule serves several functions. It protects the cell from desiccation and from toxic materials in the environment (e.g., heavy metal ions, free radicals) and promotes the concentration of nutrients at the bacterial cell surface because of its polyanionic nature. In addition, the capsule also plays a role in the adherence of bacteria to cells and mucosal surfaces. This adherence is necessary for many organisms to establish infections in appropriate hosts (discussed below). Some bacterial capsules function to protect cells from phagocytosis (in the absence of anticapsular antibodies) by polymorphonuclear leukocytes. Capsular material is usually antigenic, and the serologic detection of the capsule forms the basis of the **Quellung test** (see Chapter 12), which can be used to identify and/or subtype several important human pathogenic bacteria, including *S. pneumoniae*, *H. influenzae* type b, *Klebsiella pneumoniae*, and *Neisseria meningitidis* serogroups. The capsular material of many microorganisms is synthesized in abundance and is shed into the surrounding fluid both in vivo and in vitro.

In recent years, the capsular structure of *Staphylococcus aureus* has been an area of interest to both basic scientists and clinical microbiologists. More than 90% of *S. aureus* strains isolated in the clinical laboratory are encapsulated, and these capsules have been divided serologically into 11 types.[63] The types that have been characterized thus far (types 1, 2, 5, and 8) are complex *N*- and *O*-acetylated carbohydrates in β-1,4 and β-1,3 linkages. Capsular types 5 and 8 predominate among clinical isolates, and a predominant number of oxacillin-resistant strains express the type 5 capsular polysaccharide.[2,39] Of interest to the bench microbiologist is that capsular type 5 *S. aureus* strains also may not be reliably identified by latex agglutination coagulase tests used in clinical laboratories.[38] For this reason, latex beads coated with antibodies to type 5 and 8 capsular polysaccharides are now included in some formulations of the coagulase latex agglutination test.[40]

In some bacterial species, such as *Bacillus* and related species, the capsule is polypeptide in nature. *B. anthracis*, the cause of anthrax, is encapsulated in both infected tissue and when grown on media containing bicarbonate or in a >5% CO_2environment. Both capsule formation and toxin production are specifically "turned on" by these cultural conditions, which, interestingly, reflect the same bicarbonate and CO_2 concentrations found in tissues infected with *B. anthracis*.[91] The *B. anthracis* capsule is composed of linked, β-peptide chains of D-glutamic acid ranging from 50 to 100 residues per chain.[91] The presence of the capsule renders *B. anthracis* resistant to phagocytosis.[54]

Flagella. Bacterial flagella are long, filamentous appendages that arise at the level of the cytoplasmic membrane and extend through the cell wall into the surrounding

medium. They are responsible for cellular motility. Flagella are usually found in rod-shaped, gram-negative bacteria, although motile, gram-positive rods (e.g., *Listeria* species) and cocci (some *Enterococcus* species, *Vagococcus* species) are also found. Flagella differ in their numbers and their arrangements on cells. Bacteria with a single polar flagellum are termed **monotrichous**; those with two or more flagella originating at one pole or point are **lophotrichous;** those with a single flagellum located at two different points or poles are called **amphitrichous;** and those with two or more (a tuft) flagellae at two points or poles of the cell are called **amphi-lophotrichous.** Organisms that have flagella arising over the entire cell surface are termed **peritrichous**.

In gram-negative bacteria, flagella have been demonstrated to have a complex structure consisting of three parts: the **filament**, the **hook**, and the **basal body** (Fig. 5-12). The flagellar filament is 13 to 17 nm in diameter and is variable in length. The filament is composed of parallel subfibrils of the 30- to 40-kDa subunit protein **flagellin,** which interact to form a hollow cylinder. The filament is semirigid and forms a left-handed helix as it exits the cell. Flagellin has the capacity to self-assemble. Monomers of the protein are synthesized and passed through the lumen of the cylinder. At the growing tip of the flagellum helix, the monomer undergoes a conformational change and becomes added to the

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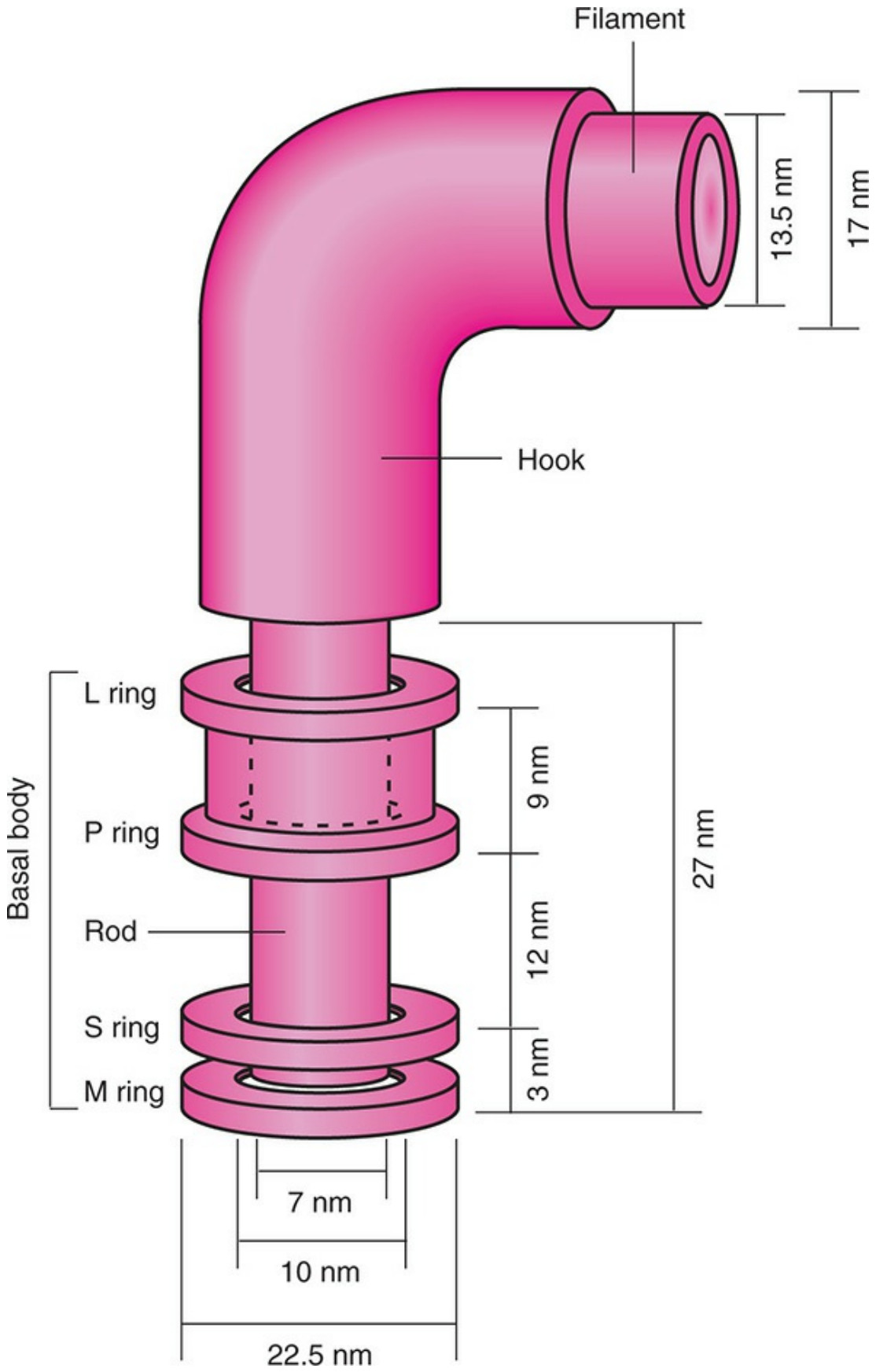

Filament
13.5 nm
17 nm
Hook
L ring
P ring
Rod
S ring
M ring
Basal body
9 nm
12 nm
3 nm
27 nm
7 nm
10 nm
22.5 nm

■ **FIGURE 5-12** Ultrastructure of a flagellum from a gram-negative bacterium.

distal end of the flagellum. The hook is composed of another distinct protein and acts as a "sleeve" from which the flagellar filament emerges. The hook permits the transmission of a rotary motion from the basal body to the filament. The **basal body** is composed of complex rings connected by a rod-shaped structure. The **M, S, P, and L rings** are anchored in the membrane, the periplasmic space, the peptidoglycan, and the lipopolysaccharide outer membrane, respectively. At least 10 proteins comprise the outer ring structure in gram-negative bacteria. The ring structure attached to the cell membrane rotates as a part of an energy-dependent reaction, causing the rigid flagellar helix to turn like a propeller. The energy for this reaction is derived by the passage of protons from the outside into the cytoplasm via the basal body. The outer rings (L and P rings) evidently function as bearings, minimizing friction and leakage of materials from the cell at the points of flagellar insertion. In gram-positive bacteria, the flagellar structure is less complex and is composed of two rings. One ring structure anchors the flagellum to the plasma membrane, while the second ring structure is embedded in the thick peptidoglycan layer.

Several species of flagellated organisms are also able to alter the expressed antigenic type of flagella that they produce; this process is known as **phase variation**. This refers to the ability of organisms to express two types of flagella alternately. Phase variation occurs by the differential expression of chromosomal genes that code for two variously structured flagellin proteins. This phenomenon was first recognized in enteric gram-negative bacteria such as *Salmonella* species, but also occurs in other species, such as *N. gonorrhoeae* (see below). Flagellar antigens in gram-negative bacilli are referred to as H antigens, from the German word "hauch," meaning "breath." Some organisms have slightly modified flagellar structures. *Vibrio* species have the typical flagellar morphology, but the flagellum is encased in a sheath derived from the outer membrane of the cell wall. In the spirochetes, the flagellum does not protrude into the environment, but lies within a sheath that is exterior to the protoplasmic cylinder of the cell body of the organism. This endoflagellum or **axial filament** arises from one pole of the cell and wraps around the cell body internal to the sheath.

Other Locomotor Organelles. Some gram-negative bacteria exhibit what is known as "gliding motility." This type of motility is most apparent when organisms are in contact with a surface. In clinical microbiology, gliding motility is seen among the *Capnocytophaga* species, which are part of the normal microbiota of the human oropharynx and are occasionally isolated from infectious processes (see Chapter 9). Studies with *Flavobacterium johnsonii* and other organisms in the *Cytophaga–Flavobacterium–Bacteroides* group have suggested that proteins or glycoproteins located within the outer membrane adhere to the substratum and that

other proteins within the cell membrane harvest proton-motive forces that cause the surface proteins to move along "tracks" within the peptidoglycan layer.[56] Other studies have also shown the presence of unique sulfonolipids in the outer membrane of gliding bacteria that are absent in nonmotile mutants of the same species. Using spontaneous, chemically induced, and transposon-induced nonmotile mutants, several unlinked genes and operons involved with gliding motility have been identified.[47]

Fimbriae (Pili). Fimbriae or pili are smaller appendages found on the surface of many gram-negative and some gram-positive bacteria. Although the terms *pili* and *fimbriae* have been used interchangeably, the latter term is now used to describe any nonflagellar hairlike appendages, while the former term is used to denote the fimbriae of gram-negative bacteria that function specifically in the transfer of DNA from one cell to another during the process of conjugation (i.e., sex pili). Fimbriae are composed of a protein called **fimbrillin** (or **pilin**), are 3 to 25 nm in diameter, and are 10 to 20 mm in length. Fimbrillin produced in gram-negative bacteria is a subunit protein of 17 to 20 kDa molecular weight. The proteins form hollow tubes that originate in the cell membrane, but that lack the basal body and hook structures of flagella. Fimbrial subunit proteins are added at the base of the structure, rather than at the tip as is seen with flagellar synthesis. Sex pili are involved in specific pair formation for exchange of genetic material during conjugation and also serve as attachment sites for bacteriophages.

Fimbriae also function as cellular organelles for attachment to cells and/or mucosal surfaces; fimbriae that serve

this attachment function are often referred to as **adhesins.** Most adhesins display lectin-like binding to terminal carbohydrate residues (e.g., mannose)—for instance, the adherence of enteric bacteria to mucosal surfaces that is mediated by type 1 (type-specific) or common pili that can be inhibited by preincubation of the bacteria with mannose. Mannose attaches to the terminal portion of the adhesin and blocks adherence; therefore, type 1 pili are termed *mannose-sensitive*. Adhesins that are not affected by mannose are termed *mannose-resistant* or *type 2 pili*. Differing lectin-like specificities are partly responsible for the tissue tropisms observed with a variety of bacterial species.

The role of fimbriae as virulence factors in gram-negative bacteria has been studied extensively in *N. gonorrhoeae* and *N. meningitidis*.[43,62] These pathogenic *Neisseria* species produce antigenically and structurally similar fimbriae that are composed of 16.5 to 21.5 kDa fimbrial subunit proteins. Virulent strains of *N. gonorrhoeae* express these surface fimbriae and are able to adhere avidly to mucosal cells in the genital

tract.[95] Fimbriated organisms are also responsible for the domed type 1 and type 2 colonies produced by freshly isolated gonococci on agar media. The loss of fimbriae on repeated subculture in vitro renders these organisms unable to initiate urogenital infection because of lack of mucosal adherence. Colonies composed of nonpiliated gonococci are larger and flatter. Gonococci possess a large number of genes that code for structurally and antigenically distinct fimbrial proteins, and these proteins undergo both **phase variation** and **antigenic variation** (see Chapter 11). In phase variation between the piliated and the nonpiliated state, the *pil* genes (fimbrial structural genes) are either not expressed or the fimbrial proteins cannot be assembled into functional fimbriae. In antigenic variation, new fimbrial types may emerge because of recombinational events among the 20 or so fimbrial genes present in the bacterial genome. Because of the ability of a single gonococcal strain to produce multiple, antigenically distinct fimbrial antigens, the use of fimbriae as candidate antigens for antigonococcal vaccines has been largely unsuccessful.

Among the gram-positive bacteria, only a limited number of species express cell-surface fimbriae, including some streptococci, corynebacteria, and *Actinomyces* species. *Actinomyces viscosus* and *Actinomyces naeslundii*, facultative gram-positive bacilli found in the oral cavity, express two types of fimbriae. Type 1 fimbriae mediate bacterial adherence to tooth surfaces via interaction with salivary acidic proline-rich proteins, while type 2 fimbriae mediate bacterial adherence to oral streptococci and various mammalian cell types, including polymorphonuclear leukocytes and erythrocytes.[21,74] These type 2 fimbriae bind to either galactose or *N*-acetylgalactosamine residues on the surface of coaggregating oral streptococci or with the oligosaccharides of mammalian cell membrane glycoproteins.[74] Unlike the fimbriae from gram-negative bacteria, fimbriae of *Actinomyces* species are covalently linked to the cell wall peptidoglycan layer.[100] The ability of oral *Actinomyces* species to adhere to buccal mucosal cells and to coaggregate with cariogenic oral streptococci facilitates biofilm formation and the initiation of dental plaque. Fimbrial coaggregates of *Actinomyces* and oral streptococci have been shown to resist phagocytosis and killing by polymorphonuclear cells, and binding of *Actinomyces* to PMNs results in the release of mediators of inflammation. Fimbria clearly play a key role in the ability of these periodontal pathogens to colonize and initiate infection.

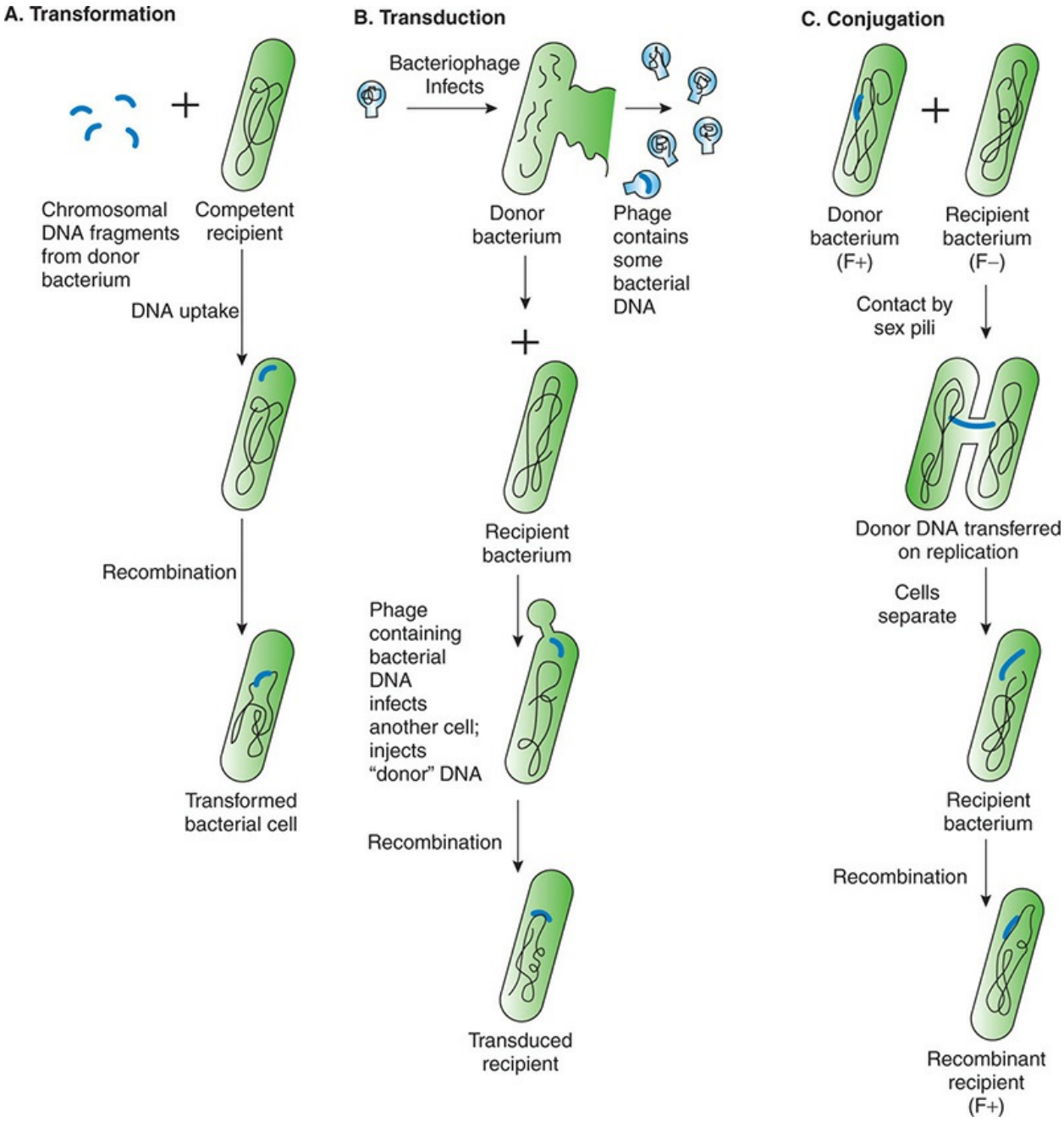
Genetic Exchange and Recombination in Bacteria

Bacterial replication occurs by binary fission, an asexual process that does not involve recombinational events and results in the generation of two daughter cells that are identical to the parent cell. Several groups of bacteria, however, do have the ability to undergo genetic exchange and recombination with other organisms. Genetic exchange between bacteria occurs by one of three general mechanisms: transformation,

transduction, and conjugation (Fig. 5-13).

Transformation involves the uptake of free DNA from the surrounding environment (Fig. 5-13A). Cells that are physiologically capable of taking up and incorporating free DNA into their genomes are termed *competent*. Competence is usually a transient state that occurs toward the late exponential phase of growth, although some organisms may be competent at all times. In competent gram-positive cells (e.g., *Bacillus subtilis*, *S. pneumoniae*), small pieces of double-stranded DNA become bound to the cell via a cell-surface receptor that is expressed during the competent period. As the DNA enters the cell, one strand is hydrolyzed by a surface-bound nuclease. Recombinational events between the single-stranded DNA and homologous regions of the bacterial chromosome result in integration of the transformed DNA into the bacterial genome. If homologous regions for the transforming DNA are lacking, the DNA strand does not integrate, genes on the particular DNA strand are not expressed, and the single-stranded DNA is degraded by endogenous restriction endonucleases. *H. influenzae*, a transformation-competent gram-negative bacterium, also possess receptors for DNA on the cell surface; DNA binding occurs via the recognition of a 10- to 14-base-pair surface-expressed nucleotide sequence that allows DNA only from closely related species to bind and enter the competent cell. Double-stranded DNA then enters the cell, but only one strand participates in the recombinational events that incorporate the transforming DNA into the genome of the recipient. Cells that normally do not express competence for genetic transformation can be made permeable to extracellular DNA ("artificially competent") by treatment with $CaCl_2$ or other salt solutions at 0°C or by **electroporation,** in which cell suspensions are exposed to an electric current that induces DNA entrance into bacterial cells.

Transduction refers to the exchange of genetic information via bacteriophages (Fig. 5-13B). Bacteriophages (or simply "phages") are viruses that infect bacteria. Some bacteriophages are **lytic;** that is, after infecting the bacterial cell, the bacteriophage regulatory genes "take over" the cellular biosynthetic machinery, resulting in the expression of phage structural genes and the production of new phage particles that are released on lysis and death of the host bacterium. With temperate bacteriophages, the genetic material of the bacteriophage becomes incorporated into the host-cell DNA as a "prophage" and replicates along with the bacterial chromosome. Such bacteriophages are also termed *lysogenic bacteriophages*, and the bacterial cell infected with this phage is



■ **FIGURE 5-13** Mechanisms of gene transfer in bacteria. Microorganisms may exchange genetic material by any of three processes: transformation (**A**), transduction via bacteriophage (**B**), and conjugation (**C**).

said to be **lysogenized.** On induction by exposure to certain chemicals (e.g., mitomycin C) or to ultraviolet irradiation, a lysogenic bacteriophage may be induced to begin production of new phages (i.e., the phage becomes "lytic"). Excision of the bacteriophage DNA from the bacterial cell genome results in some bacteriophages

containing not only "phage-specific" genes, but also host-cell genes that were located adjacent to the site of phage DNA integration in the bacterial chromosome.

The transfer of genetic information during transduction may be generalized or specialized. **Generalized transduction** refers to the accidental, random packaging of host-cell DNA into the capsid or "head" of the phage particle. Release of the mature phage particle on cell lysis and subsequent infection of another bacterial cell results in the introduction of "donor DNA" from the original host bacterium into the "recipient." Recombination of the transduced DNA with a homologous region on the chromosome of the recipient cell results in integration and subsequent expression of the transduced genes. Generalized transduction occurs with a frequency of 1 transducing phage out of 10^5 to 10^8 phage particles produced on induction from the lysogenized state, and about 1% to 2% of the total length of the host-cell genome may be transferred by this mechanism. **Specialized transduction** refers to the packaging of specific host-cell genes into the transducing prophage. This type of transduction occurs with temperate phages that have specific chromosomal integration sites, such as bacteriophage λ. Only host-cell genes that flank the integrated phage genome have the opportunity to become incorporated into the bacteriophage genome on induction from the lysogenic state. One specialized transducing phage is produced out of 10^5 to 10^6 new phage particles following induction.

Conjugation is the only mechanism of genetic exchange between bacteria that requires cell-to-cell contact

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(Fig. 5-13C). Gram-negative bacteria that are able to participate in conjugation possess a plasmid called the **F plasmid** ("fertility plasmid"). This plasmid contains an operon of genes called the *tra* region that codes for the sex pilus (or conjugative pilus) and other molecules involved in conjugation.[34] The specialized pilus functions as a vehicle for establishing contact with another bacterial cell. Once contact is established, the sex pilus apparently is retracted, thereby drawing the other cell closer. The pilus may also act as a "tube" through which DNA is passed during the conjugative process. Cells that possess F plasmids are termed F^+, while cells that lack this plasmid are termed F^-. Once contact of an F^+ cell with an F^- cell is established by the sex pilus, the circular F plasmid begins to be replicated. During this process, one of the single strands of the plasmid DNA is transferred into the recipient cell. This single strand is replicated as it enters the recipient cell, and the end result are two cells that contain complete conjugative plasmids (i.e., both become F^+ cells).

In some organisms, the F plasmid becomes integrated into the host-cell genome at specific integration sites in which homologous nucleotide sequences are present.

Establishment of a connection via the F pilus and subsequent conjugation results in the transfer of some F-plasmid genes plus genetic material from the host cell that is adjacent to the integration site of the F plasmid. Cells possessing an integrated F plasmid are termed *Hfr cells* (high-frequency recombinations). Mating between Hfr cells and F^- cells results in transfer of part of the F genome plus some host-cell genes from the donor. Recipient F^- cells usually remain F^- following conjugation since only part of the F plasmid from the donor Hfr cell is transferred to the recipient cell during the conjugative process. Therefore, these recipient cells will not possess the complete operon that is necessary for subsequent conjugation to occur. Recombination between the genetic material from the donor cell and homologous regions in the F^- recipient enables the donor DNA to become expressed in the recipient cell. The donor cell remains Hfr, since the host-cell chromosome (containing the integrated F plasmid) is replicated during the transfer of the genomic ssDNA from the Hfr to the F^- cell.

Among the gram-positive bacteria, *Enterococcus faecalis* is also able to exchange plasmids via a conjugative process. However, gene transfer is not accomplished via a pilus, but by a **coaggregation** of the organisms in response to **pheromones** produced by the donor bacterium.[65] When exposed to plasmid-free cells (or culture filtrates from plasmid-free cells), plasmid containing cells produce plasmid-encoded pheromones called **aggregation substances.** These aggregation substances are small (seven to eight amino acids) peptides. The pheromones bind to sites that are present on both recipient and donor cells, causing bacterial aggregation. Aggregation results in the establishment of the cell-to-cell connections necessary for plasmid mobilization to occur. Interestingly, enterococcal cells that contain like plasmids and similar aggregation substances respond only to pheromones from cells that have different plasmids and, therefore, produce different aggregation substances.

In addition to the mechanisms of gene transfer just described, other genetic elements also may participate in recombinational events that affect the characteristics and pathogenicity of microorganisms. **Transposable genetic elements** are pieces of DNA that can insert into different, unrelated sites on a bacterial chromosome or plasmid. Because these insertions occur randomly, mutations may be generated because of the disruption of gene sequence continuity. They have been described in *E. coli* and a wide variety of other bacteria. Unlike the mechanisms described above, regions of nucleic acid sequence homology are not required for recombination with these genetic elements to occur. Transposable genetic elements fall into two groups: insertion sequences and transposons. **Insertion sequences** (ISs) are usually small pieces of DNA that carry genes coding only for their own transposition; expression of these genes is not recognized and the insertion is usually phenotypically "silent." ISs have two major characteristics: they are small relative to other transposable elements (generally around 700 to 2,500 base-pairs in length) and only code for proteins implicated in the transposition activity (they are thus different from other transposons,

which also carry accessory genes such as antibiotic resistance genes). These proteins are usually the transposase which catalyzes the enzymatic reaction allowing the IS to move, and also one regulatory protein which either stimulates or inhibits the transposition activity. **Transposons** are larger and encode for at least one function that is recognizable as a phenotypic alteration, such as acquisition of antimicrobial resistance. Transposons described in enteric gram-negative bacteria contain genes for resistance to antimicrobial agents (e.g., aminoglycosides, tetracycline, chloramphenicol) and, among clinically significant gram-positive bacteria, transposons carrying resistance genes for macrolides and tetracycline have been described in group A β-hemolytic streptococci.[14,33]

Requirements for Bacterial Growth and Metabolism

Carbon. Bacteria can be divided into two large groups on the basis of their carbon requirement—the **lithotrophic** (or **autotrophic**) bacteria and the **organotrophic** (or **heterotrophic**) bacteria. The lithotrophic bacteria can use carbon dioxide as the sole source of carbon and synthesize from it the carbon "skeletons" for all their organic metabolites. They require only water, inorganic salts, and CO_2 for growth, and their energy is derived either from light (photolithotrophic bacteria) or from the oxidation of one or more inorganic substances (chemolithotrophic bacteria). Organotrophic bacteria are unable to use CO_2 as their sole source of carbon but require it in an organic form, such as glucose. For these heterotrophic bacteria, a portion of the organic compound that serves as an energy source is also used for the synthesis of organic compounds required by the organism. A wide variety of other substances can also be used as exclusive or partial sources of carbon by different bacterial species. Among the most versatile bacteria are *Pseudomonas* species, some of which can use more than 100 different organic compounds as their sole source of carbon and energy. The relationships among energy sources, carbon sources, and electron donors for generation of energy are summarized in Table 5-2.

Carbon Dioxide. Some bacteria are able to use atmospheric carbon dioxide as a principal source of carbon for biosynthetic reactions. The energy for catalyzing this utilization may come from light energy (**photolithotrophic bacteria**) or from oxidation of inorganic molecules (**chemolithotrophic**

TABLE 5-2 Energy and Carbon Sources of Bacteria

Type/Examples	Energy Source(s)	Carbon Source(s)	Electron Donors
Photolithotrophs Green sulfur bacteria Purple sulfur bacteria	Light	CO_2	Inorganic compounds (H_2S, S)
Photoorgano-trophs Purple nonsulfur bacteria	Light	Organic compounds (and CO_2)	Organic compounds
Chemolithotrophs Hydrogen-, sulfur, and denitrifying bacteria	Oxidation–reduction reactions	CO_2	Inorganic compounds (H_2, S, H_2S, Fe, NH_3)
Chemoorgano-trophs	Oxidation–reduction reactions	Organic compounds	Organic compounds (glucose and other carbohydrates)

bacteria). Organisms that require an organic source of carbon also require some CO_2 for certain macromolecular synthetic pathways, such as fatty acid biosynthesis. Carbon dioxide for these reactions is usually obtained from the breakdown of organic substrates occurring at the same time as the biosynthetic reactions.

Oxygen. The oxygen requirement of a particular bacterium reflects the mechanism used for energy derivation. On the basis of their oxygen requirements, bacteria may be divided into five groups. **Obligate anaerobes** grow only under condition of high reducing intensity and for which oxygen is toxic. **Aerotolerant anaerobes** are anaerobic bacteria that are not killed by exposure to oxygen. **Facultative anaerobes** are capable of growth under both aerobic and anaerobic conditions. **Obligate aerobes** have an absolute requirement for oxygen in order to grow. **Microaerophilic organisms** grow the best under lower oxygen tension; higher oxygen tensions may be

inhibitory. In obligate and facultative aerobes, the assimilation of glucose results in the terminal generation of the free radical superoxide (O_2^-). The superoxide is reduced by the enzyme superoxide dismutase to oxygen gas (O_2) and hydrogen peroxide (H_2O_2). Subsequently, the toxic hydrogen peroxide generated in this reaction is converted to water and oxygen gas by the enzyme catalase, which is found in aerobic and facultative bacteria, or by various peroxidases, which are found in several aerotolerant anaerobes.

Nitrogen. The nitrogen atoms of important biomolecules (i.e., amino acids, purines, pyrimidines) come from ammonium ions (NH_4^+). The generation of ammonium ions starts with the reduction of atmospheric N_2 to NH_4^+ (ammonium ion or ammonia, NH_3). NH_4^+ is then assimilated into more complex macromolecules by way of the key compounds **glutamate** and **glutamine**. Certain species of bacteria (*Rhizobium* species, *Azotobacter* species) and blue-green algae are able to achieve this conversion or "fix" atmospheric N_2 into a more readily usable organic form. Because of the strength of the triple bonds in N_2, nitrogen fixation requires cellular energy in the form of ATP and a powerful reductant. The process is catalyzed by a complex multienzyme system called the nitrogenase complex. In most nitrogen-fixing organisms, reduced ferredoxin is the source of electrons:

$$N_2 + 6e^- + 12\ ATP + 12\ H_2O \rightarrow 2\ NH_4^+ + 12\ ADP + 12\ Pi + 4H^+$$

The ability to fix nitrogen is accomplished primarily by the soil-dwelling bacteria mentioned above. However, some bacterial species that are involved in human disease, such as *K. pneumoniae* and certain environmental clostridia (e.g., *Clostridium pasteurianicum*) are also able to fix atmospheric nitrogen. Ammonium ions may also be generated by nitrate reduction. This is accomplished by two distinct physiologic mechanisms.**Assimilatory nitrate reduction** is a process in which nitrate is reduced to nitrite and hydroxylamine, which are then converted to ammonia for assimilation. **Dissimilatory nitrate reduction** is when nitrate serves as an alternative electron acceptor to oxygen (anaerobic respiration), with NO_2 or N_2 being the usual products. Nitrate assimilation is quite widespread in microorganisms and requires both nitrate and nitrite reductases, while dissimilatory nitrate reduction occurs only in anaerobic bacteria and facultative anaerobic bacteria growing at low oxygen tension (i.e., in a broth, for example). Ammonia generated by these mechanisms becomes incorporated into organic molecules by the action of the enzymes **glutamate dehydrogenase**, **glutamine synthetase,** and **glutamic acid synthase** (Fig. 5-14). The final products of these reactions are glutamine and glutamic acid, which then become the building blocks used in other biosynthetic reactions for the synthesis of several

amino acids, purines, pyrimidines, and other necessary nitrogenous compounds.

Growth Factors. These substances promote growth of the organism and are provided by various body fluids and tissues in vivo and in the form of yeast extract and blood and/or blood products in vitro. These factors include B-complex vitamins, minerals, certain amino acids, purines, and pyrimidines. The B-complex vitamins play a catalytic role within the cell, acting either as components of coenzymes or as prosthetic groups of enzymes. Organisms that do not require an exogenous source of a given growth factor because they are capable of synthesizing their own are referred to as **prototrophic. Auxotrophic** organisms require the addition of the growth factor to culture media before growth can occur. Small amounts of a number of inorganic ions also are required by all bacteria. In addition to nitrogen, sulfur, and phosphorus, which are present as constituents of important biologic compounds, potassium, magnesium, and calcium are often functionally associated with certain anionic polymers. Magnesium divalent cations stabilize ribosomes, cell membranes, the cell wall, and nucleic acids, and are also required for the activity of many enzymes. Potassium is also necessary for the activity of a number of enzymes, and in gram-positive bacteria the intracellular potassium concentration is influenced by the teichoic acid content of the cell wall. Most organisms also need zinc, iron, manganese, copper, and cobalt. Certain physical requirements for growth

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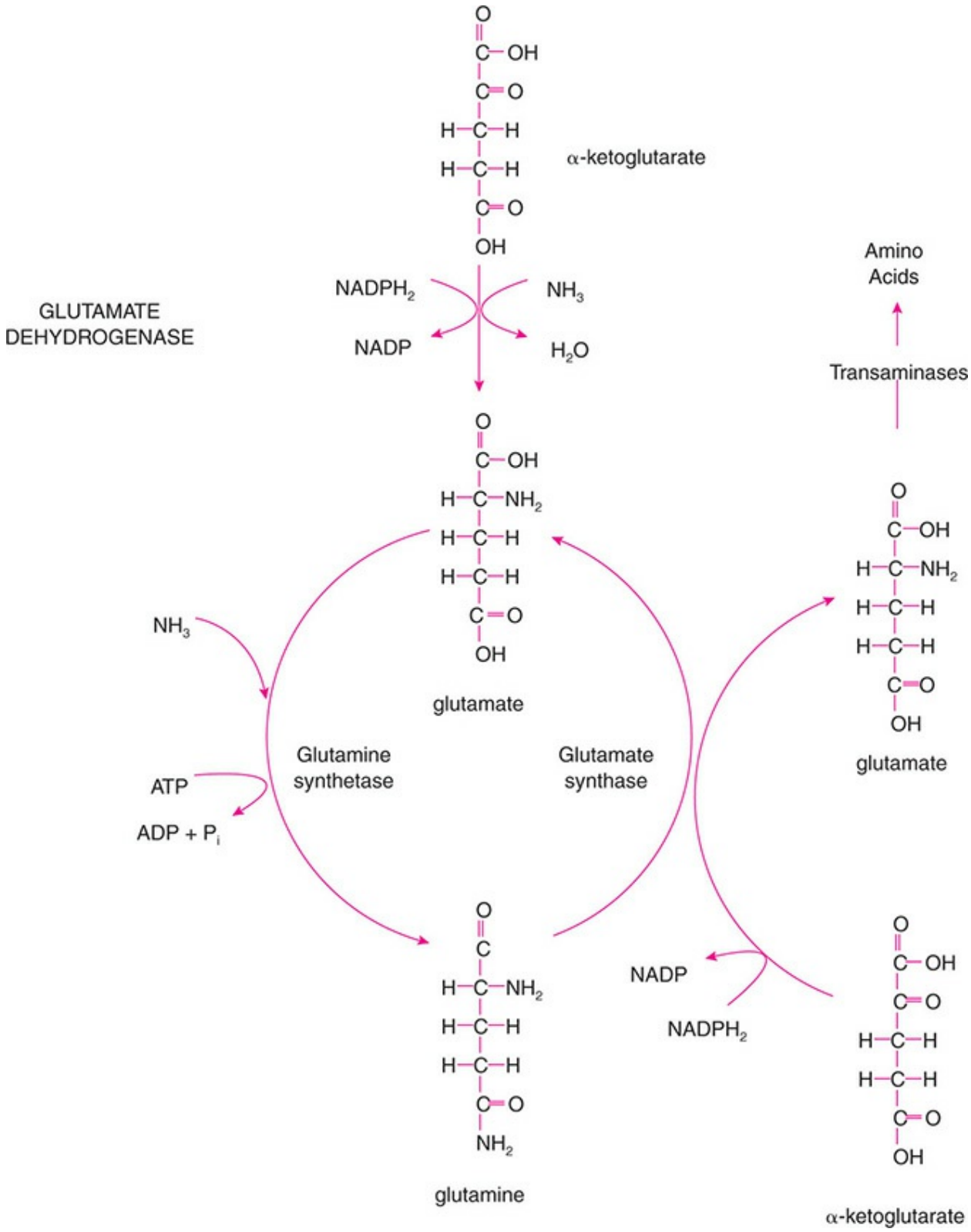

■ **FIGURE 5-14** Nitrogen assimilation and metabolism via the enzymes glutamine synthetase, glutamate dehydrogenase, and glutamate synthase. This enzymatic system results in the formation of amino acids and other compounds.

include optimal growth temperature, pH, and oxidation/reduction potential.

Kinetics of Bacterial Cell Growth

During growth in fluid culture medium, bacteria display a uniform growth curve, as expressed in logarithmic numbers of bacteria over time. A typical bacterial growth curve is shown in Figure 5-15. The lag phase is a period of physiologic adjustment and cellular preparation wherein the cell synthesizes new enzymes, cofactors, and essential metabolic intermediates, and the intracellular pools of nutrients are established. During the increasing growth phase, cell growth begins as enzymatic reaction rates begin to approach their steady-state rates. During the exponential or logarithmic growth phase, cell growth and cell division are occurring at their maximal rates. This rate is influenced by temperature, the type of carbon source being used, the rate-limiting concentrations of various essential nutrients, the types of nutrients available, and the oxygen tension or redox potential. During the declining growth phase, growth eventually ceases because of exhaustion of essential nutrients from the medium or from the accumulation of substances that are inhibitory or toxic. During the stationary phase, the number of viable cells has plateaued and the numbers of new organisms produced is equal to the numbers of cells that die

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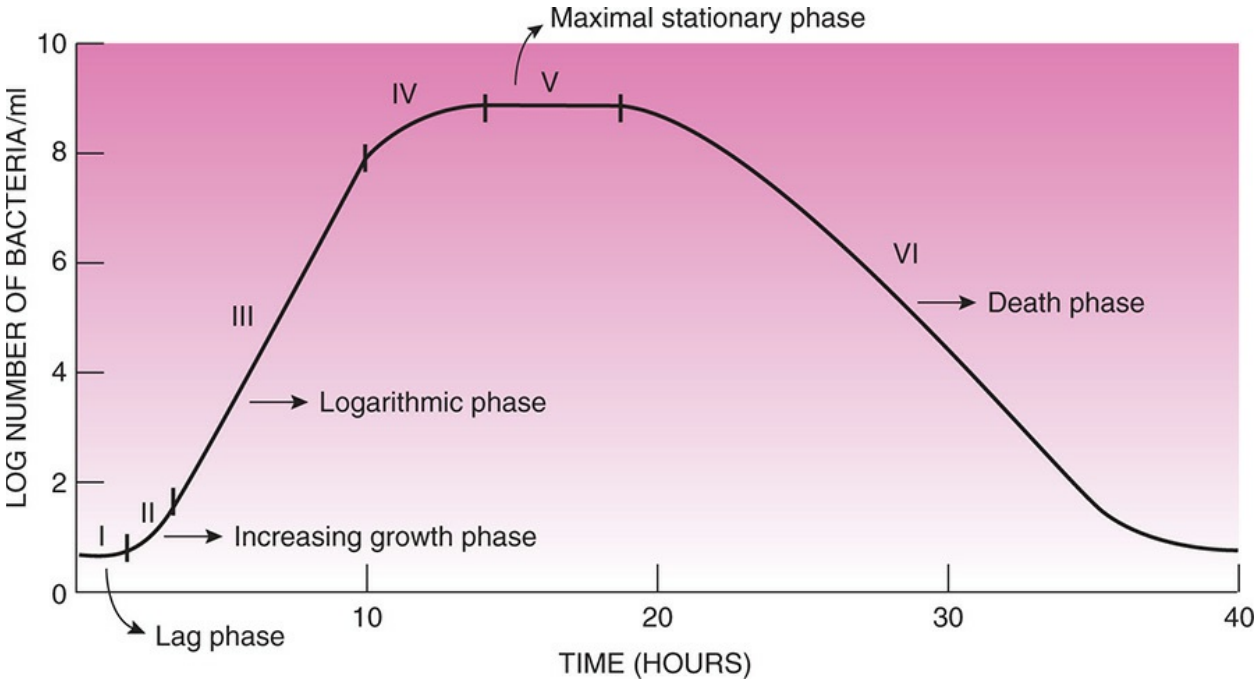

■ **FIGURE 5-15** Kinetics of bacterial growth.

because of lack of nutrients. During the death phase, cells begin to lyse and die.

Clinical laboratories most often deal with bacteria growing on solid media such as blood or chocolate agars. In general, organisms are able to grow to high numbers on solid media because the acid and other inhibitory products diffuse away from the bacterial colonies during growth, and the depletion of nutrients in the vicinity of the colonies is countered by diffusion of fresh nutrients into growth areas on the agar media. However, some organisms are extremely sensitive to slight pH shifts or metabolic by-products that cause cell lysis by themselves or that activate cellular autolysins. Periodic or frequent subculture to fresh agar media is usually sufficient for short-term preservation of organism viability on solid culture media.

General Bacterial Metabolism and Energy Generation

Fermentation. Bacterial metabolism is a dynamic balance between biosynthesis (anabolic reactions) and degradation (catabolic reactions). Catabolic reactions, in addition to providing smaller building blocks for subsequent biosynthetic processes, provide the energy necessary for biosynthetic reactions. In these processes, energy from the hydrolysis of chemical bonds is captured in the high-energy phosphate bonds of **ATP**. These bonds provide for the activation and continuation of other biochemical events. Using this energy, the bacterial cell wall, proteins, nucleic acids, and other structural and regulatory macromolecules are synthesized. Utilization of carbohydrates by bacteria and the conditions under which this utilization occurs are key characteristics for broadly characterizing bacteria. In general, many tests performed in the clinical microbiology laboratory involve the detection of the end products of bacterial metabolism in liquid culture after sufficient incubation times, either by pH indicators in the medium or by gas–liquid chromatography. The ability of a given microorganism to produce acid from a variety of carbohydrates (e.g., maltose, sucrose, mannitol, mannose, etc.) reflects the enzymatic capabilities of these organisms to initially convert such carbohydrates to glucose, which is the starting point for both aerobic and anaerobic carbohydrate catabolism.

Utilization of glucose under anaerobic conditions is termed *fermentation*. Fermentation occurs via glycolysis (also called the **hexose diphosphate** and the **Embden–Meyerhof–Parnas pathway**), with the end product being pyruvic acid or pyruvate. The glycolytic pathway from glucose to pyruvate is shown in Figure 5-16. This pathway requires two ATP molecules for initial phosphorylation of glucose to glucose-6-phosphate and subsequent phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate. During glycolysis, ATP is generated at two points in the pathway. As a result of the conversion of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid, the energy derived from the oxidation of an aldehyde group is conserved as a high-energy phosphate bond in ATP. The conversion of phosphoenolpyruvate to pyruvate results in the generation of another ATP molecule.

Therefore, four ATP molecules are produced from every molecule of glucose during glycolysis by a process called **substrate level phosphorylation,** resulting in a net gain of two molecules of ATP. In addition to ATP, reducing power is also produced by the generation of $NADH_2$ from the cofactor NAD (nicotinamide adenine dinucleotide).

Glycolysis is not the sole pathway for carbohydrate metabolism in most organisms. Many strictly aerobic bacteria use the Entner–Doudoroff pathway, which begins with glucose being converted to 6-phosphogluconic acid. This molecule is dehydrated to form 2-keto-3-deoxy-6-gluconate, and then hydrolyzed to pyruvate and glyceraldehyde-3-phosphate (Fig. 5-17). Pyruvate then enters the **Krebs tricarboxylic acid cycle** (see below), and the glyceraldehyde-3-phosphate is converted to pyruvate via the glycolytic cycle. Many bacteria use the pentose phosphate pathway, in which glucose-6-phosphate is oxidized to 6-phosphogluconic acid, which is then further oxidized to ribulose-5-phosphate (5-carbon), CO_2, and NADH (Fig. 5-18). Five-carbon monosaccharides are used in the biosynthesis of nucleic acids. Some bacteria

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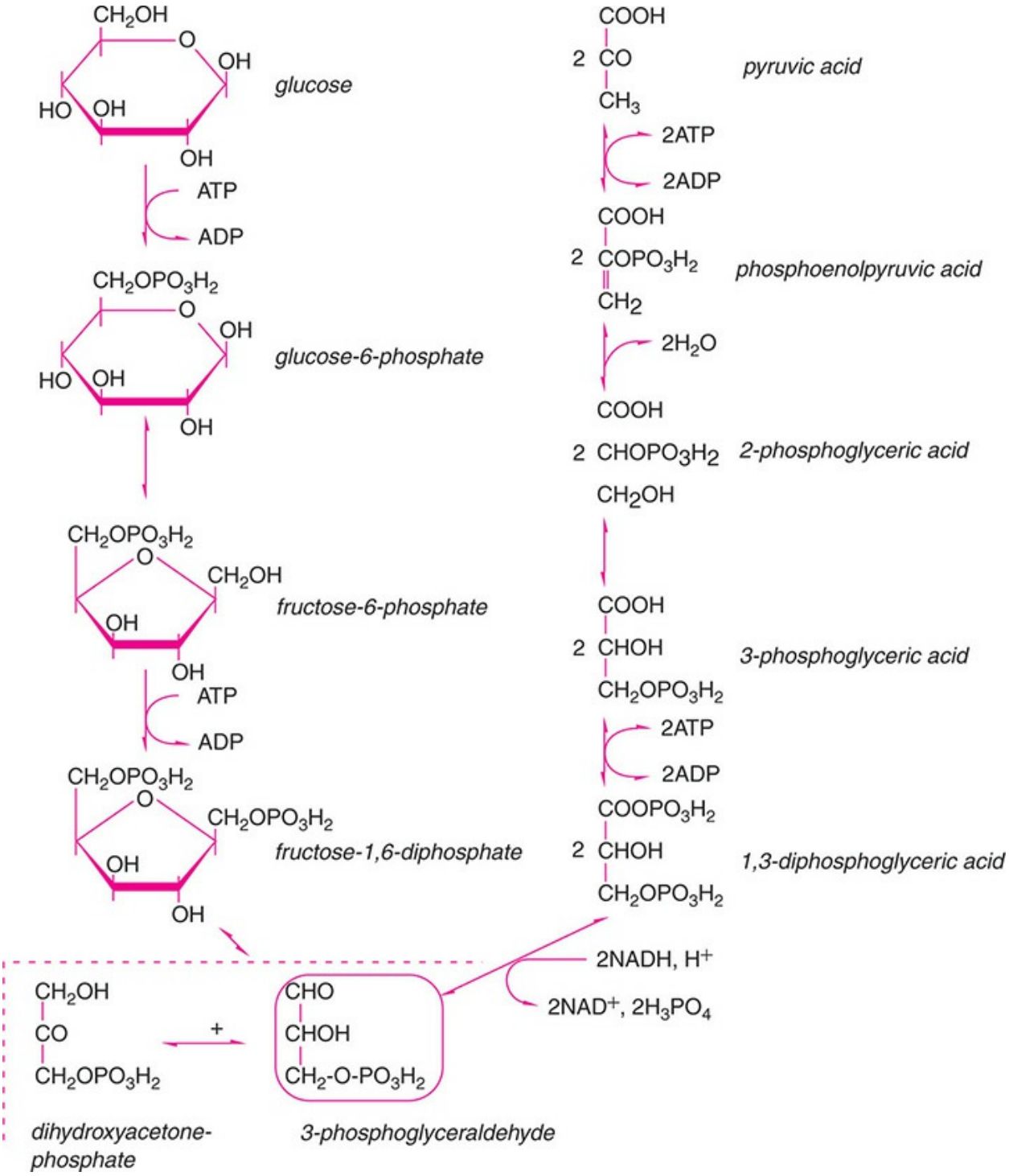

■ **FIGURE 5-16** Glycolytic pathway.

are able to metabolize 5-carbon (pentose) sugars such as xylose and arabinose. In the metabolism of 5-carbon sugars, enzymes known as transketolases and transaldolases convert these simple carbohydrates to various 3-, 4-, and 5-carbon intermediates,

which, in turn, are used as building blocks for the biosynthesis of nucleic acid precursors.

Utilization of Pyruvate. Pyruvate may enter a variety of pathways, resulting in several different end products. It is these end products that are often useful in categorizing the organisms isolated in the clinical microbiology laboratory. The metabolic pathways shown in Figures 5-19 and 5-20 include:

Pathway 1—Homolactic Fermentation (Homofermentative). In this pathway, the simplest of fermentations, the primary product of glucose fermentation is lactic acid. Some organisms that are considered homolactic fermenters also make small amounts of acetate, ethanol, and formate. The relative amounts of the latter products are dependent on the initial pH and the buffering capacity of the medium. Generally, no gas is formed. Homolactic fermentation of glucose is characteristic of the streptococci, enterococci, pediococci, and lactobacilli.[64]

Pathway 2—Heterolactic Fermentation. Pyruvate is decarboxylated to yield acetaldehyde and CO_2. The acetaldehyde is then reduced by $NADH_2$ and alcohol dehydrogenase to form ethanol. This type of pathway is seen in *Leuconostoc* species, some lactobacilli, and yeasts.

Pathway 3—Mixed Acid Fermentation. In this metabolic pathway, pyruvate is metabolized to a number of different products (acetic acid, ethanol, succinic acid, formic acid). Initially, pyruvate is cleaved in the presence of coenzyme A (CoA) to form formic acid and acetyl-CoA. Some of the acetyl-CoA is reduced to acetaldehyde and then to ethanol. The acetyl group of some acetyl-CoA is transferred to phosphate, yielding acetylphosphate, which

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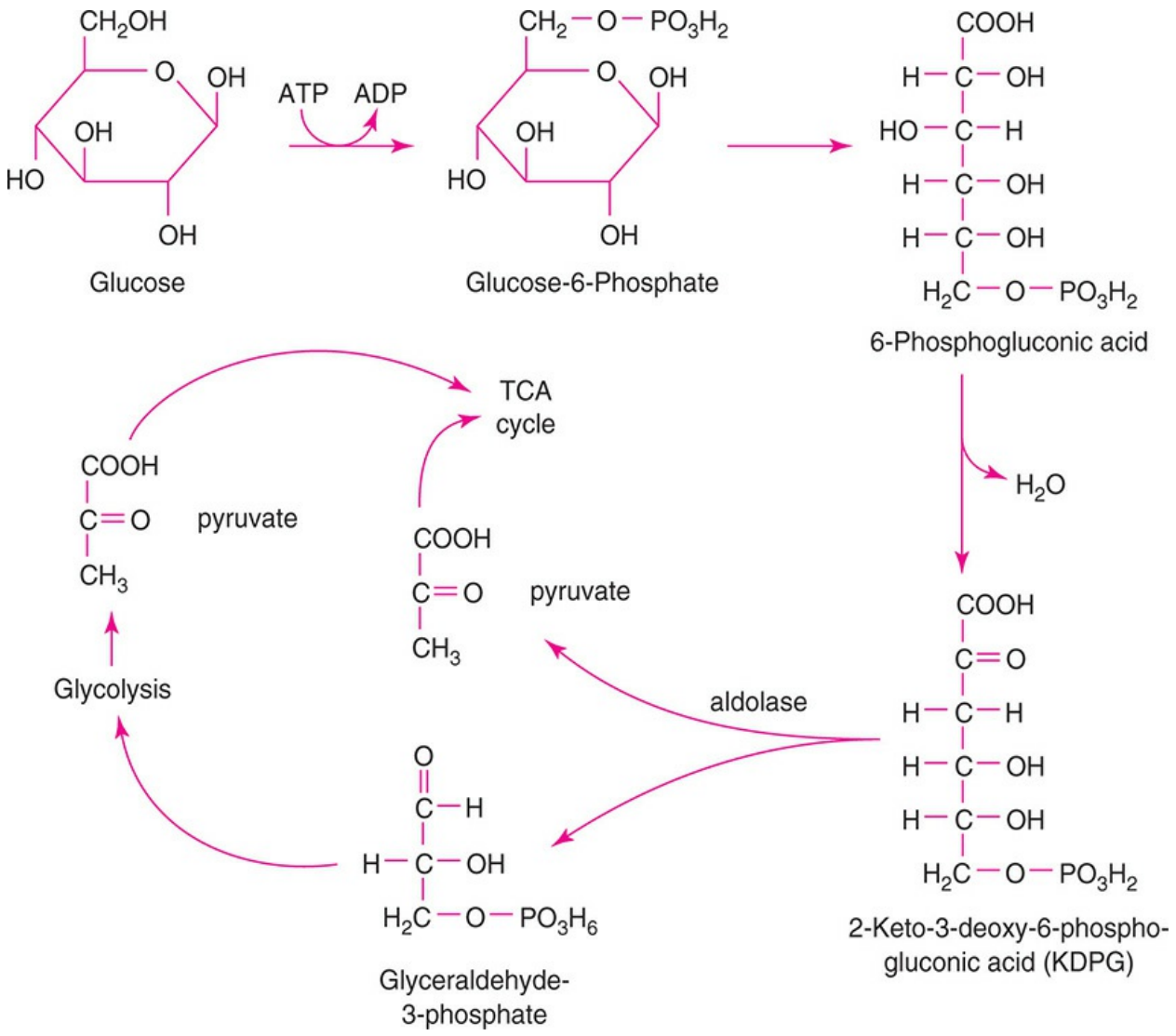
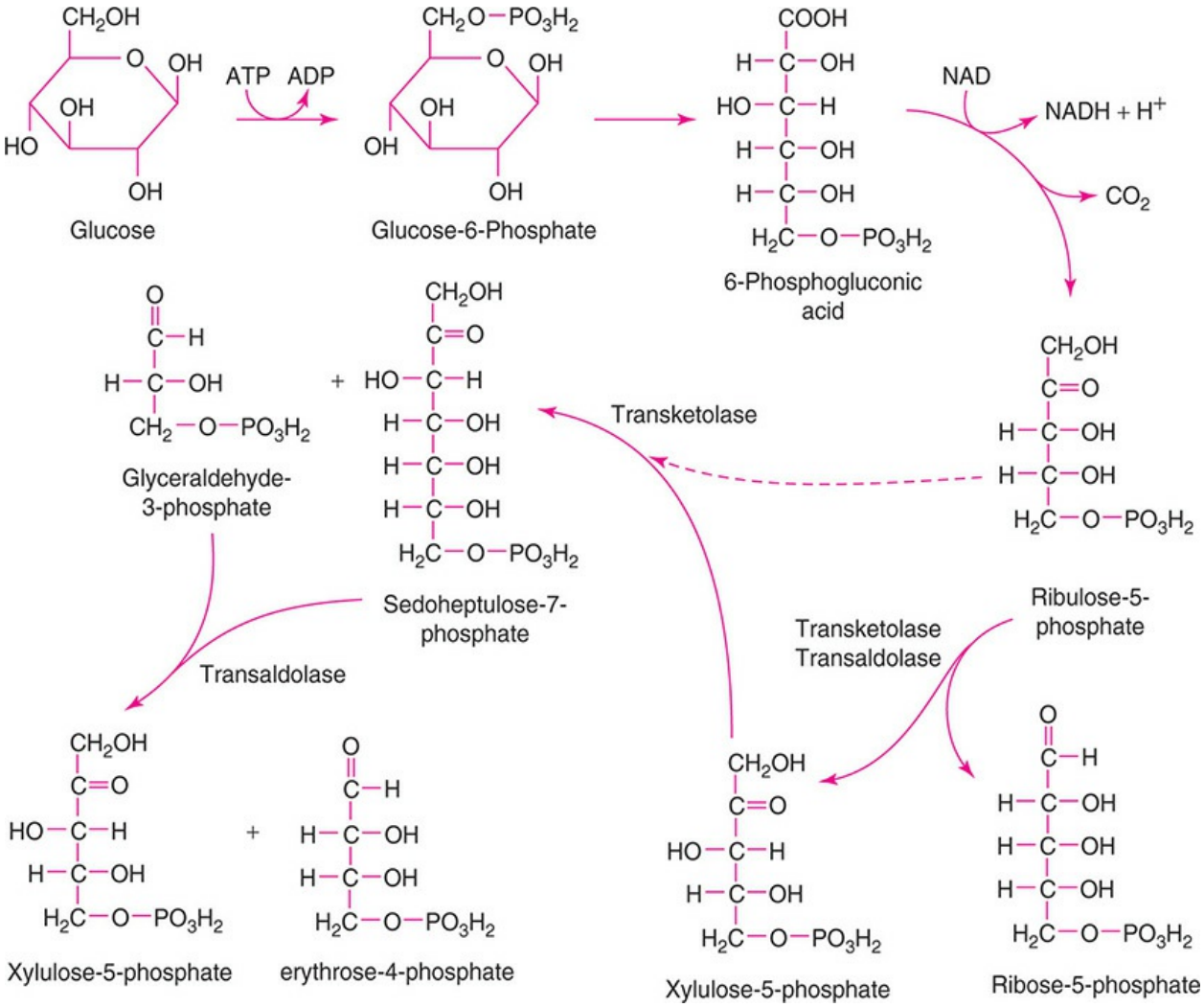


■ **FIGURE 5-17** The Entner–Doudoroff pathway.



■ **FIGURE 5-18** The pentose phosphate pathway.

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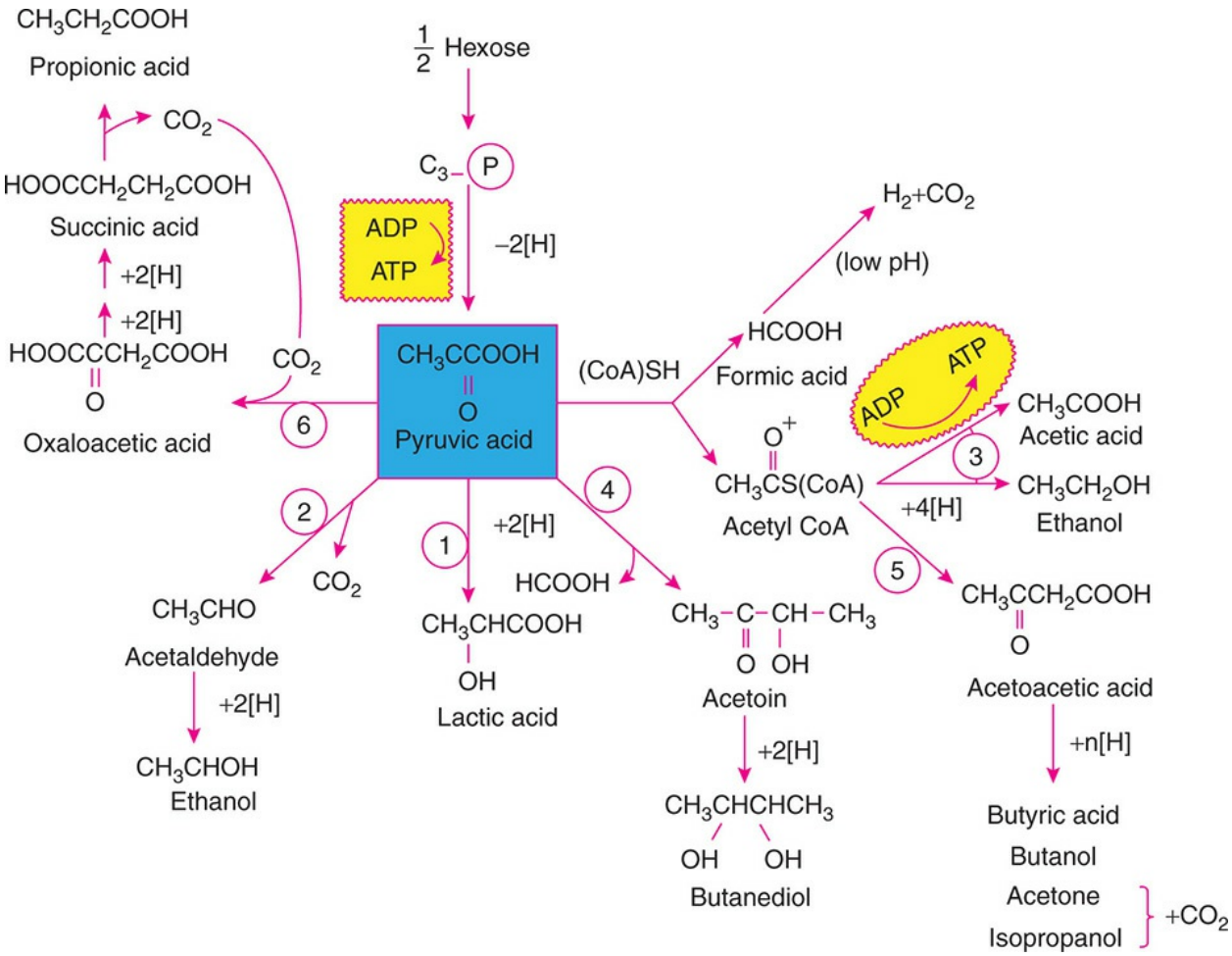

■ **FIGURE 5-19** Utilization of pyruvate formed during anaerobic fermentation.

then yields its high-energy bond to ADP, producing one ATP and acetic acid. Some bacteria are able to oxidize formate directly to hydrogen gas and CO_2. The nature and amounts of these acid products depend on the organism.

Pathway 4—Butanediol Fermentation. Bacteria that use this pathway have an enzyme that condenses two pyruvate molecules (each with 3 carbons) to CO_2 and a 5-carbon intermediate called α-acetolactate. The α-acetolactate is then enzymatically decarboxylated, resulting in acetoin (acetylmethylcarbinol) formation. In the presence of hydrogen ions, acetoin is further reduced to 2,3-butanediol. This reduction reaction is slowly reversible in air under alkaline conditions; the acetoin that is synthesized can be detected by the addition of α-naphthol. This is the basis of the **Voges–Proskauer (VP) test**, which uses α-naphthol in the presence of alkali to detect acetoin. This pathway is seen in the *Klebsiella–Enterobacter–Serratia–Hafnia* group of the family *Enterobacteriaceae*. The conversion of some of the pyruvate to 2, 3-butanediol reduces the amount of acid

relative to the mixed acid pathway described above and is responsible for **methyl-red (MR)** reaction used to separate *E. coli* and related organisms (**MR** + **/VP–**) from the *Klebsiella–Enterobacter–Serratia–Hafnia* group (**MR–/VP** +).

Pathway 5—Butanol Fermentation. This fermentation pathway is seen among members of the genus *Clostridium*. These organisms possess an enzyme that condenses two molecules of acetyl-CoA formed from pyruvate to form acetoacetyl-CoA. This molecule is reduced to in a stepwise fashion to butyryl-CoA, which can be subsequently hydrolyzed to butyric acid and ATP. Alternatively, free acetoacetate is released and decarboxylated to produce CO_2 and acetone, which may be reduced to isopropyl alcohol. In clinical microbiology laboratories performing reference work on clostridia, volatile and nonvolatile fermentation products are detected by gas–liquid chromatographic analysis of derivatives prepared from broth cultures. Detection of these metabolic end products is helpful for genus- or species-level identification of these anaerobic bacteria.

Pathway 6—Propionic Acid Fermentation. This is a cyclic-type of reaction in which oxaloacetate is formed from carbon dioxide, pyruvate, and ATP using a biotin containing coenzyme. Oxaloacetate is reduced to malate, converted to fumarate and then reduced to succinic acid (succinate). Decarboxylation of succinate results in the formation of propionic acid (propionate). This pathway is seen in anaerobic gram-negative bacilli in the genus *Bacteroides* and in *Propionibacterium* species, which are gram-positive, anaerobic, non–spore-forming bacilli.

Utilization of glucose under aerobic conditions is called respiration (Fig. 5-20). Pyruvate formed during fermentation enters the Krebs cycle, during which it is broken down to CO_2 and H_2O with the generation of ATP. Oxidative decarboxylation of pyruvate yields the high-energy intermediate called acetyl-coenzyme A (acetyl-CoA), which condenses with a molecule of oxaloacetate to form citrate and free CoA. A series of oxidative reactions ensues with the regeneration of oxaloacetate and the generation of reducing power in the forms of reduced NAD (NADH) and reduced flavin adenine dinucleotide (FADH). These high-energy compounds

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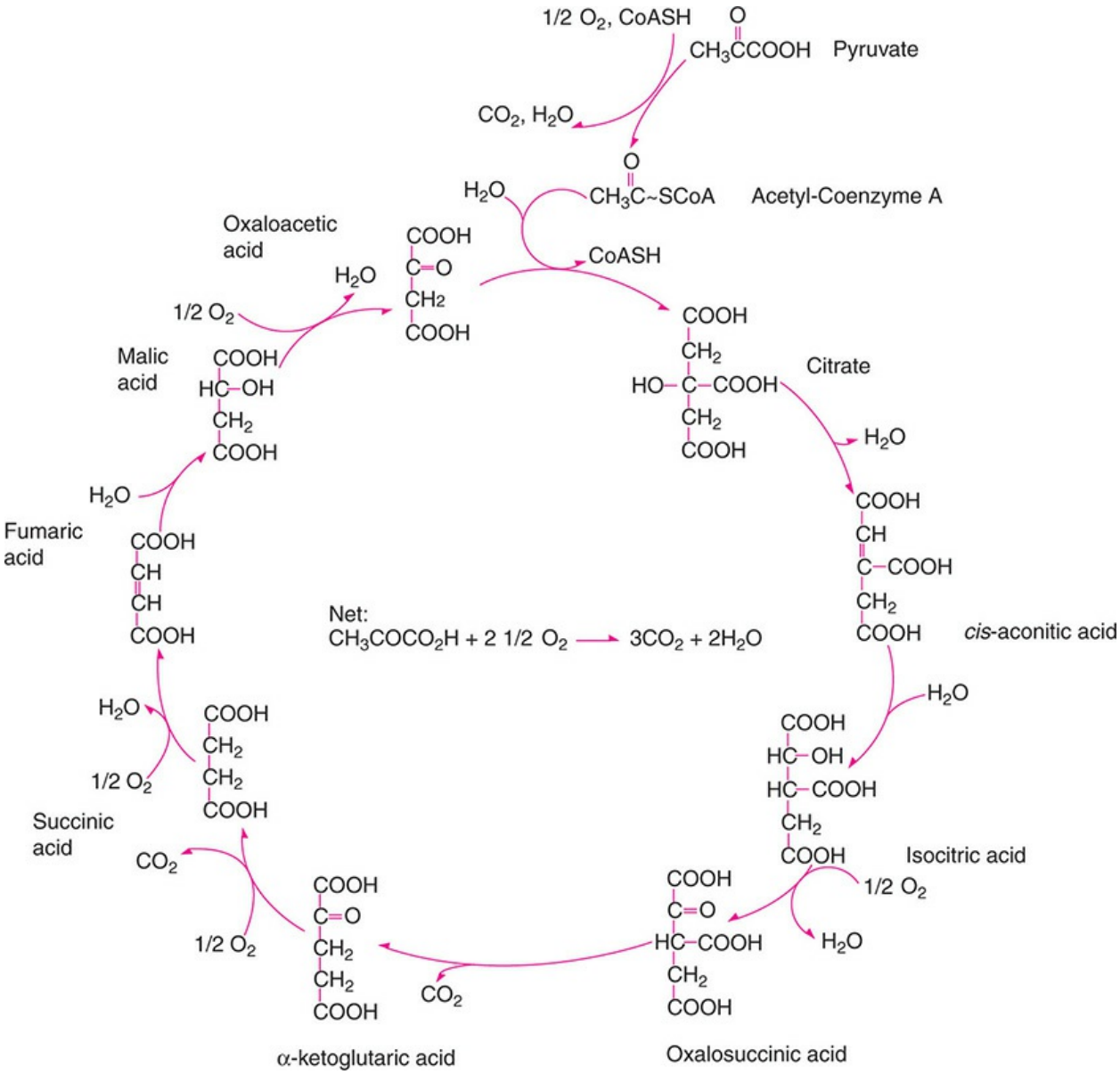

■ **FIGURE 5-20** Krebs tricarboxylic acid cycle.

subsequently enter the **electron transport chain,** which consists of alternating hydrogen and electron carriers located in sequence across the cell membrane. Transfer of protons down the electron transport chain creates a membrane potential. The energy of this potential is harnessed by a membrane-associated multienzyme system called **ATP synthase.** The subunit enzyme catalyzes the phosphorylation of ADP with inorganic phosphate. The final electron acceptor in the electron transport chain is oxygen (O_2), with the final product being H_2O. Complete oxidation of glucose via anaerobic glycolysis and the aerobic Krebs cycle results in a net gain of 38 ATP molecules per mole of glucose, as compared with the net generation of only 2 ATP

molecules per mole of glucose via the glycolytic (fermentative) pathway alone. In addition to the generation of ATP during aerobic metabolism, the Krebs cycle also provides the cell with precursors or intermediate compounds used in the biosynthesis of several other cellular components, such as purines, pyrimidines, amino acids, and lipids. The cycle also serves a catabolic function by providing a venue for the oxidative breakdown of these same macromolecules.

Bacterial Virulence Factors and Pathogenicity

Definitions and Concepts

Pathogenicity refers to the ability of an organism to cause disease. Organisms that are capable of causing disease under the appropriate circumstances are called **pathogens. Virulence** usually refers to the degree of pathogenicity within a group or species of microorganisms. The virulence of a microorganism is not generally attributable to a single factor, but is dependent on several parameters that relate to the organism, the host, and the dynamic interaction between them. This balance between the host and the "potential pathogen"

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is an area of increasing interest among microbiologists. In general, virulence encompasses two features of a pathogenic microorganism: the **infectivity** (i.e., the ability to initiate an infection) and the **severity** of the condition produced. Highly virulent, moderately virulent, and/or avirulent strains may occur within a species or group of organisms that are generally considered to be pathogenic. Infection of the host by an organism is a necessary step in the production of disease. It is important to differentiate colonization from infection. Colonization of a host with normal microbiota occurs most commonly on surfaces (e.g., skin and mucous membranes) and within luminal organs, such as the gastrointestinal tract. The colonization factors present on the surfaces of organisms that comprise the microbiota (e.g., fimbriae, lipoteichoic acids, capsules, and OMPs) are operationally the same as those used by pathogenic microorganisms. The organisms of the normal microbiota become established in and on the host early in life and persist in a dynamic state throughout the lifetime of the host. Infection with exogenous bacteria may be aborted by the presence of normal microbiota that already occupies the ecologic niche of the potential pathogen. If it does occur, infection may only be apparent by the demonstration of an antibody response with elimination of the exogenous organism. In some cases, an asymptomatic carrier state may result, or overt disease may develop.

The ability of an organism to cause disease involves both microbial and host factors.

There is currently a great deal of interest in the variety of **adaptive responses** of bacteria that result in the expression of characteristics involved in disease pathogenesis. For example, *Pseudomonas aeruginosa* is a ubiquitous soil and water organism that, because of its genetic versatility, has successfully adapted to human environments, such as hospitals, to create its own nosocomial niche. Over the years of its coexistence with humans, *P. aeruginosa* has emerged as a nosocomial and opportunistic pathogen that expresses a range of genetically determined virulence factors, including several exotoxins, hemolysins, lipases, elastases, and proteases, highly mucoid alginate capsular polysaccharides, and plasmid and transposon-mediated mechanisms of antimicrobial resistance.[93] These genetically determined adaptive responses have provided *P. aeruginosa* with the versatility to survive and actually thrive in potentially hostile environments, including the human body. Medical progress has also enabled us to appreciate the adaptive responses of organisms that are part of the normal human microbiota. When host defense mechanisms are abrogated by underlying noninfectious causes or various interventions (e.g., malignancy, diabetes mellitus, immunosuppressive therapy), endogenous organisms may also cause disease because of the phenotypic expression of characteristics that reflect a new adaptive response of the organism to its host.

In recent years, **bacterial sensing mechanisms** have become a focus of research in medical microbiology. Bacteria have developed sophisticated methods for sensing their environment and regulating the expression of genes involved in virulence.[27] **Two-component signal transduction** refers to regulatory protein pairs that function sequentially in detecting alterations in the external environment and in effecting adaptive responses in the organism. At the molecular level, this occurs by transfer of phosphate groups from sensor to effector proteins; the phosphorylation state of the effector proteins regulates the strength and duration of the response by binding to promoters in the nucleic acid that allow the expression (i.e., transcription and subsequent translation) of bacterial genes that code for virulence factors. Two-component signal transduction sensing systems have been described in enteric gram-negative bacteria, *B. fragilis*, *B. pertussis*, *M. tuberculosis*, *Vibrio* species, and *Clostridium perfringens*, among others.[27,94] Some organisms express certain virulence genes in response to threshold levels of autoinducers. These autoinducers are *N*-acylhomoserine or *N*- butyrylhomoserine lactones that are produced continually.[41] When the bacterial population reaches a certain threshold, enough autoinducer is present to activate transcription factors within the cells that coordinate the expression of virulence genes. This complex signaling mechanism, called **"quorum sensing,"** has been demonstrated in environmental bacteria (e.g., *Vibrio fischeri*, *Rhizobium leguminosarum*) and in human pathogens (i.e., *P. aeruginosa*, *Salmonella* species).[15,16,68,85] The transcription activators that respond to quorum sensing in these diverse microbial groups show significant amino acid sequence homology, suggesting

that sensing mechanisms evolved early and were disseminated widely among microorganisms that occupy various ecologic niches.

Requirements for Pathogenicity

The first step in the establishment of an infectious process resides in the ability of a microorganism to enter the host and to initiate infection. The initial contact depends on the ability of the organism to attach to and survive on the surfaces of the host. Attachment occurs via interaction of various bacterial adhesins with cell-surface receptors (see below). Some organisms will attach to epithelial cells without invasion of the deeper tissues. In such cases (e.g., *Corynebacterium diphtheriae*), toxins or enzymes elaborated by the organisms are usually responsible for the disease, which may be local or systemic. Deeper infection occurs when certain organisms attach to the mucosal epithelial cells and subsequently penetrate this barrier. Further multiplication of the organisms in the subepithelial tissues results in tissue destruction from bacterial factors (e.g., proteases, collagenases, hyaluronidases) and from the immune response (i.e., complement-mediated cytolysis, cell-mediated immune reactions). More invasive organisms may attach, penetrate the epithelial cell surfaces, multiply, and extend into the deeper tissues; eventually gaining access to the bloodstream and causing disseminated infection. Organisms such as mycobacteria and brucellae, attach, invade, multiply, and subsequently adapt themselves to a continued existence within the host, usually by inhibiting killing within the phagolysosome and living intracellularly, usually within histiocytes of the reticuloendothelial system.

Many organisms are highly specific in the types of tissues that they may infect. For example, *N. meningitidis* can be a normal inhabitant of the throat, and the asymptomatic carrier state is well characterized. Under the appropriate circumstances, these organisms may invade, causing infections of the meninges and the bloodstream. *S. pneumoniae* may also inhabit the throat and nasopharynx, yet virulent strains preferentially invade the lower respiratory tract and the bloodstream, causing pneumonia, pneumococcal sepsis

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and/or meningitis. Tissue specificities may reflect the presence of cellular receptors for bacterial surface antigens or the presence of nutrients that serve as chemoattractants for the microorganisms (e.g., certain amino acids, ions, or carbohydrates). A classic example of this nutritional dependence is seen with *Brucella abortus*, the cause of contagious abortion in cattle. This organism has a specific growth requirement for the sugar alcohol erythritol, which is present in high concentrations in the bovine uterus and placental tissue. Hence, this organism may actually “home in” on the bovine genital tract because of this nutritional predilection.

Virulence Factors of Bacteria

Bacterial virulence factors are structural components or products produced by bacteria that allow the organism to harm the host in some manner. Some virulence factors are cell-associated, while others may be extracellular. In addition, some of the factors involved in disease production are part of the anatomic or physiologic composition of the cell, and their roles as virulence factors are incidental to the life cycle of the organism.

Adhesins. In order to infect a host, microorganisms must first adhere to the mucosal surface. Bacterial adherence is usually a specific process involving bacterial cell-surface structures that are generally known as adhesins and complementary receptors on the surface of susceptible cells. Bacterial adhesins may include fimbriae, components of the bacterial capsule, lipoteichoic acids that project outside the peptidoglycan of the cell wall of gram-positive bacteria, OMPs, or other cell-surface antigens. Specific examples of well-characterized adhesins in pathogenic microorganisms have been discussed previously and include the adherence fimbriae of *N. gonorrhoeae*, mannose-sensitive (type 1) and mannose-resistant (type 2) fimbriae of uropathogenic and enteropathogenic *E. coli*, and lipoteichoic acids of β-hemolytic streptococci.[29,55,62] The human cell-surface protein fibronectin is a host receptor for some bacterial pathogens.

Aggressins. In order to survive and multiply within the host, many organisms produce a variety of substances that allow them to avoid or circumvent host defense mechanisms. These substances, termed *aggressins*, include capsules and extracellular slime substances, surface proteins and carbohydrates, enzymes, toxins, and other small molecules. The capsular structures of some bacteria enable the organisms to avoid phagocytosis by preventing interaction between the bacterial cell surface and phagocytic cells or by concealing bacterial cell-surface components that would otherwise interact with phagocytic cells or complement and lead to their ingestion.[25,58,97] Specific antibodies directed against capsular material lead to opsonization of the microorganisms. Following opsonization, encapsulated bacteria are readily and rapidly ingested and killed by phagocytic cells. Some organisms produce capsules that are structurally similar to host tissues and, therefore, are not recognized as foreign by immunosurveillance. In this manner, such organisms can evade host defenses. For example, the capsules of *E. coli* K1 and group B meningococci are composed of partially O-acetylated, α-2,8-linked, *N*- acetylneuraminic acid, which is structurally similar to the neuraminic acid found in tissue of the central nervous system. Organisms that possess capsules that behave as aggressins include *S. aureus*, *S. pneumoniae*, *N. meningitidis*, *H. influenzae* type b, *K. pneumoniae*, group A and group B β-hemolytic streptococci, and *B. anthracis*.[25,36,48,58,90,97]

Some bacteria possess surface proteins that play roles in adherence and other virulence-associated functions. For example, the M protein of group A streptococci plays many roles in the pathogenesis of infection with this organism, including adherence, resistance to phagocytosis, intracellular invasion, impairment of complement function, and lytic effects on polymorphonuclear leukocytes.[5,6,17,28,44,71,72] Protein A, a cell wall protein of *S. aureus*, is able to bind immunoglobulin (IgG) molecules by their Fc region. Since antibody-mediated phagocytosis (i.e., opsonization) is Fc receptor-dependent, protein A may interfere with this process. The presence of protein A may also inhibit the activation of complement by the staphylococcal cell wall by masking the peptidoglycan moieties that are known to have complement-activating activity. Some bacteria are able to produce proteases that are able to hydrolyze and inactivate secretory immunoglobulins (IgA).[69] This immunoglobulin acts locally to prevent bacterial adherence; hydrolysis of IgA by bacterial proteases therefore fosters mucosal colonization (see Chapter 3).

Some aggressins act after phagocytosis has occurred by interfering with phagosome/lysosome fusion and with the activity of the myeloperoxidase system. The mycobacteria and brucellae are able to adapt to an intracellular existence within host cells by elaborating substances that prevent intracellular destruction of organisms. In the mycobacteria, this may be due to the presence of cell wall–associated mycosides and sulfolipids that become incorporated into the inner aspect of the phagosome and prevent lysosome/phagosome fusion.[3,22,30] *Listeria monocytogenes* expresses a surface protein called **internalin** that binds to glycoprotein receptors on epithelial cells and allows the organism to become internalized in a membrane-bound vacuole.[66] The organism then produces a hemolysin called **listeriolysin O**, which intercalates into the membrane of the vacuole and causes the formation of pores.[53] *L. monocytogenes* then enters the cytoplasm of the cell, where it continues to grow, thereby escaping the toxic environment with the phagolysosome. *S. aureus* secretes **catalase** and **superoxide dismutase**, which inhibit organism destruction by the myeloperoxidase system of phagocytic cells. Taking up residence within phagocytic cells by these mechanisms also contributes to virulence by protecting the organisms from destruction by specific antibodies and complement. Intracellular existence also has a significant influence on therapy. Infections with organisms such as *Brucella* species and *Francisella tularensis* must be treated with antibiotics that are able to penetrate into the phagocyte and act intracellularly (e.g., tetracyclines, aminoglycosides) in order to affect these "protected" organisms.[35]

Many bacteria produce enzymes or toxins or possess cellular constituents that have direct toxic or necrotizing effects on host inflammatory cells and other components of the immune system. Leukocidin and γ-hemolysin produced by *S. aureus* cause rounding and swelling of exposed polymorphonuclear cells and macrophages, followed

by degranulation, nuclear rupture, and cell lysis.[70] The lipopolysaccharide of gram-negative bacteria may delay or attenuate the acute inflammatory

response, allowing the organism to establish itself within the host with relative ease. The lipid A portion of endotoxin, in particular, can activate complement and stimulate the release of various cytokines (i.e., interleukin [IL]-2, IL-6, IL-8, tumor necrosis factor α [TNF-α]) that lead to the clinical manifestations of endotoxic shock, which includes hypotension, disseminated intravascular coagulation, and death. Gram-positive bacteria possess cell wall peptidoglycan polymers and membrane teichoic acids that can cause a similar release of cytokines and can precipitate symptoms similar to septic shock. Some organisms have other cell-surface properties, such as the LOS and certain surface proteins that render them resistant to the bactericidal effects of normal human serum (e.g., *N. gonorrhoeae*).[67] This property may facilitate dissemination of the bacteria via the bloodstream and lymphatics, leading to systemic infection or to the establishment of infected foci at sites distant from the site of the initial infection.

The invasive properties of some bacteria are attributed to the elaboration of enzymes that act extracellularly. *S. aureus*, *S. pneumoniae*, group B streptococci, and *Propionibacterium acnes* produce an enzyme called **hyaluronate lyase** (or hyaluronidase) during the exponential growth phase. This enzyme promotes the spread of the organism through the connective tissues by depolymerizing hyaluronic acid, the ground substance responsible for cell-to-cell adhesion.[32] Group A streptococci and staphylococci also elaborate enzymes that hydrolyze fibrin clots (**streptokinase** and **staphylokinase**), which also facilitate the spread of organisms in the tissues.[23,50] *C. perfringens* and other clostridia (e.g., *C. histolyticum*, *C. septicum*, *C. sordellii*, *C. novyi*, and *C. fallax*) produce cytotoxins, cytolysins, collagenases, phospholipases, hyaluronidases, and deoxyribonucleases that allow these organisms to colonize devitalized tissue, break down the collagen matrix of muscle and connective tissue, and facilitate the extension of the organisms into these tissues to cause necrotizing fasciitis and gas gangrene.[61]

Iron has been shown to be an essential nutrient for many organisms, and a requirement for virulence in some bacterial pathogens. In this regard, successful pathogenic bacteria have evolved ways to obtain iron from the environment or from the iron-restricted milieu of host tissues, in which free iron is maintained at low concentrations by binding to transferrin and lactoferrin proteins. Microorganisms scavenge this iron by the production of **siderophores**, which are small molecules that function as high-affinity iron chelators. The production of siderophores is, therefore,

considered a virulence factor.[10] Some organisms have evolved lactoferrin- or transferrin-binding proteins, from which the iron is removed and transported into the cell.[4,31] Both *N. gonorrhoeae* and *N. meningitidis* possess outer membrane receptors for binding human transferrin, lactoferrin, and hemoglobin and periplasmic binding proteins for removal of iron from the bound carriers and transport into the cell.[1,8,51,52,88] In addition, the production of many extracellular microbial products, including **toxins**, is partially regulated at the transcriptional level by the concentrations of iron in the surrounding medium. For example, expression of the β-phage structural gene for diphtheria toxin in lysogenized *C. diphtheriae* only occurs when iron becomes the growth-limiting substrate (see below).[75]

Although plasmids themselves are not virulence factors, the genes that code for many of the bacterial cell products responsible for virulence frequently reside on bacterial plasmids. **R factors** (plasmids that contain genes coding for resistance to antimicrobial agents) may be considered virulence factors because the acquisition of resistance to antimicrobial agents fosters continued growth and spread of bacterial infections in spite of therapeutic interventions. Some bacteria also contain plasmids that code for sex pili and chromosomal mobilization. These two factors enable a microorganism to transfer genetic material (either plasmid-borne, chromosomal, or both) to other organisms. Plasmids may also bear the genes that code for colonization antigens, serum resistance, iron chelation and transport, toxin and hemolysin production, and undefined intracellular survival functions. Plasmids containing resistance genes have been described in many medically-important gram-negative (i.e., all genera of the *Enterobacteriaceae*, *Pseudomonas*, *Vibrio*, *Pasteurella*, *Campylobacter*, *Haemophilus*, *Neisseria*, and *Bacteroides*) and gram-positive (e.g., *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Bacillus*, *Clostridium*, and *Corynebacterium*) genera.

Exotoxins and Endotoxins. Toxins of microbial origin fall into two groups: exotoxins and endotoxins. **Bacterial exotoxins** are the most potent biologic toxins known and are produced predominantly by gram-positive bacteria, although some gram-negative bacteria also elaborate them. Exotoxins are usually protein in nature and are heat-labile. Because they are proteins, many can be inactivated or destroyed by proteolytic enzymes. Conversely, some exotoxins become activated only after partial hydrolysis ("nicking") by proteolytic enzymes (see below). The toxic activity of many exotoxins can be destroyed by formaldehyde treatment (toxoid development) and neutralized by specific antibodies. Exploitation of these properties led to the development of the diphtheria and tetanus toxoids that are used for active immunization against diphtheria and tetanus, respectively.

In general, bacterial exotoxins fall into two groups. The first group consists of **cytolytic toxins**, which act on cell membranes to cause pore formation and

subsequent lysis of the cell. Examples of these toxins include the seven enterotoxins of *S. aureus*, and streptolysins O and S produced by group A streptococci. The second group consists of two-subunit, A–B, or **bipartite toxins**. These toxins contain a B (or binding) subunit that attaches to a specific host-cell receptor, and an A (or active) subunit, that passes into the cell and interacts with the target. These toxins include the cholera toxin of *Vibrio cholerae*, the Shiga toxin of *Shigella dysenteriae*, pertussis toxin produced by *B. pertussis*, and diphtheria toxin produced by lysogenized *C. diphtheriae*. Certain diseases, such as tetanus, botulism, diphtheria, and cholera, are due almost entirely to the effects of the toxins on target organs and tissues.

Tetanus is caused by the systemic effects of tetanus neurotoxin, the toxin produced by *Clostridium tetani*. Tetanus usually occurs as a result of a wound infection by *C. tetani* or, rarely, from injection of materials contaminated with *C. tetani* vegetative cells or spores. Tetanus neurotoxin is released upon cell lysis after bacterial growth under anaerobic conditions (e.g., in deep puncture wounds). The toxin is translated initially as a single peptide of about 150 kDa. On release from the bacterial cell, the toxin peptide is cleaved by proteolytic enzymes to form a light (L, 50 kDa) and a heavy (H, 100 kDa) chain that are connected by a disulfide bond; under reducing

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conditions, the two peptides are split into separate L and H chains. The receptor-binding site of the intact toxin molecule resides in the H chain. The L chain is internalized and moves from the peripheral nerves to the central nervous system by retrograde axonal transport. When tetanus toxin reaches the neurons in the spinal cord, brain stem, and cerebellum, it binds to its receptor, a ganglioside containing stearic acid, sphingosine, glucose, galactose, *N*- acetylglucosamine, and *N*- acetyl neuraminic acid (sialic acid). The spasmogenic effect of the toxin is due to its action on presynaptic reflexes involving interneurons in the spinal cord. The toxin blocks the normal postsynaptic inhibition of spinal motor neurons following afferent impulses by preventing the release of inhibitory neurotransmitters (i.e., γ-aminobutyric acid, glycine). This presynaptic blockage of central neurons results in elevated muscle tone and hyperactive reflexes. Resulting sensitivity to excitatory impulses, unchecked by inhibitory mechanisms, produces the generalized spastic paralysis characteristic of tetanus.

Botulism results from the ingestion of toxins formed by *Clostridium botulinum* growing in food, the principal vehicles being improperly canned fruits and vegetables (generally home canned), condiments, and fish products. Wound botulism is a systemic intoxication resulting from growth of *C. botulinum* and toxin production in wounds, and was reported during the 1980s and 1990s as a complication of wound infections in parenteral-drug users.[18,101] *C. botulinum* can also colonize the intestinal

tract of infants and produce toxin at that site (infant botulism).[92] Toxin accumulates in cells of *C. botulinum* during spore germination and active vegetative cell growth, but is released only upon cell lysis. Organisms within *C. botulinum* cluster into three groups depending on their 16S rRNA gene sequences: *C. botulinum* types B, E, and F, *C. botulinum* types C and D, and *C. botulinum* types A, F, and B. *C. botulinum* type G clusters independently from the others and is also called *Clostridium argentinense*.[24] These groups correspond to seven serologic types of *C. botulinum* toxins (types A, B, C-1, D, E, F, and G [associated with *C. argentinense*]), and each produces an immunologically type-specific toxin.[89] Toxin types A, B, E, and F are the types known to affect humans.

Native botulinum toxins are labile proteins and are complexed with nontoxin proteins to stabilize the molecules.[73] Botulinum toxin is produced as a progenitor protein with a molecular weight of about 150 kDa. These toxins are produced as inert molecules that become activated after proteolysis, but proteolytic cleavage is internal to the peptide molecules and the toxins do not change in molecular weight following activation. The active toxin, therefore, consists of a 50-kDa light (L) chain and a 100-kDa heavy (H) chain connected by a disulfide bond. After absorption from the gastrointestinal tract, the toxin reaches susceptible neurons (at neuromuscular junctions and peripheral autonomic synapses) via the bloodstream. There it becomes bound to presynaptic terminals, where it blocks the release of acetylcholine from cholinergic motor nerve endings. The hallmark of botulism includes cranial nerve involvement, bilateral and descending weakening, and paralysis of skeletal muscles and is characterized by fatigue, dizziness, nausea, blurring of vision, slurring of speech, dilatation of pupils, urinary retention, general flaccid paralysis of skeletal muscles, and respiratory paralysis.[19]

Diphtheria is another example of an illness that is primarily due to the action of a toxin.[26] Interestingly, only strains of *C. diphtheriae* that contain a lysogenic bacteriophage (β-corynephage) are able to produce diphtheria toxin. The structural genes for the toxin (called the *tox* gene) is part of the bacteriophage genome. The expression of the *tox* gene is regulated by an iron-activated repressor and toxin production occurs only when iron becomes the growth-limiting substrate.[76] In the presence of iron (Fe^{2+}), the repressor forms active dimers with Fe^{2+}, which bind to the *tox* gene promoter and inhibit *tox* gene expression. When Fe^{2+} becomes limiting, the Fe^{2+}/repressor dimer dissociates, allowing the *tox* gene to be transcribed and translated.[37,98]

The toxin molecule is formed by *C. diphtheriae* in association with the cell membrane and is secreted from the cell as a single peptide consisting of 535 amino acids with a molecular weight of about 58 kDa. Through proteolysis, the single peptide is cleaved into two major chains, designated as A and B, that are connected by

disulfide bonds. Peptide A is a 21-kDa peptide that contains the enzymatic activity of the molecule that inhibits protein synthesis, while peptide B is a 37-kDa fragment that contains a C-terminal receptor-binding domain and an internal translocating domain. Peptide B is responsible for binding of the toxin molecule to its target receptor. On binding to the target cell via peptide B, the toxin enters the cell via receptor-mediated endocytosis. The low pH within the endosome triggers the translocation of peptide A (via interaction with the peptide B translocating domain) into the cytosol; at this time the disulfide bonds are reduced, peptide B is released, and peptide A becomes enzymatically active. Peptide A inhibits protein synthesis by the adenoribosylation of elongation factor-2, an enzyme that is required for translocation of the polypeptidyl–tRNA from the acceptor site to the donor site on the eucaryotic ribosome. The adenoribosyl group is transferred from NAD to elongation factor-2 by the peptide A toxin subunit, rendering EF-2 inactive:

$$\mathrm{NAD^+ + EF\text{–}2} \xrightarrow[\text{A}]{\text{toxin peptide}} \text{adenoribosyl phosphate:}$$

$$\text{EF-2 complex} + \text{nicotinamide} + \mathrm{H^+}$$

Treatment of the intact toxin with formalin renders a toxoid that cannot be split into A and B subunits. Therefore, the toxoid lacks the ability to catalyze its toxic intracellular effects, yet it retains antigenicity. Immunity to diphtheria is generally mediated by the presence of antibodies against the toxin.

All the signs and symptoms of cholera caused by *V. cholerae* result from the rapid loss of fluid from the gastrointestinal tract. Increased electrolyte secretion is caused by a protein enterotoxin. The 84-Da enterotoxin consists of a binding subunit (subunit B) composed of five identical 11.5-kDa monomers and a biologically active 27-kDa subunit (subunit A). The mode of action of cholera toxin is described in Chapter 8.

Endotoxins on the other hand, are produced only by gram-negative bacteria and consist primarily of LPS. LPS, as described earlier, is a structural component of the gram-negative outer membrane, containing the somatic (O) antigenic determinants. Endotoxins are heat-stable, are not detoxified by formaldehyde treatment, and are only partially neutralized by specific antibodies. Compared with many of

the exotoxins, endotoxins are of relatively low toxicity. Although endotoxin may escape into the surrounding fluids (as “blebs” on the surface of gram-negative bacteria), the whole cell generally retains the major portion of the toxic activity. The biologic and toxic activities of endotoxin are broad. Nanogram amounts of endotoxin cause fever in humans and the release of endogenous pyrogen. Larger doses cause hypotension,

lowered polymorphonuclear leukocyte and platelet counts from increased margination of these cells toward the walls of small blood vessels, hemorrhage, and sometimes disseminated intravascular coagulation due to the activation of clotting mechanisms. Endotoxin is also mitogenic for B lymphocytes and stimulates the release of several cytokines from macrophages.

Bacterial Superantigens. Another group of extracellular protein toxins, such as those produced by *S. aureus* or group A β-hemolytic streptococci are able to bind to structural peptides on host T-lymphocyte receptors and macrophage class II histocompatibility antigens. This binding induces the release of cytokines. Various *S. aureus* extracellular proteins, such as toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins A and B, and group A streptococcal toxins act as "superantigens" and induce high levels of T-cell proliferation, prompt IL-2 and TNF-α release and cause a shock syndrome.[79] Superantigens produced by various bacteria will be discussed further in subsequent chapters dealing with specific bacterial genera.

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CHAPTER 6

The *Enterobacteriaceae*

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Characteristics for Presumptive Identification

- Screening Characteristics
  - Carbohydrate Utilization
  - Cytochrome Oxidase Activity
  - Nitrate Reduction

Culture Media Used for Detection of Carbohydrate Fermentation

- Use of Kligler's Iron Agar and Triple Sugar Iron Agar
- Biochemical Principles

Selection of Primary Isolation Media

- Chemicals and Compounds Used in Selective Media
- Selective Isolation Media
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- Enrichment Media
- Guidelines for Choosing Selective Isolation Media

Differential Identification Characteristics
- Indole Production
- Methyl Red Test
- Voges–Proskauer Test
- Citrate Utilization
- Urease Production
- Decarboxylation of Lysine, Ornithine, and Arginine
- Phenylalanine Deaminase Production
- Hydrogen Sulfide Production
- Motility

Taxonomy of the *Enterobacteriaceae*
- Classification of *Enterobacteriaceae* by Tribes
- Key Identification Characteristics for the Most Common Species
  - Tribe *Escherichieae*
  - Tribe *Edwardsielleae*
  - Tribe *Salmonelleae*
  - Tribe *Citrobactereae*
  - Tribe *Klebsielleae*
  - Tribe *Proteeae*
  - Tribe *Yersinieae*
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- Miscellaneous New Genera of *Enterobacteriaceae*
  - Identification Characteristics of Newer *Enterobacteriaceae*
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Quick Screening Methods for Rapid Identification
- Commercial Screening Kits
- Chromogenic Agar Media

Classic Identification Systems
- Checkerboard Matrix
- Branching Flow Diagrams
- Computer-Aided Schemes

Numeric Coding Systems
- Reading Octal Codes in Numeric Code Registers
- Estimated Frequency of Occurrence

Calculation of Likelihood
Resolving Discrepancies
Packaged Kit Identification Systems
Overview of Packaged Systems
Specific Identification Systems
API 20E
BBL Crystal Enteric/Nonfermenter ID System
RapID onE System
Biolog GN2 Microplate
Microscan System
Sensititre System
Semiautomated and Automated Identification Systems
MicroScan Walkaway
Vitek System
Sensititre Gram-Negative AutoIdentification System
The Phoenix System
The OmniLog ID System
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrophotometry

Gram-negative bacilli belonging to the *Enterobacteriaceae* are the most frequently encountered bacterial isolates recovered from clinical specimens. Widely dispersed in nature, these organisms are found in soil and water, on plants, and, as the family name indicates, within the intestinal tracts of humans and animals. Before the advent of antibiotics, chemotherapy, and immunosuppressive measures, the infectious diseases caused by the *Enterobacteriaceae* were relatively well defined. Diarrheal and dysenteric syndromes, accompanied by fever and septicemia in classic cases of typhoid fever, were known to be caused by *Salmonella* and *Shigella* species. Classic cases of pneumonia, characterized by the production of brick-red or "currant jelly" sputum, were known to be caused by Friedlander's bacillus (*Klebsiella pneumoniae*). *Escherichia coli*, *Proteus* species, and various members of the *Klebsiella–Enterobacter* group were commonly recovered from traumatic wounds contaminated with soil or vegetative matter or from abdominal wound incisions following gastrointestinal surgery.

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Thus, members of the *Enterobacteriaceae* may be incriminated in virtually any type of infectious disease and recovered from any specimen received in the laboratory. Immunocompromised or debilitated patients are highly susceptible to hospital-acquired infections, either after colonization with environmental strains or following invasive procedures, such as catheterization, bronchoscopy, colposcopy, or surgical biopsies, in which mucous membranes are traumatized or transected.

Endotoxic shock is one potentially lethal manifestation of infection with gram-negative bacteria, including the *Enterobacteriaceae*. Endotoxin is a complex pharmacologically active lipopolysaccharide that is contained within the cell wall of gram-negative species. This lipopolysaccharide is structured in three layers: (1) an outer variable carbohydrate portion that determines O-antigenic specificity (e.g., various *Salmonella* serotypes), (2) a middle core polysaccharide that is structurally similar among species, and (3) a central, highly conserved lipid moiety called lipid-A. The biologic effects of endotoxin have been demonstrated experimentally: small quantities injected intravenously into animals produce fever, leukopenia, capillary hemorrhage, hypotension, and circulatory collapse—symptoms that are, to a large extent, the same as those seen in humans with gram-negative sepsis.

The limulus lysate assay, which uses a reagent prepared from the amebocytes of the horseshoe crab (*Limulus polyphemus*), has been used with varying success in the diagnosis of endotoxic shock.[206] The lysate undergoes gelation when in contact with even trace amounts of endotoxin. More promising in the diagnosis of gram-negative sepsis is the development of monoclonal antibodies that can be used in enzyme-linked immunoassay or other techniques for the detection of lipid-A. The pharmacologic effects of endotoxin can be attributed primarily to lipid-A. It is highly antigenic and has determinants common to all strains of gram-negative bacilli. Thus, the detection of circulating lipid-A in patients with gram-negative sepsis, using a monoclonal antibody, could establish a diagnosis so that presumptive therapy could be started before the causative organism is recovered and identified.

Microbiologists must be alert to the emergence of any *Enterobacteriaceae* that are resistant to multiple antibiotics. Virtually all resistance seen to β-lactam antibiotics in *Enterobacteriaceae* is mediated by acquired or chromosomal β-lactamases. Certain members of the *Enterobacteriaceae* possess chromosomally determined inducible AmpC β-lactamases. These include the so-called MYSPACE organisms: *Morganella morgannii, Yersinia enterocolitica, Serratia marcescens, Providencia* spp., *Aeromonas* spp. *Citrobacter freundii* complex, and *Enterobacter* spp.[315] Antibiotic resistance may also evolve in formerly susceptible clinical isolates through the transfer of plasmids known as R factors or R plasmids. These large R plasmids encode for genes that produce β-lactamase enzymes capable of hydrolyzing penicillins, first-, second-, and third-generation cephalosporins and aztreonnam, and have therefore been designated extendedspectrum β-lactamases or ESBLs.[507] Detecting these

resistant strains is not only important in treating the patient from whom the isolate is recovered but also has important implications for surveillance of nosocomial infections. The mechanisms by which resistance may develop and methods used for detection of resistance mechanisms are discussed in more detail in Chapter 17. Specific clinical syndromes of individual genera and species are discussed in later sections of this chapter.

Characteristics for Presumptive Identification

What are the initial clues that an unknown isolate recovered from a clinical specimen may belong to the *Enterobacteriaceae*? In specimens other than feces, a Gram-stained preparation may reveal short, plump, gram-negative bacillary or coccobacillary cells, ranging from 0.5 to 2 fm wide to 2 to 4 fm long (see Image Plate 6-1A). However, species differentiation cannot be made on the basis of only Gram stain morphology.

Characteristic colonial morphology of an organism growing on a solid medium may provide a second clue. Typically, members of the *Enterobacteriaceae* produce relatively large, dull gray, dry, or mucoid colonies on sheep blood agar, the latter suggesting encapsulated strains of *K. pneumoniae* (see Image Plate 6-1B and C). Hemolysis on blood agar is variable and indistinctive. Colonies appearing as a thin film or as waves (a phenomenon known as **swarming**) suggest that the organism is motile and probably a *Proteus* species (see Image Plate 6-1D). Colonies that appear red on MacConkey agar or have a green sheen on eosin methylene blue (EMB) agar (see Image Plate 6-2) indicate that the organism is capable of forming acid from lactose in the medium.

Differentiation of the *Enterobacteriaceae*, however, is based primarily on the presence or absence of different enzymes coded by the genetic material of the bacterial chromosome. These enzymes direct the metabolism of bacteria along one of several pathways that can be detected by special media used in in vitro culture techniques. Substrates on which these enzymes can react are incorporated into the culture medium, together with an indicator that can detect either the utilization of the substrate or the presence of specific metabolic products. By selecting a series of media that measure different metabolic characteristics of the microorganisms to be tested, a biochemical profile can be determined for making a species identification.

Screening Characteristics

Definitive identification of the members of the *Enterobacteriaceae* may require a battery of biochemical tests. Considerable time loss and possible misidentification can be avoided if a few preliminary observations are made to ensure that the organism being tested belongs to this group. If the organism is a gram-negative organism of

another group, it may be necessary to use a different set of characteristics than that commonly used for the identification of the *Enterobacteriaceae.* With few exceptions, all members of the *Enterobacteriaceae* demonstrate the following characteristics:

- Glucose is fermented (see Image Plate 6-1E and F)
- Cytochrome oxidase is negative (see Image Plate 6-1G)
- Nitrate is reduced to nitrite (see Image Plate 6-1H).

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Carbohydrate Utilization. It is common for laboratory microbiologists to refer to all carbohydrates as sugars. This is convenient in an operational sense, although it is understood that polyhedral alcohols, such as dulcitol and mannitol, or cationic salts of acetate or tartrate are not carbohydrates and thus are not truly sugars in a chemical sense.

The term **fermentation** is also used somewhat loosely in reference to the utilization of carbohydrates by bacteria, with terms such as **lactose-fermenters** and **non–lactosefermenters**. By definition, fermentation is an oxidation–reduction metabolic process that takes place in an anaerobic environment, and instead of oxygen, an organic substrate serves as the final hydrogen (electron) acceptor. In bacteriologic test systems, this process is detected by observing color changes in pH indicators as acid products are formed. Acidification of a test medium may occur through the degradation of carbohydrates by pathways other than fermentation, or there may be ingredients other than carbohydrates in some media that result in acid end products. Although most bacteria that metabolize carbohydrates are facultative anaerobes, the utilization may not always be under strictly anaerobic conditions, as is observed in the production of acid products by bacterial colonies growing on the surface of agar media. Even though all tests used to measure an organism's ability to enzymatically degrade a "sugar" into acid products may not be "fermentative," these terms will be used in this textbook for convenience.

Basic Principles of Fermentation. Pasteur's mid-19th century studies of the action of yeasts on wine provide the basis for our present understanding of carbohydrate fermentation. Pasteur observed that certain contaminating bacterial species produced a drop in the pH of wine (a carbohydrate substrate) from the production of a variety of acids. Full descriptions of the fermentative pathways by which a monosaccharide such as glucose is degraded evolved soon thereafter. Through a series of enzymatic glycolytic cleavages and transformations, the glucose molecule is split into a series of three carbon compounds, the most important of which is pyruvic acid. The chemical sequence by which glucose is converted to pyruvic acid is known as the **Embden–Meyerhof pathway** (EMP; Fig. 6-1). Many bacteria, including all *Enterobacteriaceae*, ferment glucose through the EMP to form pyruvic acid; the

manner in which pyruvic acid is further used, however, varies among bacterial species. The alternative fates of pyruvic acid are the result of a variety of fermentation pathways, yielding quite different products (Fig. 6-1).

Bacteria are differentiated by the carbohydrates they metabolize and the types and quantities of acids produced. These differences in enzymatic activity serve as one of the important characteristics by which the different species are recognized. It is important for students of microbiology to understand that in the glycolytic formation of pyruvic acid, adenosine triphosphate (ATP) is generated at the expense of the reduction of nicotinamide adenine dinucleotide (NAD) to $NADH_2$. For each glucose molecule that is fermented to form pyruvic acid, four hydrogen ions are consumed through the reduction of two NAD to two $NADH_2$. Because the total NAD in the cell is very limited, fermentation would cease

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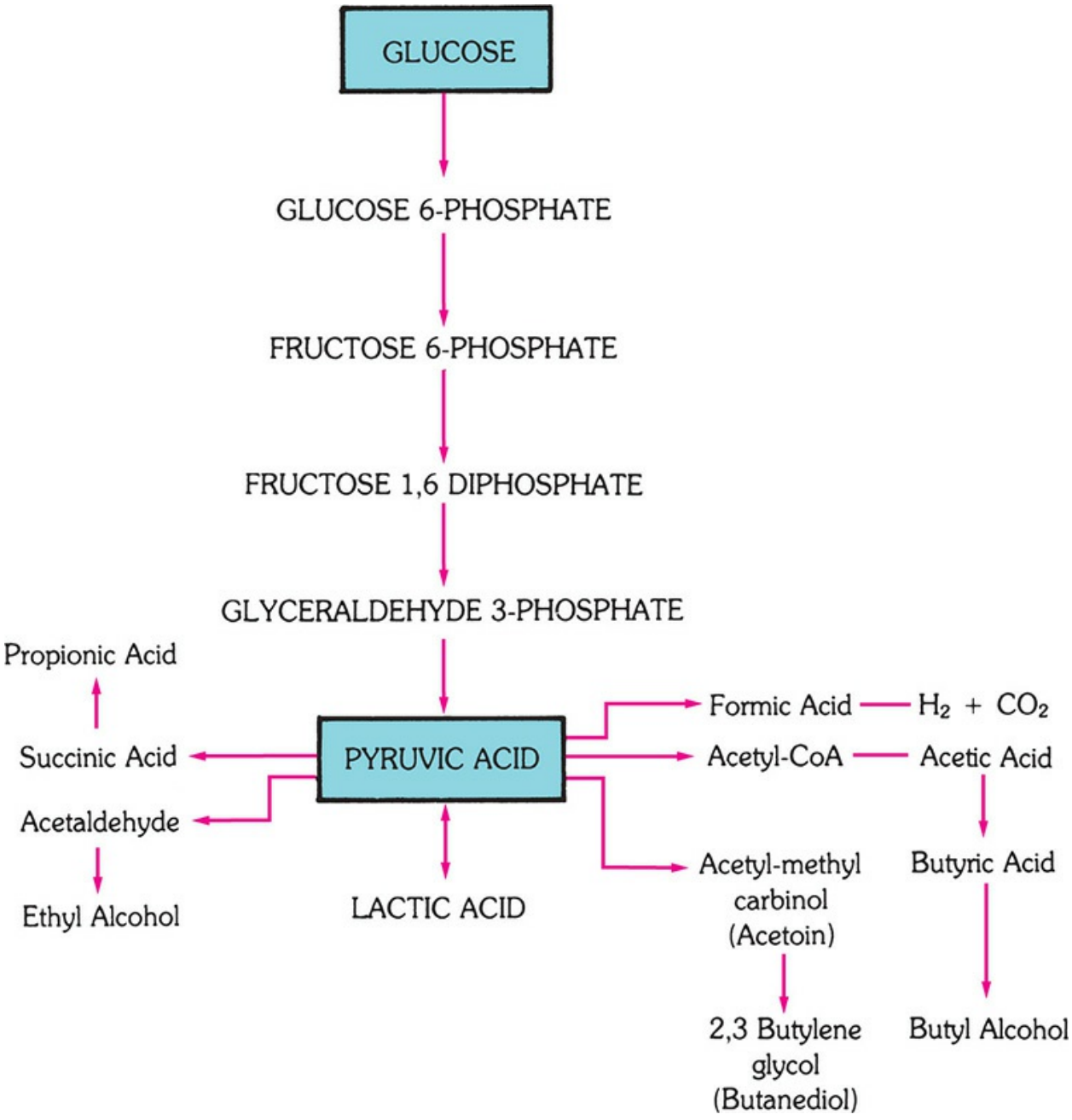
very rapidly if the $NADH_2$ were not reoxidized in the further metabolism of pyruvic acid. Figure 6-2 depicts the fermentation of three molecules of glucose by means of two alternative pathways. For example, glucose fermentation by *E. coli* occurs by means of the mixed acid fermentation pathway and results in the production of large quantities of acetic, lactic, and formic acids, with a marked drop in the pH of the test medium. This is detected by a positive methyl red test (Fig. 6-2). On the other hand, the *Klebsiella–Enterobacter–Hafnia–Serratia* group metabolizes pyruvic acid primarily through the butylene glycol pathway, producing acetyl methyl carbinol (acetoin) and a positive Voges–Proskauer (VP) test (Fig. 6-2). Note that the principal end products in this latter pathway are alcohols, with only a small amount of acid produced; thus the methyl red test is usually negative for this group of organisms.



■ **FIGURE 6-1** Fermentation of glucose to form pyruvate (EMP) and the alternative fates of pyruvic acid.

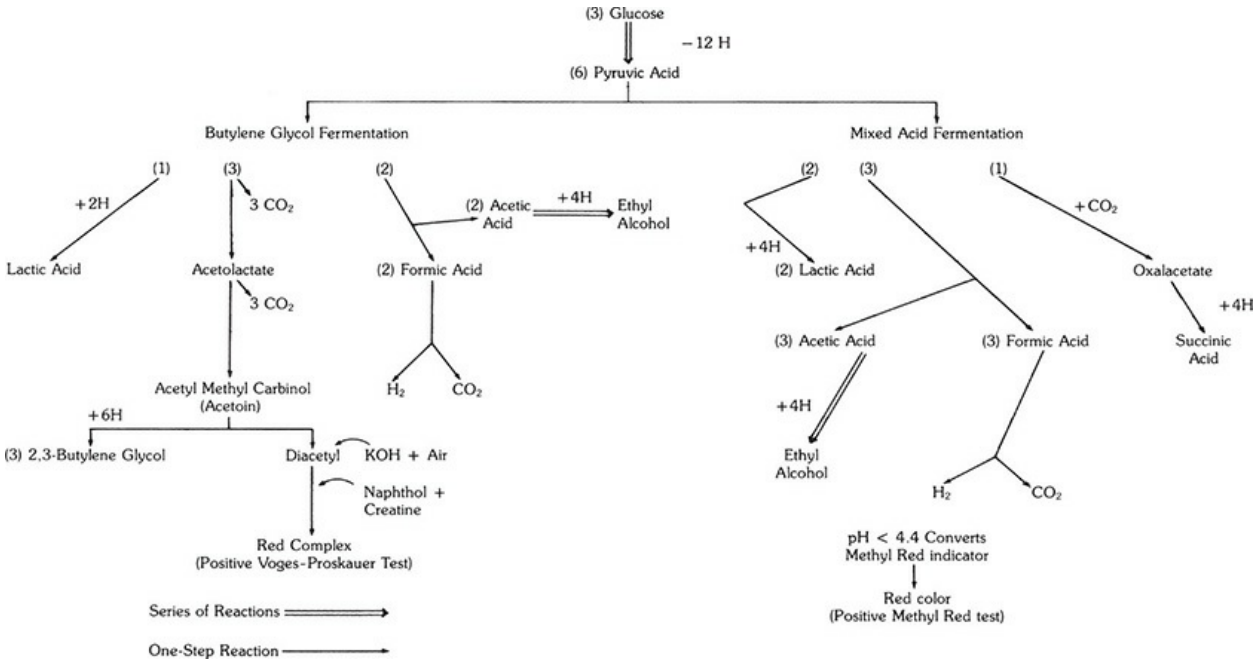
The gas resulting from fermenting bacteria is primarily a mixture of hydrogen and carbon dioxide formed through the cleavage of formic acid. It is an accepted rule of thumb that any bacterium that forms gas in carbohydrate test medium must first form acid, which is self-evident from the EMP scheme shown in Figure 6-1. Gas is best detected by using a broth carbohydrate-fermentation medium into which small inverted Durham tubes have been placed (see Image Plate 6-1F). Even trace amounts

of gas, which collect as bubbles under the Durham tubes, can be detected. Some species of *Enterobacteriaceae* lack the enzyme formic dehydrogenase and cannot cleave formic acid and, as a result, do not form even trace amounts of CO_2 (e.g., most species of *Shigella*). Conversely, organisms that use the butylene glycol pathway (i.e., are VP-positive) produce copious amounts of CO_2 (Fig. 6-2). Therefore, when a large amount of gas is observed, one should consider members of the *Klebsiella–Enterobacter–Hafnia–Serratia* group as the likely identification. The formation of ethyl alcohol by microorganisms is of utmost commercial importance in the manufacture of alcoholic beverages and organic reagents; however, it is of limited usefulness in the laboratory identification of bacteria.

The bacterial fermentation of lactose is more complex than that of glucose. Lactose is a disaccharide composed of glucose and galactose, connected through an oxygen linkage known as a **galactoside bond.** On hydrolysis, this bond is severed, releasing glucose and galactose. For a bacterium to metabolize lactose, two enzymes must be present: (1) β-galactoside permease, permitting the transport of a β-galactoside, such as lactose, through the bacterial cell wall; and (2) β-galactosidase, the enzyme required to hydrolyze the β-galactoside bond once the disaccharide has entered the cell. The final acid reaction results from the degradation of glucose as shown in Figure 6-3.

Because lactose fermentation ultimately proceeds by way of glucose degradation through the EMP, it follows that any organism incapable of metabolizing glucose cannot form acid from lactose. This explains why glucose is omitted from the formulas of primary isolation media such as MacConkey agar and EMB agar: if it is not omitted, the ability to detect the lactose-fermenting capability of the test bacteria would be lost. In the test medium, the end point of lactose fermentation is the detection of acid production. A non–lactose-fermenting organism is one that lacks either one or both of the two enzymes required for lactose metabolism or lacks the ability to attack glucose. So-called late lactose-fermenters are believed to be organisms that exhibit β-galactosidase activity but show sluggish β-galactoside permease activity.

β-Galactosidase and the ONPG Test. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) is a compound structurally



■ **FIGURE 6-2** Mixed acid and butylene glycol pathways of glucose fermentation.

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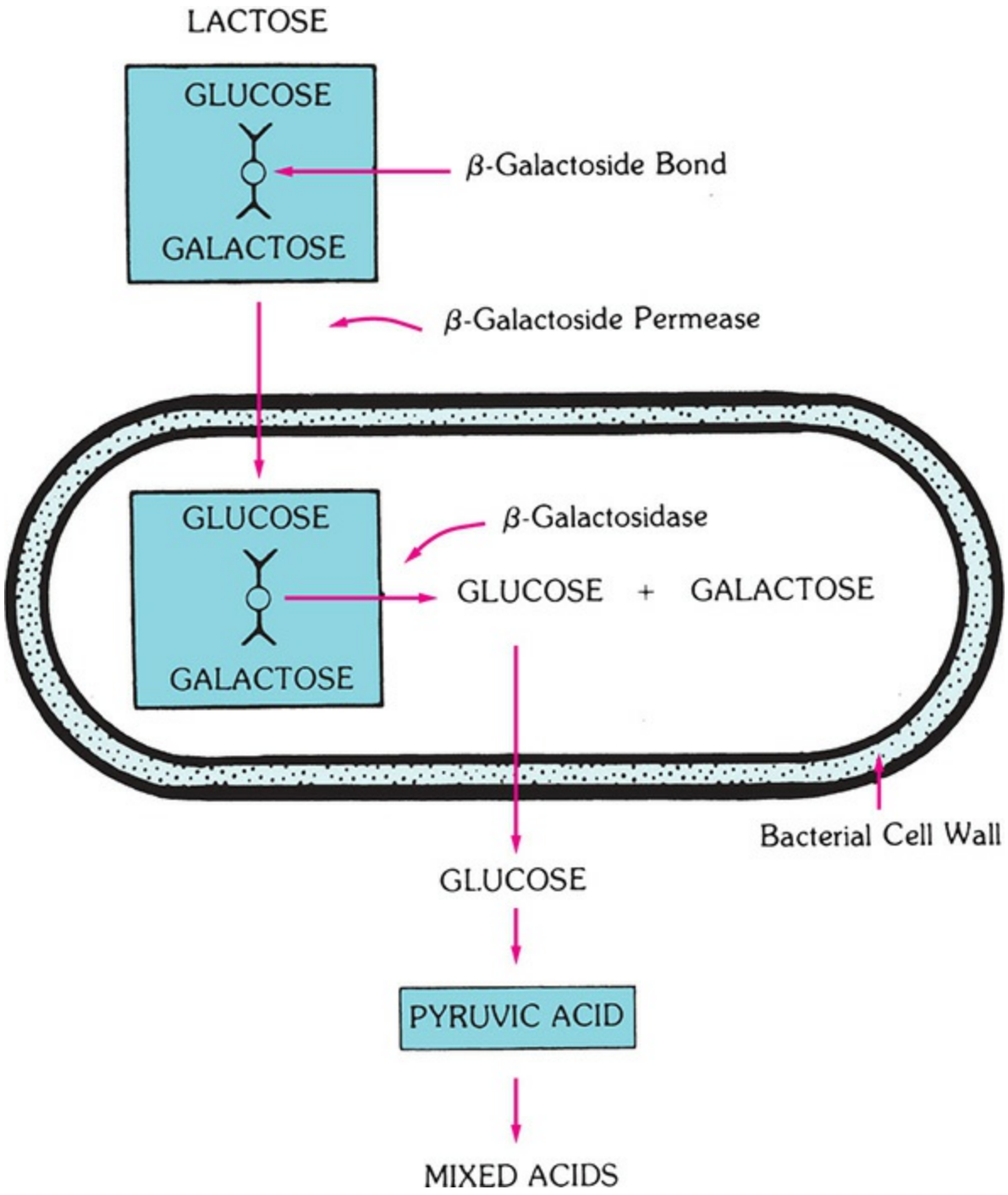

■ **FIGURE 6-3** Bacterial fermentation of lactose: Lactose, a disaccharide composed of molecules of glucose and galactose joined by a β-galactoside bond, diffuses through the bacterial cell wall under the action of β-galactoside permease. If the bacterium produces β-galactosidase, the lactose is hydrolyzed to produce glucose and galactose. The glucose is then metabolized as illustrated in Figure 6-1.

similar to lactose except that the glucose has been replaced by an *o*-nitrophenyl group.

This rather ingenious manipulation of the molecule forms the basis for the ONPG test, which is outlined in Chart 6-1. This test detects the enzyme β-galactosidase far more quickly than does the test for lactose fermentation previously described. This is helpful in identifying late lactose-fermenters that are deficient in β-galactoside permease. ONPG permeates the bacterial cell more readily than lactose and, under the action of β-galactosidase, is hydrolyzed into galactose and *o*-nitrophenol (see Chart 6-1). *o*-Nitrophenol is a chromophore that is colorless when bound to D-galactopyranoside but is yellow in its free (unbound) form (see Image Plate 6-4A).

ONPG test tablets that can be easily reconstituted by adding a small amount of water are available commercially and are convenient for use in the laboratory. Organisms with strong β-galactosidase activity may produce a positive test within a few minutes after inoculation of the medium. The ONPG test is most helpful for detection of β-galactosidase activity in late lactose-fermenters, such as some strains of *E. coli*, in which differentiation from species of *Shigella* (except certain strains of *Shigella sonnei*) may otherwise be difficult. The test is also helpful in distinguishing some strains of *Citrobacter* species and *Salmonella* ser. Arizonae (ONPG-positive) from most *Salmonella* species (ONPG-negative). The ONPG test is not a substitute for the determination of lactose fermentation because only the enzyme β-galactosidase is measured.

Cytochrome Oxidase Activity. Any organism that displays cytochrome oxidase activity following the procedure and test conditions outlined in Chart 1-5 is excluded from the *Enterobacteriaceae*. The developing color reaction must be interpreted within 10 to 20 seconds because many organisms, including selected members of the *Enterobacteriaceae*, may produce delayed false-positive reactions. Both oxidase-positive and oxidase-negative control organisms should be tested if there is difficulty in interpreting the cytochrome oxidase reaction. The commercial cytochrome oxidase droppers are used most often because of their convenience. The color reactions are clearly visible within 10 seconds. If metal inoculating loops or wires are used in the laboratory for transferring bacteria to the oxidase reagent, those made from stainless steel or Nichrome may produce false-positive reactions, owing to trace amounts of iron oxide on the flamed surface of the metal. This problem can be circumvented by using plastic or platinum inoculating loops or by using wooden applicator sticks or cotton swabs to perform the oxidase test. Tetramethyl- *p*-phenylenediamine, rather than the dimethyl derivative, is most commonly used because the reagent is more stable, more sensitive, and less toxic (see Chart 1-5 and Image Plate 6-1G).

Nitrate Reduction. All *Enterobacteriaceae*, with the exception of certain biotypes of *Pantoea agglomerans* and certain species of *Serratia* and *Yersinia*, reduce nitrate to nitrite. Because a variable period of incubation (3 to 24 hours, depending on the system used) is required to perform the nitrate reduction test, it is not commonly used

to prescreen unknown bacterial isolates. Rather, the test is used in most laboratories either to confirm the correct classification of an unknown microorganism or as an aid in determining the identification of bacterial species. Details of the nitrate reduction test are presented in Chart 6-2.

Any basal medium that supports the growth of the organism and contains a 0.1% concentration of potassium nitrate (KNO_3) is suitable for performing this test. Nitrate broth and nitrate agar in a slant are the media forms most commonly used in clinical laboratories. Because the enzyme nitrate reductase is maximally active under anaerobic conditions, Zobell[688] has recommended the use of semisolid agar. Semisolid media also enhance the growth of many bacterial species and provide the anaerobic environment needed for enzyme activation. The addition of zinc dust to all negative reactions, as shown in Chart 6-2, should be a routine procedure. Most organisms capable of reducing nitrates will do so within 24 hours; some may produce detectable quantities within 2 hours. A rapid nitrate test has been described by Schreckenberger and Blazevic.[586] Both α-naphthylamine and sulfanilic acid are relatively unstable, so their reactivity should be determined at frequent intervals by testing with positiveand negative control organisms. The diazonium compound that forms from the reaction of the reduced nitrate and reagents is also relatively unstable, and the color tends to fade; accordingly, readings should be made soon after the reagents are added (see Image Plate 6-1H).

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Culture Media Used for Detection of Carbohydrate Fermentation

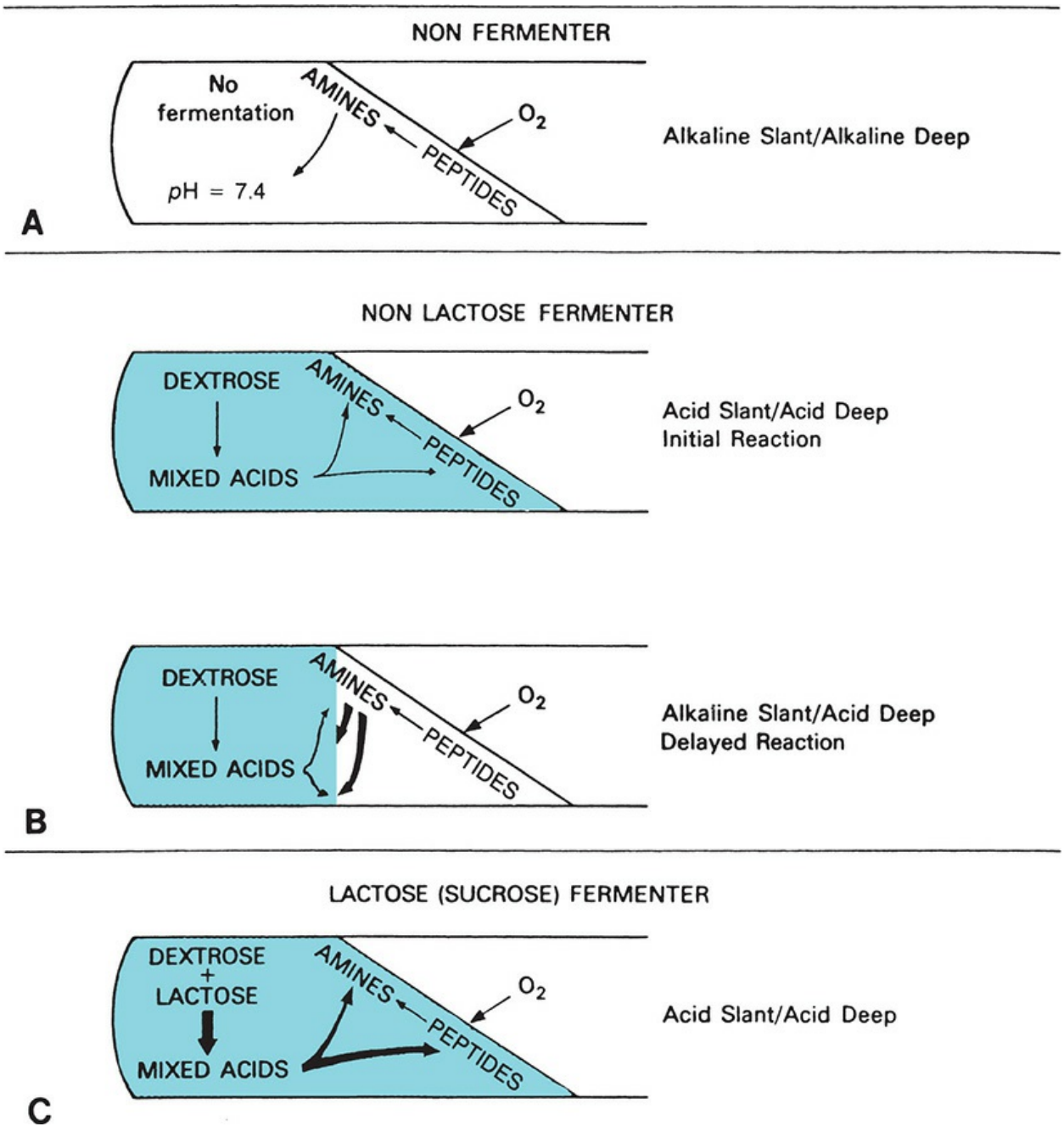
A variety of liquid or agar media can be used to measure the ability of a test organism to fermentatively utilize carbohydrates. The carbohydrate to be tested, such as glucose, is filter-sterilized and added aseptically to a basal medium to a final concentration of 0.5% to 1.0%. The formula of a typical basal fermentation medium contains trypticase (BBL), 10 g; sodium chloride, 5 g; phenol red, 0.018 g; and distilled water to equal 1 L. Trypticase is a hydrolysate of casein that serves as a source for carbon and nitrogen; sodium chloride is an osmotic stabilizer; and phenol red is a pH indicator that turns yellow when the pH of the medium drops below 6.8. Image Plate 6-1F illustrates acid fermentation reactions in purple broth medium. All of the *Enterobacteriaceae* grow well in this type of medium, and the base formula used is a matter of personal preference. In addition to producing a pH color shift in fermentation culture media, the production of mixed acids, notably butyric acid, often

results in a pungent, foul odor from the culture medium. When such an odor is detected, one should immediately be suspicious of the presence of one of the *Enterobacteriaceae* (in addition, the anaerobic bacteria produce characteristic metabolic products with distinctive odors).

Use of Kligler's Iron Agar and Triple Sugar Iron Agar

In practice, microorganisms that are capable of fermenting glucose are commonly detected by observing the reactions they produce when grown on Kligler's iron agar (KIA) or triple sugar iron (TSI) agar (Fig. 6-4; see Image Plate 6-1E). If an organism cannot ferment glucose, then an alkalineslant–alkaline-butt (no change) reaction is observed (see Fig. 6-4A), indicating a lack of acid production and failure of the test organism to ferment any of the sugars present. This reaction alone is sufficient to exclude an organism from the *Enterobacteriaceae*. The formula for KIA is listed in Box 6-1 (the formula for TSI is identical except that 10 g of sucrose is added).

Several observations are important in studying the formulas of KIA and TSI. The incorporation of four protein derivatives—beef extract, yeast extract, peptone, and proteose peptone—makes KIA and TSI nutritionally very rich. The lack of inhibitors permits the growth of all but the most fastidious bacterial species (excluding the obligate anaerobes). For this reason, KIA and TSI agar can be used only when testing a bacterial species selected from a single colony recovered on primary or selective agar plates. Glucose and lactose (and sucrose in TSI medium) are evenly distributed throughout both the slant and butt (deep) portion of the tube. However, lactose is present in a concentration 10 times



■ **FIGURE 6-4** Three general types of reactions produced by bacteria growing on KIA: **A.** Nonfermentative bacilli that are unable to produce acids from the fermentation of glucose or lactose; there is no change in the medium (represented by *white*). **B.** Initial acidification of both the deep and the slant of the medium (*shaded area*) by bacteria that ferment glucose, but the slant reverts back to alkaline pH as alkaline amines are formed from the oxidative decarboxylation of peptides (derived from protein in the medium) near the surface. **C.** Complete permanent acidification of both the deep and the slant of the tube by lactose-fermenting bacteria.

BOX 6-1

Kligler's Iron Agar

Beef extract, 3 g
Yeast extract, 3 g
Peptone, 15 g
Proteose peptone, 5 g
Lactose, 10 g
Glucose, 1 g
Ferrous sulfate, 0.2 g
Sodium chloride, 5 g
Sodium thiosulfate, 0.3 g
Agar, 12 g
Phenol red, 0.024 g
Distilled water to equal 1 L
Final pH, 7.4

that of glucose (similarly, the ratio of sucrose to glucose is 10:1 in TSI medium). This 10:1 ratio is important to the understanding of the biochemical principles discussed later. Ferrous sulfate as a hydrogen sulfide detector is somewhat less sensitive than other ferric or ferrous salts; therefore, there may be discrepancies in the hydrogen sulfide readings between KIA and TSI and other test media (see Image Plate 6-4B). The phenol red indicator is yellow below a pH of 6.8. Because the pH of the uninoculated medium is buffered at 7.4, relatively small quantities of acid production result in a visible color change.

Biochemical Principles

The biochemical principles underlying the reactions observed in KIA or TSI agar are illustrated in Figure 6-4. Note that the molten agar is allowed to solidify in a slant. This configuration results in essentially two reaction chambers within the same tube. The **slant** portion, exposed throughout its surface to atmospheric oxygen, is aerobic; the lower portion, called the **butt** or the **deep,** is protected from the air and is relatively anaerobic. It is important when preparing the media that the slant and the deep are kept equal in length, approximately 3 cm (1.5 in.) each, so that this two-chamber effect is preserved.

KIA and TSI tubes are inoculated with a long, straight wire. The well-isolated test colony recovered from an agar plate is touched with the end of the inoculating needle, which is then stabbed into the deep of the tube, extending to within 3 to 5 mm of its bottom. When the inoculating wire is removed from the deep of the tube, the slant surface is streaked with a back-and-forth motion. Inoculated tubes are placed into an incubator at 35°C for 18 to 24 hours. The color photographs shown in Image Plates 6-1E and 7-1A reveal the reactions that are described in Box 6-2. Thus, as shown in Figure 6-4A and in Plate 7-1A, without carbohydrate fermentation, no acids are formed, and the amine production in the slant together with the alkaline buffers produce a red color throughout the medium. The bacteria that produce this type of reaction are known as **nonfermenters** (see Chapter 7).

BOX 6-2

Reactions on KIA

Alkaline Slant/Alkaline Deep (K/K)

No carbohydrate fermentation. This is characteristic of nonfermentative bacteria, such as *Pseudomonas aeruginosa*.

Alkaline Slant/Acid Deep (K/A)

Glucose fermented; lactose (or sucrose for TSI medium) not fermented. This is characteristic of non–lactose-fermenting bacteria, such as *Shigella* species.

Alkaline Slant/Acid (Black) Deep (K/A/H_2S)

Glucose fermented; lactose not fermented, hydrogen sulfide produced. This is characteristic of non–lactose-fermenting, hydrogen sulfide-producing bacteria, such as *Salmonella* species, *Citrobacter* species, and *Proteus* species.

Acid Slant/Acid Deep (A/A)

Glucose and lactose (or sucrose with TSI) fermented. This is characteristic of lactose-fermenting coliforms, such as *Escherichia coli* and the *Klebsiella–Enterobacter* species.

If the KIA tube is inoculated with a glucose-fermenting organism that cannot utilize lactose, only a relatively small quantity of acid can be obtained from the 0.1% concentration of glucose in the medium. Initially, during the first 8 to 12 hours of incubation, even this amount of acid may be sufficient to convert both the deep and

the slant color to yellow. Within the next few hours, however, the glucose supply is completely exhausted, and the bacteria begin oxidative degradation of the amino acids within the slant portion of the tube where oxygen is present. This results in the release of amines that soon counteract the small quantities of acid present in the slant; by 18 to 24 hours, the entire slant reverts to an alkaline pH, and the color returns to red. In the deep (anaerobic portion) of the tube, however, amino acid degradation is insufficient to counteract the acid formed, and the medium remains yellow. Thus, the alkaline-slant–acid-deep reaction on KIA (or TSI) is an important initial indicator that the test organism is a non–lactose-fermenter (see Fig. 6-4B and Image Plate 6-1E).

If the KIA tube is inoculated with a lactose-fermenting organism, then, even though the glucose is completely used up after the first 8 to 12 hours, fermentation continues as the organism is able to use lactose (present in 10 times the concentration of glucose). Consequently, when the tube is examined at the end of 18 to 24 hours, acid production from fermentation of lactose is still occurring, and both the slant and the deep appear yellow, resulting in an acid-slant–aciddeep reaction (see Fig. 6-4 *C* and Image Plate 6-1E).

Many microbiologists prefer TSI over KIA because the addition of sucrose to the formula helps screen for *Salmonella*

and *Shigella* species, because neither of these (except rare strains) metabolizes either lactose or sucrose. Therefore, any acid–acid reaction of TSI indicates that either lactose, sucrose, or both have been fermented, excluding *Salmonella* and *Shigella.* It should also be remembered that *Y. enterocolitica* ferments sucrose, but not lactose; thus, on TSI, the reaction will be acid–acid (similar to coliforms such as *E. coli*), but on KIA, the reaction will be alkaline–acid (similar to a non–lactose-fermenter). Consequently, when screening stool specimens for *Salmonella*, *Shigella*, and *Yersinia*, some might argue that KIA is preferable to TSI.

For the detection of hydrogen sulfide, which is colorless, the medium must include an indicator. Sodium thiosulfate is the source of sulfur atoms in most media used for hydrogen sulfide production. Iron salts (ferrous sulfate and ferric ammonium citrate) incorporated in the culture media then react with hydrogen sulfide to produce an insoluble black precipitate (ferrous sulfide). An acid environment is required for an organism to produce hydrogen sulfide and, therefore, a source of hydrogen ions must be provided. Because the deep of the KIA and TSI tubes becomes acidic with glucose fermentation (hydrogen ions increase), the blackening is often first seen or confined there, particularly with non–lactosefermenting bacteria (see Image Plate 6-1E). Thus, it follows that a black deep should be read as acid even if the usual yellow color is obscured by the black precipitate. KIA and TSI are less sensitive in the detection of hydrogen sulfide than other iron-containing media, such as sulfide indole motility

(SIM) medium (see Image Plate 6-4B).

If an organism can be excluded from the *Enterobacteriaceae* before an extended battery of biochemical tests is set up, considerable time and labor will be saved. It is recommended that either a KIA or a TSI slant be set up on all isolates suspected of being one of the *Enterobacteriaceae* at the same time that differential test media of kit systems are set up. Even if an organism is a fermenter and is suspected of being one of the *Enterobacteriaceae*, a cytochrome oxidase test should be performed to exclude organisms belonging to other genera of fermenting bacteria, such as *Aeromonas, Plesiomonas, Vibrio*, and *Pasteurella* species, that are oxidase-positive.

Selection of Primary Isolation Media

Selective culture media must be used to recover the significant species of bacteria from specimens that may harbor a mixture of microorganisms. To make rational selections, microbiologists must know the composition of each formula and the purpose and relative concentration of each chemical or compound that is included. For example, it is not sufficient to know that bile salts are included in the formulas of a number of selective media to inhibit the growth of grampositive and some of the more fastidious gram-negative bacteria species. For example, *Salmonella–Shigella* (SS) agar contains about five times the concentration of bile salts compared with MacConkey agar and is more inhibitory to *E. coli* and more selective for the recovery of *Salmonella* species from stool cultures.

For the recovery of the *Enterobacteriaceae* from clinical specimens that potentially harbor mixed bacteria, three general types of media are available: (1) nonselective media for primary isolation (e.g., blood agar); (2) selective or differential agars (e.g., MacConkey and Hektoen enteric agars); and (3) enrichment broths. Tables 6-1 and 6-2 compare different media commonly used in clinical practice. The formulas are complex and include ingredients that not only inhibit the growth of certain bacterial species (selective), but also detect several biochemical characteristics that are important in making a preliminary identification of the microorganisms present in the specimen (differential).

Chemicals and Compounds Used in Selective Media

Box 6-3 lists the general types of chemicals and compounds used in selective media, including brief comments on the function of each.

Selective Isolation Media

In 1905, MacConkey[403] first described a selective differential medium (neutral red–

bile salt agar) that he used to isolate gram-negative enteric bacilli from specimens containing mixtures of bacterial species. He incorporated lactose and the indicator neutral red into this medium to provide a visual means for detecting lactose utilization by the test organism. At that time, all non–spore-forming, gram-negative bacilli were still referred to as enteric organisms; however, microbiologists had recognized that certain species were more pathogenic to humans than others. The carbohydrate-utilization patterns of several species of bacteria were already known by the turn of the century, and the fermentation of lactose, in particular, was recognized as an important marker for differentiating certain enteric pathogens. Holt–Harris and Teague[297] in 1916 described a medium with eosin and methylene blue as indicators for differentiating between lactose-fermenting and non–lactosefermenting colonies. Sucrose was included in the medium to detect members of the coliform group that ferment sucrose more readily than lactose.

MacConkey and EMB agars are only moderately inhibitory and are designed primarily to prevent growth of gram-positive bacteria from mixed cultures. Many species of fastidious gram-negative organisms are inhibited as well; however, all *Enterobacteriaceae* grow well. Table 6-1 compares the formulas, inhibitory ingredients, and key differential characteristics for MacConkey and EMB agars. A new formulation of MacConkey agar, MacConkey III, was developed by researchers at Becton–Dickinson to improve recovery of CO_2-sensitive *Enterobacteriaceae*. Although aerobic incubation is recommended for MacConkey agar, it has become common in many clinical microbiology laboratories to incubate all primary plates including MacConkey agar in a 5% CO_2 incubator in order to keep all plates together. The problem with this is that some strains of *Enterobacteriaceae* may fail to grow or show partial inhibition when incubated in CO_2 because of a decrease in pH of the medium. In one study, MacConkey III was shown to

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TABLE 6-1 Selective Differential Media for Recovery of *Enterobacteriaceae*

Medium	Formulation		Purpose and Differential Ingredients	Reactions and Interpretation
MacConkey agar (see Image Plates 6-2*A* and *B*)	Peptone Polypeptone Lactose Bile salts Sodium chloride Agar Neutral red Crystal violet Distilled water to Final pH, 7.1	17 g 3 g 10 g 1.5 g 5 g 13.5 g 0.03 g 0.001 g 1 L	MacConkey agar is a differential-plating medium for the selection and recovery of the *Enterobacteriaceae* and related enteric gram-negative bacilli. The bile salts and crystal violet inhibit the growth of gram-positive bacteria and some fastidious gram-negative bacteria. Lactose is the sole carbohydrate. Lactose-fermenting bacteria produce colonies that are in varying shades of red, owing to the conversion of the neutral red indicator dye (red below pH 6.8) from the production of mixed acids. Colonies of non–lactose-fermenting bacteria appear colorless or transparent.	Typical strong lactose fermenters, such as species of *Escherichia, Klebsiella,* and *Enterobacter*, produce red colonies surrounded by a zone of precipitated bile. Slow or weak lactose fermenters, such as *Citrobacter, Providencia, Serratia,* and *Hafnia*, may appear colorless after 24 hours or slightly pink in 24–48 hours. Species of *Proteus, Edwardsiella, Salmonella*, and *Shigella*, with rare exceptions, produce colorless or transparent colonies. Representative colonies, showing these various reactions, are shown in Image Plate 6-2.
Eosin methylene blue (EMB) agar (see Image Plates 6-2*C* through *F*)	Peptone Lactose Sucrose[a] Dipotassium, PO_4 Agar Eosin Y Methylene blue Distilled water to Final pH, 7.2	10 g 5 g 5 g 2 g 13.5 g 0.4 g 0.065 g 1 L	EMB agar is a differential-plating medium that can be used in place of MacConkey agar in the isolation and detection of the *Enterobacteriaceae* or related coliform bacilli from specimens with mixed bacteria. The aniline dyes (eosin and methylene blue) inhibit gram-positive and fastidious gram-negative bacteria. They combine to form a precipitate at acid pH, thus also serving as indicators of acid production. Levine EMB, with only lactose, gives reactions more in parallel with MacConkey agar; the modified formula also detects sucrose fermenters.	Typical strong lactose-fermenting colonies, notably *Escherichia coli*, produce colonies that are green-black with a metallic sheen. Weak fermenters, including *Klebsiella, Enterobacter, Serratia*, and *Hafnia*, produce purple colonies within 24–48 hours. Non–lactose-fermenters, including *Proteus, Salmonella*, and *Shigella*, produce transparent colonies. *Yersinia enterocolitica*, a nonlactose, sucrose fermenter, produces transparent colonies on Levine EMB and purple-to-black colonies on the modified formula. See Image Plate 6-2.

[a]Modified Holt–Harris–Teague formula. Sucrose is not contained in Levine EMB agar.

TABLE 6-2 Highly Selective Media for Recovery of *Enterobacteriaceae* From Gastrointestinal Specimens

Medium	Formulation		Purpose and Differential Ingredients	Reactions and Interpretation
Salmonella–Shigella (SS) agar (see Image Plates 6-3*G* and *H*)	Beef extract Peptone Lactose Bile salts Sodium citrate Sodium thiosulfate Ferric citrate Agar Neutral red Brilliant green Distilled water to Final pH, 7.4	5 g 5 g 10 g 8.5 g 8.5 g 8.5 g 1 g 12.5 g 0.025 g 0.033 g 1 L	SS agar is a highly selective medium formulated to inhibit the growth of most coliform organisms and permit the growth of species of *Salmonella* and *Shigella* from environmental and clinical specimens. The high bile salts concentration and sodium citrate inhibit all gram-positive bacteria and many gram-negative organisms, including coliforms. Lactose is the sole carbohydrate and neutral red is the indicator for acid detection. Sodium thiosulfate is a source of sulfur. Any bacteria that produce hydrogen sulfide gas are detected by the black precipitate formed with ferric citrate (relatively insensitive).High selectivity of SS agar permits use of heavy inoculum.	Any lactose-fermenting colonies that appear are colored red by the neutral red. Rare strains of *Salmonella arizonae* are lactose fermenting, and colonies may simulate *Escherichia coli.* Growth of species of *Salmonella* is uninhibited, and colonies appear colorless with black centers, owing to hydrogen sulfide gas production. Species of *Shigella* show varying inhibition and colorless colonies with no blackening. Motile strains of *Proteus* that appear on SS agar do not swarm. See Image Plate 6-3

Hektoen enteric (HE) agar (see Image Plates 6-3E and F)	Peptone Yeast extract Bile salts Lactose Sucrose Salicin Sodium chloride Sodium thiosulfate Ferric ammonium citrate Acid fuchsin Thymol blue Agar Distilled water to Final pH, 7.6	12 g 3 g 9 g 12 g 12 g 2 g 5 g 5 g 1.5 g 0.1 g 0.04 g 14 g 1 L	HE agar is a recent formulation devised as a direct-plating medium for fecal specimens to increase the yield of species of *Salmonella* and *Shigella* from the heavy numbers of normal flora. The high bile salt concentration inhibits growth of all gram-positive bacteria and retards the growth of many strains of coliforms. Acids may be produced from the carbohydrates, and acid fuchsin reacting with thymol blue produces a yellow color when the pH is lowered. Sodium thiosulfate is a sulfur source, and hydrogen sulfide gas is detected by ferric ammonium citrate (relatively sensitive).	Rapid lactose fermenters (such as *E. coli*) are moderately inhibited and produce bright-orange to salmon-pink colonies. *Salmonella* colonies are blue-green, typically with black centers from hydrogen sulfide gas. *Shigella* appear greener than *Salmonella*, with the color fading to the periphery of the colony. *Proteus* strains are somewhat inhibited; colonies that develop are small, transparent, and more glistening or watery in appearance than species of *Salmonella* or *Shigella*. See Image Plate 6-3.
Xylose lysine deoxycholate (XLD) agar (see Image Plates 6-3A through D)	Xylose Lysine Lactose Sucrose Sodium chloride Yeast extract Phenol red Agar Sodium deoxycholate Sodium thiosulfate Ferric ammonium citrate Distilled water to Final pH, 7.4	3.5 g 5 g 7.5 g 7.5 g 5 g 3 g 0.08 g 13.5 g 2.5 g 6.8 g 0.8 g 1 L	XLD agar is less inhibitory to growth of coliform bacilli than HE agar and was designed to detect shigellae in feces after enrichment in gram-negative broth. Bile salts in relatively low concentration make this medium less selective than the other two included in this table. Three carbohydrates are available for acid production, and phenol red is the pH indicator. Lysine-positive organisms, such as most *Salmonella* species, produce initial yellow colonies from xylose utilization and delayed red colonies from lysine decarboxylation. Hydrogen sulfide detection system is similar to that of HE agar.	Organisms such as *E. coli* and *Klebsiella–Enterobacter* species may use more than one carbohydrate and produce bright yellow colonies. Colonies of many species of *Proteus* are also yellow. Most species of *Salmonella* produce red colonies, most with black centers from hydrogen sulfide gas. *Shigella*, *Providencia*, and many *Proteus* species use none of the carbohydrates and produce translucent colonies. *Citrobacter* colonies are yellow with black centers; many *Proteus* species are yellow or translucent with black centers; salmonellae are red with black centers. See Image Plate 6-3.

provide superior recovery and colony size in both air and CO_2 incubation to a comparator MacConkey agar.[355]

Deciding whether to use MacConkey or EMB agar is largely a matter of personal preference, because bacterial species that use lactose can be differentiated on both. MacConkey agar contains neutral red as the pH indicator and, as a result, lactose-metabolizing colonies appear pink from the production of mixed acids (see Image Plate 6-2A and B). Strong acid-producing bacteria, such as *E. coli*, form deep red colonies with a diffuse pink precipitate in the agar surrounding the colonies caused by precipitation of the bile salts in the medium that occurs at a low pH (see Image Plate 6-2A). Weaker acid-producing bacteria form light-pink colonies or colonies that are clear at the periphery and have pink centers, with the agar surrounding the colonies remaining clear (see Image Plate 6-1C). On EMB agar, strong acid-producing bacteria form colonies that have a metallic sheen (see Image Plate 6-2C and D). The appearance of the sheen, caused by precipitation of dye in the colonies, is highly suggestive of *E. coli*, although other strong acid producers, such as *Y. enterocolitica*, may have a similar appearance.

Highly Selective Isolation Media Used Primarily for Gastrointestinal Specimens

Media are made highly selective by the addition of a variety of inhibitors to their formulas, generally in higher concentrations than in MacConkey and EMB agars. These media are used primarily to inhibit the growth of *E. coli* and other "coliforms," but they allow *Salmonella* and *Shigella* species to grow out from stool specimens.

Several selective media formulated for use in clinical laboratories are discussed here. The most commonly used are SS agar, xylose lysine deoxycholate (XLD) agar, and HE agar. These are described in Table 6-2.

Deciding which of these selective media to use for the recovery of enteric pathogens from fecal specimens depends both on personal preference and on the species to be selected. In general, these media are used in the clinical laboratory for the recovery of *Salmonella* and *Shigella* species from diarrheal stool specimens, or in public health laboratories to investigate possible fecal contamination of food and water supplies. Virtually all species of *Salmonella* grow well in the presence of bile salts, which explains why the gallbladder often serves as one reservoir for human carriers. Bile

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BOX 6-3

Chemicals and Compounds Used in Selective Media

Protein hydrolysates (e.g., peptones, meat infusion, tryptones, and casein): Proteins are cleaved by acids or enzymes into amino acids and peptides that can be used by bacteria to provide the carbon and nitrogen needed for bacterial metabolism.

Carbohydrates: A variety of disaccharides (e.g., lactose, sucrose, and maltose), hexoses (dextrose), and pentoses (xylose) are included in selective media for two purposes: (1) to provide a ready source of carbon for energy and (2) to serve as substrates in biochemical reactions for identification of unknown organisms.

Buffers: Balanced monosodium and disodium or potassium phosphates are most commonly used. Buffers provide (1) a stable pH for optimal growth of microorganisms and (2) a standard reference pH for those media in which acid or alkaline reactions are used to identify microorganisms.

Enrichments (e.g., blood, serum, vitamin supplements, and yeast extracts): Growth supplements are added to media to recover fastidious organisms. Enrichments are less commonly used for the recovery of the *Enterobacteriaceae* because most members of this group grow without them.

Inhibitors: Various compounds may serve to inhibit the growth of certain undesired bacterial species, thus making the medium selective: (1) aniline dyes (e.g., brilliant green and eosin), (2) heavy metals (e.g., bismuth), (3) chemicals (e.g., azide, citrate, deoxycholate, selenite, and phenylethyl alcohol), and (4) antimicrobial agents (e.g., neomycin, colistin, vancomycin, and chloramphenicol). Their relative

concentrations are important in determining the selectivity of the medium in which they are contained.

pH indicators: Fuchsin, methylene blue, neutral red, phenol red, and bromcresol purple are commonly used indicators in test media to measure pH shifts resulting from bacterial metabolism of given substrates.

Miscellaneous indicators: Other indicators may be included to detect specific bacterial products (e.g., ferric and ferrous ions for the detection of hydrogen sulfide).

Miscellaneous compounds and chemicals: Agar, a gelatinous extract of red seaweed, is commonly added to a medium, in varying concentrations, as a solidifying agent. Concentrations of 1% to 2% are used for plating media; concentrations of 0.05% to 0.3% are used for semisolid motility media; and trace amounts are added to anaerobic broth media to prevent convection currents and oxygen penetration. Sodium thiosulfate is commonly added to provide a source of sulfur.

salts are added to selective media because other species of enteric bacilli, including some of the more fastidious strains of *Shigella,* grow poorly or not at all. SS and HE agars contain relatively high concentrations of bile salts and are well adapted for recovering *Salmonella* species from specimens heavily contaminated with other coliform bacilli. However, because of its inhibitory effect on the recovery of certain strains of *Shigella* species, the routine use of SS agar as a single selective medium for isolation of enteric pathogens from stool specimens is not recommended.

XLD agar contains lactose, sucrose, and xylose[627]; thus, microorganisms that ferment these carbohydrates form yellow colonies (see Image Plate 6-3A). Bacteria incapable of fermenting these carbohydrates do not produce acids and form colorless colonies (see Image Plate 6-3B). Organisms that produce hydrogen sulfide form black pigment beginning in the center of the colonies (see Image Plate 6-3C). XLD agar also contains lysine. This is important because many species of *Salmonella* will ferment xylose and, therefore, will initially produce yellow colonies on XLD, but because these same species also decarboxylate lysine, the colonies will revert to pink after the small amount of xylose in the medium is used up. Lactose and sucrose, added in excess, prevent lysine-positive coliforms from similarly reverting. Because the decarboxylation of lysine results in the formation of strongly alkaline amines, a light-pink halo may appear around the colonies on XLD agar (see Image Plate 6-3C). Black colonies without a pink halo are more suggestive of a hydrogen sulfideproducing strain of *Proteus* species (see Image Plate 6-3D).

The carbohydrates in HE agar are lactose, sucrose, and salicin.[354] Microorganisms capable of fermenting these carbohydrates also form yellow colonies (see Image Plate 6-3E); asaccharolytic strains produce colonies that are translucent or light green (see

Image Plate 6-3F). Lactoseand sucrosenegative bacteria that acidify salicin may produce orange colonies. HE agar also contains ferric salts; thus hydrogen sulfide-producing colonies appear black.

Bismuth sulfite and brilliant green agars are highly selective media that are not commonly used in clinical laboratories. They are difficult to prepare, and their shelf life is very short (48 to 72 hours). These media are specifically designed to recover *Salmonella typhi* from fecal specimens and are particularly useful when screening numerous patients in endemic areas or during an epidemic. *Salmonella* species (*S. typhi* in particular) can be suspected on these media because of the propensity to produce colonies with a black sheen.

Enrichment Media

As the name indicates, an enrichment medium is used to enhance the growth of certain bacterial species and inhibit

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the development of unwanted microorganisms. Enrichment media are most commonly used in clinical laboratories for the recovery of *Salmonella* and *Shigella* species from fecal specimens. Enrichment broths are particularly helpful in the recovery of organisms from the stools of *Salmonella* carriers or from patients with light *Shigella* infections in whom the number of organisms may be as low as 200 per gram of feces. (*E. coli* and other enteric bacilli may reach massive concentrations, as high as 10^9 per gram of feces.)

Enrichment media work on the principle that *E. coli* and other gram-negative organisms, which constitute the normal fecal flora, are maintained in a prolonged **lag** phase by the inhibitory chemicals in the broth. *Salmonella* and *Shigella* species are far less inhibited, enter into a log phase of growth, and are more readily recovered from fecal samples. However, after several hours, the enrichment media no longer suppress the growth of *E. coli* and other enteric organisms, which will ultimately overgrow the culture. Thus, for maximal recovery of *Salmonella* and *Shigella* species from fecal samples, it is recommended that the enrichment broth be subcultured within 8 hours.

The two most commonly used enrichment media are selenite broth and gram-negative broth. Selenite broth is more inhibitory to the growth of *E. coli* and other enteric gramnegative bacilli than is gram-negative broth. Thus, selenite broth is best adapted for the recovery of *Salmonella* or *Shigella* species from heavily contaminated specimens, such as feces or sewage. However, gram-negative broth is used with greater frequency in clinical laboratories because it is less inhibitory to the growth of many of the more fastidious strains of *Shigella* species. Enrichment of fecal specimens in gramnegative broth for 4 to 6 hours and then subculturing to HE or XLD

agar is the optimal technique for the recovery of *Shigella* species in suspected cases of bacillary dysentery. The formulas and salient characteristics of these two enrichment media are summarized in Table 6-3.

Guidelines for Choosing Selective Isolation Media

The media listed in Tables 6-1 through 6-3 and the several combinations in which they can be used may be somewhat confusing. The following is a guide for selecting media that may be optimal in the recovery of the *Enterobacteriaceae* from clinical specimens.

For specimens other than feces or rectal swabs, a combination of MacConkey or EMB agar and a blood agar is usually sufficient. Media with greater inhibitory properties are not routinely required because the concentration of commensal flora or contaminating organisms is relatively low in most nonenteric specimens. Subculturing to a more inhibitory medium can be done in instances in which it appears necessary.

For fecal specimens or rectal swabs, it is necessary to select only one medium from each of the groups listed in Tables 6-1 and 6-2. The approach outlined in Box 6-4 is suggested.

Differential Identification Characteristics

Although a preliminary identification of the *Enterobacteriaceae* is possible based on colony characteristics and biochemical reactions on primary isolation media, further species identification requires the determination of additional phenotypic characteristics that reflect the genetic code and unique identity of the organism being tested. It is the purpose of this discussion to review the salient features of the tests that measure these phenotypic characteristics and are commonly used in clinical laboratories. This orientation is necessary so that laboratory personnel can develop a fundamental understanding of the principles behind these procedures in order to recognize and correct any biochemical inconsistencies, problems with mixed cultures, or faulty techniques. It is not possible to discuss the variety of differential tests and numerous schemes available for the final species identification of the *Enterobacteriaceae*. However, several of the tests widely used in clinical laboratories to measure the metabolic characteristics by which all but a few rare or atypical species of the *Enterobacteriaceae* can be identified are listed in Box 6-5. Carbohydrate utilization and ONPG activity have been discussed previously.

TABLE 6-3 Enrichment Broths for Recovery of *Enterobacteriaceae*

Broth	Formulation		Purpose and Differential Ingredients	Reactions and Interpretation
Selenite broth	Peptone Lactose Sodium selenite Sodium phosphate Distilled water to Final pH, 7.0	5 g 4 g 4 g 10 g 1 L	Selenite F broth is recommended for the isolation of salmonellae from specimens—such as feces, urine, or sewage—that have heavy concentrations of mixed bacteria. Sodium selenite is inhibitory to *Escherichia coli* and other coliform bacilli, including many strains of *Shigella*. The medium functions best under anaerobic conditions, and a pour depth of at least 5 cm (2 in.) is recommended.	Within a few hours after inoculation with the specimen, the broth becomes cloudy. Because coliforms or other intestinal flora may overgrow the pathogens within a few hours, subculture to *Salmonella–Shigella* (SS) agar or bismuth sulfite is recommended within 8–12 hours. Overheating of the broth during preparation may produce a visible precipitate, making it unsatisfactory for use.
Gram-negative (GN) broth	Polypeptone peptone Glucose D-mannitol Sodium citrate Sodium deoxycholate Dipotassium phosphate Monopotassium phosphate Sodium chloride Distilled water to Final pH, 7.0	20 g 1 g 2 g 5 g 0.5 g 4 g 1.5 g 5 g 1 L	Because of the relatively low concentration of deoxycholate, GN broth is less inhibitory to *E. coli* and other coliforms. Most strains of *Shigella* grow well. The deoxycholate and citrate are inhibitory to gram-positive bacteria. The increased concentration of mannitol over glucose limits the growth of *Proteus* species, nonetheless encouraging growth of *Salmonella and Shigella* species, both of which are capable of fermenting mannitol.	GN broth is designed for the recovery of *Salmonella* and *Shigella* species when they are in small numbers in fecal specimens. The broth may become cloudy within 4–6 hours of inoculation, and subculture to HE agar or XLD agar within that time is recommended.

BOX 6-4

Selecting a Medium for Fecal Specimen or Rectal Swab

1. Inoculate the specimen directly to a MacConkey or EMB agar plate for primary isolation of all species of enteric gram-negative bacilli.
2. Directly inoculate either an XLD or an HE agar plate for the selective screening of *Salmonella* or *Shigella* species.
3. Enrich a small portion of the specimen by heavily inoculating either selenite or GN broth. If selenite is used, subculture to HE agar within 8 to 12 hours; if GN is used, subculture within 4 hours. *Note*: In the author's laboratory, this step is not routinely performed unless screening asymptomatic patients for presence of a carrier state.
4. Incubate all plate cultures at 35°C for 24 to 48 hours. Select suspicious colonies for definitive biochemical or serologic testing.

BOX 6-5

Tests Used to Measure

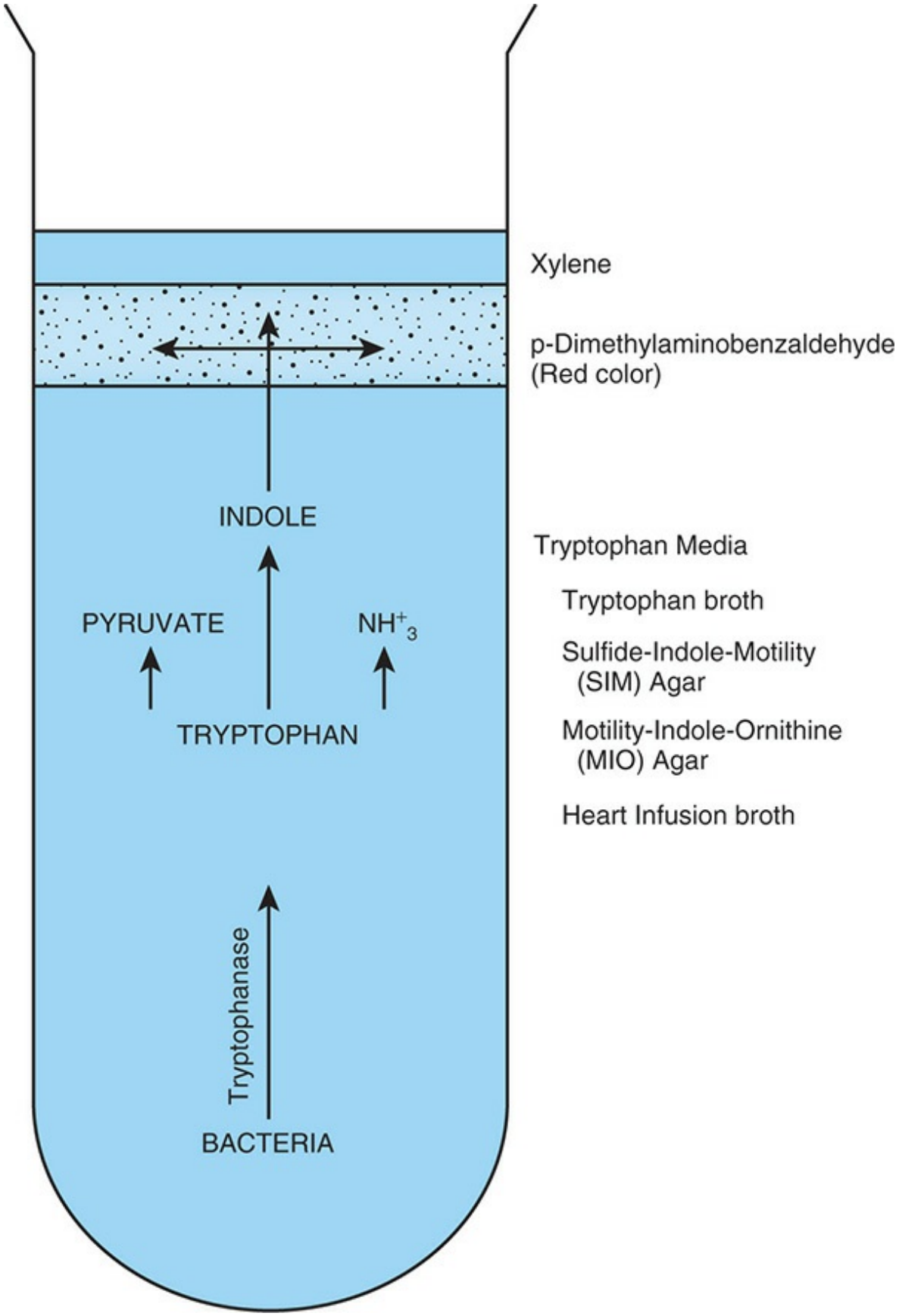
Metabolic Characteristics of *Enterobacteriaceae*

Carbohydrate utilization
o-Nitrophenyl-β-D-galactopyranoside (ONPG) activity
Indole production
Methyl red
Voges–Proskauer test (production of acetyl methyl carbinol [acetoin])
Citrate utilization
Urease production
Decarboxylation of lysine, ornithine, and arginine
Phenylalanine deaminase production
Hydrogen sulfide production
Motility

Indole Production

Indole is one of the degradation products from the metabolism of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of cleaving tryptophan, thereby producing indole, pyruvic acid, and ammonia. Indole can be detected in tryptophan test medium by observing the development of a red color after adding a solution containing *p*-dimethylaminobenzaldehyde (e.g., Ehrlich's or Kovac's reagent). The biochemistry and details of the indole test are schematically illustrated in Figure 6-5 and Chart 1-4, respectively. A color reproduction is shown in Image Plate 6-4C.

The choice between Ehrlich's and Kovac's reagents is one of personal preference. Ehrlich's reagent is more sensitive and is preferred when testing nonfermentative bacilli or anaerobes in which indole production is minimal. Because indole is soluble in organic compounds, xylene or chloroform should be added to the test medium before adding Ehrlich's reagent. This extraction step is less critical for Kovac's reagent because amyl alcohol is used for the diluent (ethyl alcohol is used with Ehrlich's reagent).


Xylene
p-Dimethylaminobenzaldehyde
(Red color)
INDOLE
Tryptophan Media
Tryptophan broth
Sulfide-Indole-Motility
(SIM) Agar
Motility-Indole-Ornithine
(MIO) Agar
Heart Infusion broth
PYRUVATE
NH+3
TRYPTOPHAN
Tryptophanase
BACTERIA

■ **FIGURE 6-5** Formation of indole by tryptophanaseproducing bacteria growing on a culture medium containing tryptophan. Indole is one of the immediate degradation products (in addition to pyruvic acid and ammonia) resulting from the deamination of tryptophan. Indole can be extracted from the aqueous phase of the medium by chloroform and detected by the addition of Ehrlich's reagent (dimethylaminobenzaldehyde).

Methyl Red Test

A simplified schema showing only two alternative pathways (mixed acid and butylene glycol) for the metabolism of the pyruvate formed from the fermentation of glucose is shown in Figure 6-2. Bacteria that follow primarily the mixed acid fermentation route often produce sufficient acid to maintain a pH below 4.4 (the acid color breakpoint of the methyl red indicator). The methyl red test provides a valuable characteristic for identifying bacterial species that produce strong acids from glucose.

The details of the methyl red test are shown in Chart 6-3. The test, as originally described, requires 48 to 72 hours of incubation before a valid result can be obtained, an amount of time unacceptable to most clinical microbiology laboratories. Barry and coworkers[38] have described a modification that can be read in 18 to 24 hours. A 0.5-mL aliquot of broth is used with a relatively heavy inoculum of the test organism. One or two drops of methyl red reagent are added after 18 to 24 hours of incubation at 35°C, and the development of red color indicates a positive test (see Image Plate 6-4C). The Barry modification is as accurate as the test originally described and saves a significant amount of time.

Voges–Proskauer Test

The details of the VP test, shown in Chart 6-4, are based on the conversion of acetyl methyl carbinol (acetoin) to diacetyl through the action of potassium hydroxide and atmospheric oxygen. Diacetyl is converted into a red complex under the catalytic action of α-naphthol and creatine (see Image Plate 6-4D).

Note, in Figure 6-2, that the formation of acetoin and butylene glycol is an alternative pathway for the metabolism of pyruvic acid. Bacteria that use this pathway, such as certain strains within the *Klebsiella–Enterobacter–Serratia–Hafnia* group, produce only small quantities of mixed acids that may be insufficient to lower the pH of the methyl red medium enough to produce a color change. Consequently, most species of the *Enterobacteriaceae* that are VP-positive, with rare exceptions, are methyl red-negative and vice versa (see Image Plate 6-4C and D).

Citrate Utilization

The principle of the citrate-utilization test (Chart 6-5) is to determine the ability of an organism to use sodium citrate as the sole source of carbon for metabolism and growth. The original formula, described by Koser[365] in 1923, was a broth medium containing sodium ammonium phosphate, monopotassium phosphate, magnesium sulfate, and sodium citrate. Proteins and carbohydrates were omitted as carbon and nitrogen sources. The end point of the Koser test was either the presence or lack of visible turbidity after incubation of the test organism: this end point was actually a measure of the organism's ability to use carbon from sodium citrate to produce sufficient growth to become visible. It was soon recognized, however, that nonspecific turbidity could occur in Koser's medium. Simmons[597] resolved this problem by adding agar and bromthymol blue to the Koser formula, which provided a more sensitive color end point. Simmon's citrate agar medium is poured into a test tube and slanted. A light inoculum from a colony of growth of the test organism is streaked onto the surface of the agar slant. If the inoculum is too heavy, preformed organic compounds within the cell walls of dying bacteria may release sufficient carbon and nitrogen to produce a false-positive test result. When inoculating a series of tubes of differential culture media with an unknown organism, it is important that the citrate medium be streaked first to prevent carryover of proteins or carbohydrates from the other media.

The production of a blue color in the test medium after 24 hours of incubation at 35°C indicates the presence of alkaline products and a positive citrate-utilization test result (see Image Plate 6-4D). If carbon is used from sodium citrate, nitrogen is also extracted from the ammonium phosphate contained in the medium, releasing ammonia. Occasionally, visible growth is detected along the streak line before conversion of the medium to a blue color. This visible growth also indicates a positive test result. Malonate, acetate, and mucate are other anionic radicals commonly used to determine the ability of bacteria to use these simple compounds as a sole source of carbon.

The acronym IMViC (indole, methyl red, VP, and citrate) was once used by sanitarians and epidemiologists to refer to the tests needed to detect fecal contamination of food and water. Public health officials have used *E. coli* for many years to indicate fecal contamination. *Enterobacter aerogenes* produces colonies on primary isolation media that often cannot be distinguished from *E. coli*. However, the recovery of *E. aerogenes* from food and potable water does not necessarily signify fecal contamination because the organism is widespread in soil, grasses, and vegetative matter. Therefore, a set of biochemical characteristics was needed to differentiate the two organisms. The IMViC tests were adopted to serve this purpose. Most strains of *E. coli* are indoleand methyl red-positive, with VP and citrate being negative. *E. aerogenes* typically produces exactly opposite reactions (see Image Plates 6-4C and D). Although the individual characteristics included in the IMViC battery are still used

in bacterial identification systems, they are used only infrequently as a specific test set.

In view of the complexity of differentiating over 100 species of *Enterobacteriaceae* presented in the next section, it is interesting that there was a time when the major decisions in microbiology were relatively simple and could be made on the basis of only four easy-to-perform biochemical tests.

Urease Production

Microorganisms that possess the enzyme urease hydrolyze urea, releasing ammonia and producing a pink–red color change in the medium (see Image Plate 6-4E). The details of the urease test are shown in Chart 6-6.

Important differences between Stuart's urea broth and Christensen's urea agar should be noted. Stuart's broth is heavily buffered with phosphate salts at a pH of 6.8. Relatively large quantities of ammonia must be formed by the test organism before the buffer system is overcome and the medium's pH is elevated above 8.0 to produce a color change in the indicator. Stuart's broth, therefore, is virtually selective for *Proteus* species.

Christensen's urea agar[142] is less buffered than Stuart's urea broth and contains peptones and glucose. This enriched

medium supports the growth of many species of bacteria that cannot grow in Stuart's broth, and the decreased buffer capacity permits the detection of smaller amounts of ammonia. Organisms that produce less urease, such as certain species of *Klebsiella, Enterobacter, Brucella*, and *Bordetella bronchiseptica*, can be tested with Christensen's urea agar. For many of these species, a positive urease reaction is first detected by a pink-to-red color change in the slant portion of the agar (see Image Plate 6-4E). The slant initially turns red because the alkaline reaction, resulting from the splitting of small quantities of urea, is augmented by the amines formed from the oxidative decarboxylation of the amino acids in the airexposed portion of the medium.

Decarboxylation of Lysine, Ornithine, and Arginine

Many species of bacteria possess enzymes capable of decarboxylating specific amino acids in the test medium. The decarboxylase enzymes remove a molecule of CO_2 from an amino acid to form alkaline-reacting amines. The following are the amino acids most commonly tested and their amine degradation products:

Lysine → cadaverine

Ornithine → putrescine

Arginine → citrulline

A number of test systems have been described to measure this property, based on either detection of an alkaline pH shift in the test medium or direct measurement of the reaction products. For example, the amines resulting from the decarboxylation reaction can be detected with Ninhydrin reagent after extraction from the broth culture with chloroform. This is the relatively sensitive Carlquist reaction,[89] most commonly used for detecting the weak decarboxylase activity of many of the nonfermentative gram-negative bacilli and certain species of anaerobic bacteria.

The decarboxylase activity of the *Enterobacteriaceae* is most commonly measured in clinical microbiology laboratories with Møller decarboxylase broth.[443] The details of this test are shown in Chart 6-7. The end point of the reaction is the production of an alkaline pH shift in the medium and the development of a blue–purple color after incubation with the test organism (see Image Plate 6-4F). Note, in the Møller formula, included in Chart 6-7, that the medium is buffered at pH 6.0. This is more acidic than most culture media. This low pH is necessary because the decarboxylase enzymes are not optimally active until the pH of the medium drops below 5.5. The drop from 6.0 to 5.5 results from the growing bacteria that metabolize the small amount of glucose in the medium to produce mixed acids. A control tube, devoid of amino acid, must always be included when performing the decarboxylase test to ensure that this initial drop in pH has occurred. A yellow color change in the bromcresol purple indicator of the control tube shows acidification. Pyridoxal phosphate is included in the medium and acts as a coenzyme to further enhance the decarboxylase activity.

Many microbiologists prefer Falkow lysine broth[217] over the Møller medium because the Falkow test depends on only an alkaline shift in pH indicator, and neither an anaerobic nor an acid environment is required. However, this medium cannot be used to detect lysine decarboxylase activity in certain members of the *Klebsiella–Enterobacter–Serratia–Hafnia* group. They produce acetyl methyl carbinol, which interferes with the final alkaline pH shift, leading to false-negative interpretations. Modifications in this medium form the basis of the motility indole ornithine (MIO)[199] semisolid agar used in clinical microbiology laboratories. Rapid methods for detecting ornithine[226] and lysine[76] decarboxylase activity in members of the *Enterobacteriaceae* have also been described.

Edwards and Fife[201] described a solid lysine decarboxylase medium based on the Falkow formula, which includes ferric ammonium citrate and thiosulfate for the detection of hydrogen sulfide. This medium is lysine iron agar (LIA), used in many laboratories as an aid in the identification of *Salmonella* species, most of which are both hydrogen sulfidepositive and lysine decarboxylase-positive. A black deep and a purple slant with LIA are virtually indicative of *Salmonella* species. Another advantage of LIA is that *Proteus* and *Providencia* species, both of which deaminate, rather than

decarboxylate, amino acids, can be detected by the development of a red color in the slant of the tube (see Image Plate 6-4G).

The lysine decarboxylase test is useful in differentiating lactose-negative *Citrobacter* species (0% positive) from *Salmonella* species (98% positive). Almost all strains of *S. sonnei* (more than 98%) possess ornithine decarboxylase activity, whereas only a few strains of *S. boydii* (2.5%) show such activity; *S. dysenteriae* and *S. flexneri* are ornithine-negative. The ornithine decarboxylase test is perhaps most useful in differentiating *Klebsiella* species (most species are negative) from *Enterobacter* species (most strains are positive).

Phenylalanine Deaminase Production

The phenylalanine deaminase determination is useful in the initial differentiation of *Proteus, Morganella*, and *Providencia* species from other gram-negative bacilli. Only members of these genera, and a few relatively rare isolates of the *Enterobacter* group, possess the enzyme responsible for the oxidative deamination of phenylalanine. The test is easily performed, as outlined in Chart 6-8. Phenylpyruvic acid may be detected in as little as 4 hours if a heavy inoculum is used; however, 18 to 24 hours of incubation are generally recommended. The phenylalanine test medium uses yeast extract as the source for carbon and nitrogen. Meat extracts or protein hydrolysates contain varying amounts of naturally occurring phenylalanine that can lead to inconsistent results. The development of a green color after addition of the ferric chloride reagent is immediate and easy to visualize (see Image Plate 6-4E).

Hydrogen Sulfide Production

The ability of certain bacterial species to liberate sulfur from sulfur-containing amino acids or other compounds in the form of H_2S is an important characteristic for their identification. The media most commonly used for the detection of H_2S and the sources for sulfur and the sulfide indicators are listed in Table 6-4. The sequence of steps leading to the production and detection of H_2S in a test system is outlined in Box 6-6.

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TABLE 6-4 Media for the Detection of Hydrogen Sulfide (H_2S)

Media	Sulfur Source	H_2S Indicator
Bismuth sulfite	Peptones plus sulfite	Ferrous sulfate

Citrate sulfide agar	Sodium thiosulfate	Ferric ammonium citrate
Deoxycholate citrate agar	Peptones	Ferric citrate
Lysine iron agar	Sodium thiosulfate	Ferric ammonium citrate
Kligler's iron agar	Sodium thiosulfate	Ferrous sulfate
Triple sugar iron agar	Sodium thiosulfate	Ferrous sulfate
Lead acetate agar	Sodium thiosulfate	Lead acetate
Salmonella–Shigella agar	Sodium thiosulfate	Ferric citrate
Sulfide–indole–motility medium	Sodium thiosulfate	Peptonized iron
Xylose–lysine–deoxycholate or Hektoen enteric	Sodium thiosulfate	Ferric ammonium citrate

The differences in detecting H_2S production in the different media result from alteration in one or more of these conditions. H_2S detected in one medium may not be detected in another, and it is necessary to know the test system used when interpreting identification charts. SIM medium is more sensitive than KIA for the detection of H_2S, presumably because of KIA's semisolid consistency, lack of carbohydrates to suppress H_2S formation, and use of peptonized iron as the indicator (see Image Plate 6-4B). KIA, on the other hand, is more sensitive than TSI agar because sucrose is believed to suppress the enzyme mechanisms responsible for H_2S production. Lead acetate is the most sensitive indicator and should be used whenever bacteria that produce only trace amounts of H_2S are tested. Unfortunately, lead acetate, when incorporated in culture media, also inhibits the growth of many fastidious bacteria, specifically the ones that may require a sensitive detection system. These organisms can be tested for production of H_2S by draping a lead acetate-impregnated filter paper strip under the cap of a culture tube of KIA medium. In this way, the extreme sensitivity of the lead acetate indicator can be used without incorporating it directly into the medium.

With all H_2S detection systems, the end point is an insoluble, heavy metal sulfide, which produces a black precipitate in the medium or on the filter paper strip. Because hydrogen ions must be available for H_2S formation, the blackening is first seen in test media in which acid formation is maximal, that is, along the inoculation line, within the

deeps of slanted agar media, or in the centers of colonies growing on agar surfaces.

BOX 6-6

How H_2S Is Produced

1. Release of sulfide from cysteine or thiosulfate by bacterial enzymatic action
2. Coupling of sulfide (S^{2-}) with a hydrogen ion (H^1) to form H_2S
3. Detection of H_2S by iron, bismuth, or lead to produce insoluble, heavy metal sulfides that appear as a black precipitate

Motility

Bacterial motility is another important determinant in making a final species identification. Bacteria move by means of flagella, the number and location of which vary among the different species. Flagellar stains are available for this determination (see Chapter 7).

Bacterial motility can be observed directly by placing a drop of culture broth medium on a microscope slide, adding a coverslip and viewing it under a microscope using a high-dry objective (40×). This technique is used primarily for detecting the motility of bacteria species that do not grow well in semisolid agar media. However, *Enterobacteriaceae* do grow well, and tubes containing semisolid agar are most commonly used.

Motility media have agar concentrations of 0.4% or less. At higher concentrations, the gel is too firm to allow the organisms to spread freely. Combination media, such as SIM medium[404] or MIO agar[199] have found wide use in clinical microbiology laboratories because more than one characteristic can be measured in the same tube. The motility test must be interpreted first because the addition of an indole reagent may obscure the results. Because SIM medium and MIO agar have a slightly turbid background, interpretations may be somewhat difficult with bacterial species that grow slowly in these media. In these cases, a motility test medium (Box 6-7) is recommended because it supports the growth of most fastidious bacteria and has a crystal-clear appearance.

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BOX 6-7

Motility Test Medium (Edwards and Ewing)

Beef extract, 3 g
Peptone, 10 g
Sodium chloride, 5 g
Agar, 4 g
Distilled water to equal 1 L
Final pH, 7.3

The motility test is interpreted by making a macroscopic examination of the medium for a diffuse zone of growth flaring out from the line of inoculation (see Image Plate 6-4H). The use of tetrazolium salts in motility medium has been advocated as an aid in the visual detection of bacterial growth. Tetrazolium salts are colorless but are converted into insoluble red formazan complexes by the reducing properties of growing bacteria. In a motility test medium containing tetrazolium, the development of this red color helps trace the spread of bacteria from the inoculation line. However, these salts may inhibit certain fastidious bacteria and cannot be

Taxonomy of the *Enterobacteriaceae*

The application of new technologies to study the taxonomy of microorganisms has led to a rapid increase in the number of genera and species of bacteria that fit the general criteria for *Enterobacteriaceae*. In 1972, Edwards and Ewing[200] described 11 genera and 26 species belonging to the *Enterobacteriaceae*. In 1985, Farmer and associates[220] described 22 genera comprising 69 species and 29 enteric groups. In this chapter, 37 genera and 148 species, biogroups, and unnamed enteric groups of *Enterobacteriaceae* are described.

Classification of Enterobacteriaceae by Tribes

The division of the *Enterobacteriaceae* into tribes is not used in the current edition of *Bergey's Manual* or in the Centers for Disease Control and Prevention (CDC) classification because the authors believe that the use of tribes is of no diagnostic significance and of questionable taxonomic significance. This argument has validity from the perspective of pure classification. Yet, for the users of this book who are new to the field of microbiology and who must orient themselves to the complex and confusing *Enterobacteriaceae*, the tribe concept proposed by Ewing[212] has certain

teaching and learning advantages. We agree with Ewing that his scheme, although imperfect, represents a good compromise between practical and ideal taxonomies.

The tribe concept provides both students and practitioners with a convenient method of grouping together the major genera within the family that share similar biochemical reactions and are of similar diagnostic importance. We believe it is important that practicing microbiologists maintain a base of knowledge that is firmly grounded in the morphology, physiology, and biochemistry of medically important bacteria. Furthermore, certain phenotypic patterns that allow easy subgrouping and clustering of related species must be committed to memory. This orientation is especially important if microbiologists use semiautomated and automated commercial systems with computer-assisted identification, because it serves as a quality control to validate instrument-generated information. The use of the tribe concept as an approach to learning the *Enterobacteriaceae* serves these goals well, and is the approach chosen for use in this textbook for teaching the key features of the established genera of the *Enterobacteriaceae*. The current species included in the established genera, sorted by tribes, are listed in Table 6-5.

Key Identification Characteristics for the Most Common Species

Table 6-6 shows the key identification characteristics used in separating the established genera of the *Enterobacteriaceae* into seven tribes. Students should study this table and learn to categorize an unknown isolate into one of these tribes on the basis of reactions seen with these key tests. The following observations are made to assist students in identifying the most common species.

The members of the *Escherichieae* have the following key reactions: indole-positive, methyl red-positive, VP-negative, citrate-negative (the classic example of mixed acid fermenters). They are negative for all the other key biochemical tests: hydrogen sulfide, phenylalanine deaminase, and urea. Note from Table 6-6 that *Shigella* species are similar to *Escherichia* species except that they are negative for CO_2 gas and motility.

The *Edwardsielleae* are similar to *Escherichieae* except for the property of being hydrogen sulfide-positive. Students may wish to think of *Edwardsiella tarda* as hydrogen sulfidepositive *E. coli*. *Salmonelleae* resemble *Edwardsielleae* except that they are indole-negative and citrate-positive. *Citrobacter freundii* is similar to *Salmonella* except for being lysinenegative. *C. koseri* differs from *C. freundii* by being hydrogen sulfide-negative and indole-positive.

The *Klebsielleae* are composed of the VP-positive members of the *Enterobacteriaceae.* As shown in Table 6-6 and illustrated in Figure 6-2, most members of this tribe produce copious amounts of CO_2, so much so that the deep portion of KIA and TSI slants is often pushed halfway up the tube. Notice that the *Klebsiella* species are nonmotile and that *Pantoea* is triple-decarboxylase-negative

(lysine-negative, arginine-negative, and ornithine-negative).

The *Proteeae* are separated from all others by virtue of being phenylalanine deaminase-positive, a feature unique to this tribe. The urea reaction for the species in the genus *Proteus* and *Morganella* as well as one of the *Providencia* species (*P. rettgeri*) is strongly positive. Both species of *Proteus* listed in Table 6-6 are hydrogen sulfide-positive and exhibit swarming motility.

The *Yersinieae* represented here by the most commonly isolated species, *Y. enterocolitica*, are very similar to members

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TABLE 6-5 Important Recent Changes in the Established Genera of the *Enterobacteriaceae*

New Designation	Previous Designation	Comments
Tribe I: *Escherichieae*		
Escherichia coli		Sorbitol (+) except for serotype 0157:H7. All other species are sorbitol (−).
Escherichia coli inactive	Alkalescens-Dispar	Anaerogenic, lactose-negative (or delayed), and nonmotile.
Escherichia albertii	New species	Associated with diarrheal disease in Bangladeshi children. Indole-negative, ferments D-mannitol but not D-xylose. Separated from *H. alvei* by negative VP test.
Escherichia fergusonii	Enteric Group 10	Found in blood, urine, and feces. Indole (+), Sorbitol (−), LAO (+, −, +), lactose (−) but ONPG (+).
Escherichia hermannii	Enteric Group 11	Wounds and feces most common sources. Yellow pigmented, indole (+), sorbitol (−), LAO (−, −, +).
Escherichia vulneris	Enteric Group 1 API group 2 Alma group 1	Most strains from human wounds. Over half the strains are yellow pigmented.
Shigella		The four species of *Shigella* and *E. coli* form a single species on the basis of DNA hybridization. *S. dysenteriae* (Group A), *S. flexneri* (Group B), and *S. boydii* (Group C) are biochemically similar and must be separated by serologic methods. *S. sonnei* is ornithine (+).
Tribe II: *Edwardsielleae*		
Edwardsiella tarda	*Edwardsiella anguillimortifera* Asakusa Group	Produce indole and abundant H_2S, ferment glucose and maltose but not mannitol, lactose, sucrose, or arabinose. Found in cold-blooded animals. Opportunistic human pathogen. May cause wound infections and diarrhea.
Edwardsiella tarda biogroup 1		Indole (+), H_2S (−), mannitol, sucrose, and arabinose positive. Found in snakes, not a human clinical isolate.
Edwardsiella hoshinae		Indole (−), H2S (−), isolated from birds, reptiles, and water. Several isolates from human feces.
Edwarsiella ictaluri		No human isolates, causes enteric septicemia in catfish.
Tribe III: *Salmonelleae*		
Salmonella	*S. cholerae-suis* *S. typhi* *S. enteritidis*	All subgroups (subgenera) of *Salmonella* and *Arizona* are considered to belong to the same species. Organisms are now reported by genus and serotype, omitting reference to species.
Tribe IV: Citrobactereae		
Citrobacter amalonaticus	*Levinea amalonaticus*	H_2S (−), indole (+), adonitol (−), malonate (−). Primarily found in human feces, very rarely isolated from blood.
Citrobacter braakii	*Citrobacter* genomospecies 6	H_2S and indole are variable, adonitol (−), malonate (−). Isolated from human stools, urine, wounds, and from animals and food.
Citrobacter farmeri	Citrobacter amalonaticus biogroup 1	Found primarily in human feces. Stains of biogroup 1 ferment sucrose, raffinose, alpha-methyl-D-glucoside, and melibiose and are citrate (−). *C. amalonaticus* usually have the opposite reactions.
Citrobacter freundii		H_2S (+), indole (−), adonitol (−), malonate (−). Found in urine, throat, sputum, blood, and wounds.
Citrobacter gillenii	*Citrobacter* genomonspecies 10	H_2S is variable, indole (−), adonitol (−), malonate (+). Found in human stool and food.
Citrobacter koseri	*Levinea malonatica* *Citrobacter diversus*	H_2S (−), indole (+), adonitol (+), malonate (+). Found in urine, throat, nose, sputum, and wounds. Rare cause of neonatal meningitis.
Citrobacter murliniae	*Citrobacter* genomospecies 11	H_2S is variable, indole (+), adonitol (−), malonate (−). Found in human stools and blood.
Citrobacter rodentium	*Citrobacter* genomospecies 9	H_2S (−), indole (−), adonitol (−), malonate (+). This organism has been isolated only from rodents.

Citrobacter sedlakii	*Citrobacter* genomonspecies 8	H_2S (−), indole (+), adonitol (−), malonate (+). Found in human stools, blood, and wounds.
Citrobacter werkmanii	*Citrobacter* genomospecies 7	H_2S (+), indole (−), adonitol (−), malonate (+). Found in human stools and urine and from food.
Citrobacter youngae	*Citrobacter* genomonspecies 5	H_2S and indole are variable, adonitol (−), malonate (−). Isolated from human stools and blood and from animals and food.
Tribe V: Klebsielleae		
Klebsiella granulomatis	*Calymmatobacterium granulomatis*	Cannot be cultured on conventional culture media. Causative agent of granuloma inguinale.
Klebsiella pneumoniae subsp. *ozaenae*	*Klebsiella ozaenae*	Biochemically inactive strain of *K. pneumoniae*. Causes atrophic rhinitis, a condition called ozena.
Klebsiella pneumoniae subsp. *rhinoscleromatis*	Klebsiella rhinoscleromatis	Biochemically inactive strain of *K. pneumoniae*. Causes a granulomatous disease known as rhinoscleroma.
Klebsiella alba	New species	Plant and soil isolates
Klebsiella singaporensis	New species	Plant and soil isolates
Klebsiella variicola	New species	Plant and soil isolates
Raoultella ornithinolytica	*Klebsiella* Group 47 *Klebsiella ornithinolytica*	MIO (−, +, +). Isolated from blood, urine, sputum, and wounds.
Raoultella planticola	*Klebsiella* Species 2 *Klebsiella trevisanii Klebsiella planticola*	Water and plant isolates. Rare human clinical isolates. May be difficult to separate from ornithine-negative strains of *R. ornithinolytica*.
Raoultella terrigena	*Klebsiella terrigena*	Soil and water isolates
Cronobacter sakazakii	Yellow-pigmented *Enterobacter cloacae* *Enterobacter sakazakii*	LAO (−, +, +). Bright yellow pigment at 35°C. May cause meningitis, brain abscesses, and bacteremia in neonates.
"*Enterobacter agglomerans* complex"	*Erwinia herbicola* *Erwinia milletiae*	Heterogeneous group of organisms representing over 13 DNA hybridization groups (HG). HG XIII has been transferred to the new genus Pantoea as *Pantoea agglomerans*. Organisms that are LAO negative (referred to as "triple decarboxylase negative") and are yellow pigmented were usually identified as *E. agglomerans* in the past.
Enterobacter asburiae	Enteric Group 17 atypical *Citrobacter*	Biochemically similar to *E. cloacae*. Nonmotile, VP (−), (79% + after 2 days), urea (+) (delayed). Isolated from variety of human sources: blood, urine, wounds, respiratory tract, feces.
Enterobacter aerogenes		LAO (+, −, +). Common clinical isolate.
Enterobacter cancerogenus	*Erwinia cancerogena* *Enterobacter taylorae* Enteric Group 19	Includes organisms formerly classified as *E. taylorae*. LAO (−, +, +), adonitol, inositol, sorbitol, raffinose, and melibiose all negative. Isolated from a variety of clinical sources including blood and spinal fluid. Majority of strains isolated from clinical specimens.
Enterobacter cloacae subsp. *cloacae*	*Enterobacter cloacae*	LAO (−, +, +). Common clinical isolate.
Enterobacter cloacae subsp. *dissolvens*	Enterobacter dissolvens Erwinia dissolvens	Separation of these subspecies by phenotypic testing is challenging, and separation using MALDI-TOF is not yet possible. Laboratories should report as "*E. cloacae* complex."
Enterobacter hormaechei	Enteric Group 75	LAO (−, +, +). Biochemically closest to *E. cancerogenus* except is urea (+), sucrose (+), and esculin (−). Isolates reported from blood, wounds, and sputum.
Enterobacter kobei	Enteric Group 69 Japanese NIH Group 21	Closely related to *E. cloacae*. Majority isolated from clinical specimens.
Kosakonia cowanii	Japanese NIH Group 42 *Enterobacter cowanii*	LAO (−, −, −): phenotypically resemble *P. agglomerans*

Lelliottia nimipressuralis	*Erwinia nimipressuralis* *Enterobacter nimipressuralis*	Closely related to *E. cloacae*. Not found in human clinical specimens.
Pluralibacter gergoviae	atypical *Enterobacter aerogenes* *Enterobacter gergoviae*	LAO (+, −, +). Strong urease (+). Found in environment and urine and respiratory tract of humans. Rare isolates have been recovered from blood.
Pluralibacter pyrinus	*Erwinia pirina* *Enterobacter pyrinus*	Urease (+), most closely resembling *P. gergoviae*, is differentiated by its growth in KCN broth, acid production from myoinositol, and lack of acid production from raffinose. Causes brown leaf spot disease of pear trees.
Hafnia alvei	*Enterobacter hafniae*	Lactose (−), LAO (+, −, +), grows at 35°C but biochemically more active at 25°C. Found in clinical specimens, especially feces, occasionally from blood, sputum, urine, and wounds.
Hafnia alvei biogroup 1	"*Hafnia protea*" *Obesumbacterium proteus* biogroup 1	Not a human clinical isolate. Occurs in breweries, where it grows in beer wort.
Pantoea agglomerans	*Enterobacter agglomerans* HG XIII *Erwinia herbicola* *Erwinia milletiae*	LAO (−, −, −) some may be yellow pigmented. Isolated from plant surfaces, seeds, and water, as well as from humans (wound, blood, urine, internal organs) and animals.
Pantoea ananatis	*Pantoea ananas* *Enterobacter agglomerans* HG VI *Erwinia ananas* *Erwinia uredovora*	Plant pathogen, causes pineapple rot
Pantoea citrea	New species	Isolated from Mandarin oranges in Japan
Pantoea dispersa	*Enterobacter agglomerans* HG III	Isolated from plant surfaces, seeds, humans, and the environment. Separated from *P. agglomerans* by negative salicin reaction.
Pantoea punctata	New species	Isolated from Mandarin oranges in Japan
Pantoea stewartii subsp. *indologenes*	*Erwinia stewartii*	Cause of leaf spot on foxtail millet and pearl millet
Pantoea stewartii subsp. *stewartii*	*Erwinia stewartii*	Causative agent of Stewart's bacterial wilt of corn
Pantoea terrea	New species	Isolated from soil in Japan
Serratia entomophila		Resembles *S. marcescens* (arabinose −). Insect pathogen, no human isolates reported.
Serratia ficaria		Natural habitat is figs and fig wasps. Has been very rarely reported from human clinical specimens.
"*Serratia*" *fonticola*		Not really a species of *Serratia*. A water organism, rarely isolated from human specimens, mostly wounds.
Serratia grimesii	see *Serratia liquefaciens* group	Isolated from environment and human clinical specimens. Cannot be differentiated from other members of "*S. liquefaciens* group" by commonly used tests.
"*Serratia liquefaciens* group"	*Enterobacter liquefaciens*, Different biogroups within the species *S. liquefaciens*	Consists of several DNA hybridization groups including species now named *Serratia proteamaculans*, and *S. grimesii*. Cannot be separated by currently utilized biochemical tests. Differ from *S. marcescens* by being L-arabinose (+). Report as "*S. liquefaciens* group."
Serratia marcescens		DNase (+), gelatin (+), L-arabinose (−) (other species are positive). Common clinical isolate. Red pigment produced by some strains.
Serratia odorifera biogroup 1 *Serratia odorifera* biogroup 2		Dirty, musty odor like potatoes. Two biogroups. Biogroup 1 is ornithine, sucrose, and raffinose positive, and is predominantly isolated from sputum. Biogroup 2 is negative for these three reactions and has been recovered from blood and CSF.
Serratia plymuthica	*Bacterium plymuthica*	May have red pigment. Isolated from soil, water, sputum. Extremely rare in clinical specimens.

Serratia proteamaculans subsp. *proteamaculans*	see *Serratia liquefaciens* group	Cannot be separated from other members of "*S. liquefaciens* group" by currently utilized biochemical tests. Differ from *S. marcescens* by being L-arabinose (+).
Serratia quinivorans	*Serratia proteamaculans* subsp. *quinovora* see *Serratia liquefaciens* group	Isolated from plants, wild rodents, insects, and water, but not yet from human clinical specimens.
Serratia rubidaea		Red pigment produced. Rarely isolated in humans.
Tribe VI: *Proteeae*		
Proteus hauseri	*Proteus vulgaris* biogroup 3, DNA group 3	H_2S (+), indole (+), salicin and esculin are (−).
Proteus mirabilis		H_2S (+), indole (−), ornithine (+). Common clinical isolate.
Proteus myxofaciens		No human isolates reported. Isolated only from living and dead gypsy moths.
Proteus penneri	*Proteus vulgaris* biogroup 1	Closely related to *P. vulgaris*, except that indole (−), salicin (−), esculin (−), and chloramphenicol resistant.
Proteus vulgaris	*Proteus vulgaris* biogroup 2	H_2S (+), indole (+), ornithine (−) Common clinical isolate. Indole, salicin, and esculin are positive.
Proteus vulgaris biogroup 3		Consists of four separate genetic species and are designated DNA groups 3, 4, 5, and 6. Indole (+), but salicin and esculin are (−). DNA group 3 has recently been named *Proteus hauseri*.
Morganella morganii subsp. *morganii*	*Proteus morganii*	H_2S (−), lysine, ornithine and motility all variable, trehalose (−). Causes urinary tract infections and is cultured from many other body sites. Contains four biogroups designated A through D.
Morganella morganii subsp. *sibonii*	*Morganella morganii* biogroup 1	H_2S (−), lysine, ornithine variable, trehalose and motility (+). Contains three biogroups designated E through G.
Morganella morganii subsp. 3		
Providencia alcalifaciens	*Providencia alcalifaciens* biogroup 1,2	Urea (−), adonitol (+), inositol (−). Generally isolated from diarrheic stools, particularly children.
Providencia heimbachae		Not a human clinical isolate. Found in penguin feces and an aborted cow fetus.
Providencia rettgeri	*Proteus rettgeri*	Urea (+), adonitol (+), inositol (+). Mostly isolated from urine of hospitalized and catheterized specimens.
Providencia rustigianii	*Providencia alcalifaciens* biogroup 3, *Providencia friedericiana*	Urea (−), adonitol (−), inositol (−). Rarely found in clinical specimens, mostly from human feces.
Providencia stuartii	*Providencia alcalifaciens* biogroup 4	Urea (v), adonitol (−), inositol (+). Isolated most often from urine, less often from wounds, burns, and bacteremias. May cause nosocomial outbreaks.
Tribe VII: *Yersinieae*		
Yersinia aldovae	*Yersinia enterocolitica*-like Group X2	Biochemically similar to *Y. enterocolitica*. Isolated from surface water, drinking water, and fish
Yersinia bercovieri	*Yersinia enterocolitica* biogroup 3B	Biochemically similar to *Y. enterocolitica*. Isolated from human feces, water, soil, and raw vegetables.
Yersinia enterocolitica		May cause diarrhea, terminal ileitis, mesenteric lymphadenitis, arthritis, and septicemia in humans.
Yersinia frederiksenii	Biogroup of *Y. enterocolitica*	Mainly found in water, sewage, and fish. Occasionally found in human feces, blood, and sputum. Rarely associated with gastrointestinal illness.
Yersinia intermedia	Biogroup of *Y. enterocolitica*	Found in fresh water, sewage, and aquatic animals. Human isolates have been from stool, blood, wounds, and urine. Probably not a cause of gastrointestinal illness.
Yersinia kristensenii	Biogroup of *Y. enterocolitica*	Found in water, soil, and animals. Human isolates have been from stool, blood, and urine. No evidence that it can cause gastrointestinal illness.

Yersinia mollaretii	*Yersinia enterocolitica* biogroup 3A	Biochemically similar to *Y. enterocolitica*. Isolated from human feces, drinking water, meat, and raw vegetables.
Yersinia pestis	*Pasteurella pestis* *Yersinia pseudotuberculosis* subsp. *pestis*	Causative agent of plague.
Yersinia pseudotuberculosis		May cause mesenteric lymphadenitis, diarrhea, and septicemia in humans.
Yersinia rohdei		Biochemically similar to *Y. enterocolitica*. Isolated from dog feces, water, and human feces.
"Yersinia" ruckeri	"Red mouth bacterium"	Will probably be moved to a new genus. Fish pathogen. Human isolates are extremely rare.

IMViC, indole, methyl red, Voges–Proskauer, citrate; LAO, lysine, arginine, ornithine; MIO, motility, indole, ornithine; PAD, phenylalanine deaminase; HG, hybridization group; (+), >90% of strains positive; (−), >90% of strains negative; (v), variable.

TABLE 6-6 Key Identification Characteristics for the Most Common *Enterobacteriaceae*

	KIA	GAS	H_2S	MR	VP	IND	CIT	PAD	URE	MOT	LYS	ARG	ORN	ONPG
Tribe I: *Escherichieae*														
Genus: Escherichia														
E. coli	A/A	+	−	+	−	+	−	−	−	+	+	−/+	+/−	+
Genus: Shigella														
Groups A, B, C	Alk/A	−	−	+	−	−/+	−	−	−	−	−	−	−	−
S. sonnei	Alk/A	−	−	+	−	−	−	−	−	−	−	−	+	+
Tribe II: *Edwardsielleae*														
Genus: Edwardsiella														
E. tarda	Alk/A	+	+	+	−	+	−	−	−	+	+	−	+	−
Tribe III: *Salmonelleae*														
Genus: Salmonella	Alk/A	+	+	+	−	−	+	−	−	+	+	+/−	+	−
Tribe IV: *Citrobactereae*														
Genus: Citrobacter														
C. freundii	A/A; Alk/A	+	+	+	−	−	+	−	+/−	+	−	+/−	−/+	+
C. koseri	Alk/A	+	−	+	−	+	+	−	+/−	+	−	+/−	+	+
Tribe V: *Klebsielleae*														
Genus: Klebsiella														
K. pneumoniae	A/A	++	−	−	+	−	+	−	+	−	+	−	−	+
K. oxytoca	A/A	++	−	−	+	+	+	−	+	−	+	−	−	+
Genus: Enterobacter														
E. aerogenes	A/A	++	−	−	+	−	+	−	−	+	+	−	+	+
E. cloacae	A/A	++	−	−	+	−	+	−	+/−	+	−	+	+	+
Genus: Hafnia														
H. alvei	Alk/A	+	−	−/+	+	−	−	−	−	+	+	−	+	+
Genus: Pantoea														
P. agglomerans	A/A; Alk/A	−/+	−	−/+	+/−	−/+	+/−	−/+	−/+	+	−	−	−	+

Genus: Serratia														
S. marcescens	Alk/A	+	−	−/+	+	−	+	−	−	+	+	−	+	+
Tribe VI: *Proteeae*														
Genus: Proteus														
P. vulgaris	Alk/A	+/−	+	+	−	+	−/+	+	++	+[a]	−	−	−	−
P. mirabilis	Alk/A	+	+	+	+/−	−	+/−	+	++	+[a]	−	−	+	−
Genus: Morganella														
M. morganii	Alk/A	+	−	+	−	+	−	+	++	+	−	−	+	−
Genus: Providencia														
P. rettgeri	Alk/A	−	−	+	−	+	+	+	++	+	−	−	−	−
P. stuartii	Alk/A	−	−	+	−	+	+	+	−/+	+/−	−	−	−	−
P. alcalifaciens	Alk/A	+/−	−	+	−	+	+	+	−	+	−	−	−	−
Tribe VII: *Yersinieae*														
Genus: Yersinia														
Y. enterocolitica	Alk/A	−	−	+	−	+/−	−	−	+/−	−[b]	−	−	+	+

[a]Swarming motility demonstrated on noninhibitory media.
[b]Nonmotile at 36°C, motile at 22°C.
KIA, Kligler's iron agar; H_2S, hydrogen sulfide; MR, methyl red; VP, Voges–Proskauer; IND, indole; CIT, citrate; PAD, phenylalanine deaminase; URE, urease; MOT, motility; LYS, lysine; ARG, arginine; ORN, ornithine; ONPG, o-nitrophenyl-β-D-galactopyranoside; ++, strong positive reaction; +, 90% or more strains positive; −, 90% or more strains negative; +/−, 50% to 90% of strains positive; −/+, 50% to 90% of strains negative; shaded areas indicate key reactions; A/A, acid slant/acid butt; Alk/A, alkaline slant, acid butt.

of the *Escherichieae*, except that *Y. enterocolitica* is usually urea-positive. Students may wish to think of *Y. enterocolitica* as urea-positive *E. coli*. Note that the motility for *Y. enterocolitica* is negative at 36°C but positive at 22°C.

Key reactions for students to remember are found in Box 6-8. When it is necessary to confirm the identification of a common *Enterobacteriaceae* with an unusual biochemical pattern or when an uncommon species is suspected, it will be necessary to consult Table 6-7, which gives the reactions of all named species of *Enterobacteriaceae* to 48 biochemical substrates.

Tribe *Escherichieae*. The two genera within this tribe are *Escherichia* and *Shigella*. On first reflection, these two groups of bacteria would not appear to be related because of differences in growth characteristics and appearance on the enteric isolation media (*E. coli* characteristically ferments lactose, *Shigella* species do not). *E. coli* is generally biochemically active compared with Shigella species, which tend to be inert. However, *E. coli* and *Shigella* species are closely related genetically; in fact, all four species of *Shigella* and *E. coli* form a single species on the basis of DNA hybridization studies.[64] However, because *Shigella* species are associated with a specific disease spectrum (bacillary dysentery) and because specific typing antisera for separating *E. coli* from *Shigella* are commercially available, *Shigella* species will continue to be classified in a separate genus, at least for now. Students should note,

however, that certain late lactose-fermenting, nonmotile, and biochemically inactive strains of *E. coli* can be difficult to differentiate from *Shigella* species, and rare strains of *Shigella*

BOX 6-8

Key Facts to Remember for Identifying *Enterobacteriaceae*

Hydrogen Sulfide-Positive

Edwardsiella tarda
Salmonella species
Citrobacter freundii
Proteus vulgaris
Proteus mirabilis

Voges–Proskauer-Positive

Klebsiella species
Enterobacter species
Hafnia species
Pantoea species
Serratia species

Phenylalanine Deaminase-Positive

Proteus species
Providencia species
Morganella species

Nonmotile at 36°C

Shigella species
Klebsiella species
Yersinia species (motile at 22°C)

TABLE 6-7 Biochemical Reactions of the Named Species and Unnamed Groups of the Family *Enterobacteriaceae*[a,b]

Organism	Indole production	Methyl red	Voges–Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D–Glucose, acid	D–Glucose, gas	Lactose fermentation	Sucrose fermentation	D–Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	myo–Inositol fermentation
Budvicia aquatica	0	93	0	0	80	33	0	0	0	0	27	0	0	0	100	53	87	0	60	0	0	0	0
Buttiauxella agrestis	0	100	0	100	0	0	0	0	0	100	100	0	80	60	100	100	100	0	100	0	100	0	0
Buttiauxella brennerae	0	100	0	0	0	0	0	0	0	33	100	0	100	100	100	100	0	0	100	0	100	67	0
Buttiauxella ferragutiae	0	100	0	0	0	0	0	100	0	80	60	0	40	0	100	100	0	0	100	0	100	0	0
Buttiauxella gaviniae	0	100	0	20	0	0	0	0	20	0	80	0	60	100	100	40	60	0	100	0	100	100	0
Buttiauxella izardii	0	100	0	0	0	0	0	0	0	100	100	0	67	100	100	100	100	0	100	0	100	0	0
Buttiauxella noackiae	33	100	0	33	0	0	100	0	67	0	100	0	100	100	100	100	0	0	100	0	100	0	0
Buttiauxella warm-boldiae	0	100	0	33	0	0	100	0	0	0	100	0	33	100	100	100	0	0	100	0	100	0	67
Cedecea davisae	0	100	50	95	0	0	0	0	50	95	95	0	86	91	100	70	19	100	100	0	99	0	0
Cedecea lapagei	0	40	80	99	0	0	0	0	80	0	80	0	100	99	100	100	60	0	100	0	100	0	0
Cedecea neteri	0	100	50	100	0	0	0	0	100	0	100	0	65	100	100	100	35	100	100	0	100	0	0
Cedecea species 3	0	100	50	100	0	0	0	0	100	0	100	0	100	0	100	100	0	50	100	0	100	0	0
Cedecea species 5	0	100	50	100	0	0	0	0	50	50	100	0	100	0	100	100	0	100	100	0	100	0	0
Citrobacter amalonati-cus	100	100	0	95	5	85	0	0	85	95	95	0	99	1	100	97	35	9	100	1	30	0	0
Citrobacter braakii	33	100	0	87	60	47	0	0	67	93	87	0	100	0	100	93	80	7	100	33	0	0	0
Citrobacter farmeri	100	100	0	10	0	59	0	0	85	100	97	0	93	0	100	96	15	100	100	2	9	0	0
Citrobacter freundii	33	100	0	78	78	44	0	0	67	0	89	0	89	11	100	89	78	89	100	11	0	0	0
Citrobacter gillenii	0	100	0	33	67	0	0	0	33	0	67	0	100	100	100	100	67	33	100	0	0	0	0
Citrobacter koseri (C. diversus)	99	100	0	99	0	75	0	0	80	99	95	0	0	95	100	98	50	40	99	40	15	99	0

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0	80	0	100	0	93	0	0	0	0	0	0	27	0	20	27	0	0	0	100	0	93	0	0	0
0	100	100	100	100	100	100	100	0	0	100	100	0	60	100	60	0	0	0	100	0	100	0	100	0
0	100	100	33	100	100	100	100	0	0	100	100	67	67	67	0	0	0	0	100	0	100	0	100	0
100	100	0	100	100	100	100	100	40	0	100	0	0	0	60	0	0	0	0	100	0	100	0	100	0
0	100	0	100	60	100	100	100	0	0	100	0	80	0	80	40	0	0	0	100	0	100	0	100	0
0	100	33	100	100	100	100	100	0	0	100	67	0	33	100	67	0	0	0	100	0	100	0	100	0
0	100	0	100	100	100	100	100	33	0	100	0	0	0	100	100	0	0	0	100	0	100	0	100	0
0	100	0	100	100	100	100	100	0	0	100	0	0	0	0	0	0	0	0	100	0	100	0	100	0

0	0	10	0	100	100	100	100	5	0	45	0	100	0	0	0	0	91	0	100	0	90	0	100	0
0	0	0	0	100	0	100	100	0	0	100	0	100	0	0	0	60	100	0	100	0	99	0	100	0
100	0	0	0	100	100	100	100	0	0	100	0	100	0	0	0	0	100	0	100	0	100	0	100	0
0	0	100	0	100	100	100	100	50	0	100	100	100	0	0	0	50	100	0	100	0	100	0	100	0
100	0	100	0	100	100	100	100	0	0	100	100	100	0	0	0	50	50	0	100	0	100	0	100	0
99	99	5	100	99	99	100	100	2	0	5	0	0	60	96	96	86	0	0	99	0	97	0	100	0
100	100	7	100	100	100	100	73	33	0	0	80	0	87	100	93	53	0	0	100	0	80	0	100	0
98	100	100	100	100	100	100	100	75	0	0	100	0	65	100	93	80	0	0	100	0	100	0	100	0
100	100	44	100	100	89	100	44	11	0	0	100	0	100	100	100	44	0	0	100	0	89	0	100	0
100	100	0	100	100	100	100	67	0	0	0	67	0	67	67	100	0	0	0	100	0	67	0	100	0
99	99	0	99	100	100	100	99	40	0	1	0	98	99	95	90	75	0	0	100	0	99	0	100	0

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Citrobacter murliniae	100	100	0	100	67	67	0	0	67	0	100	0	100	0	100	100	67	33	100	100	33	0	0
Citrobacter rodentium	0	100	0	0	0	100	0	0	0	100	0	0	0	100	100	100	100	0	100	0	0	0	0
Citrobacter sedlakii	83	100	0	83	0	100	0	0	100	100	100	0	100	100	100	100	100	0	100	100	17	0	0
Citrobacter werkmanii	0	100	0	100	100	100	0	0	100	0	100	0	100	100	100	100	17	0	100	0	0	0	0
Citrobacter youngae	15	100	0	75	65	80	0	0	50	5	95	0	95	5	100	75	25	20	100	85	10	0	5
Cronobacter sakazakii	11	5	100	99	0	1	50	0	99	91	96	0	99	18	100	98	99	100	100	5	99	0	75
Edwardsiella hoshinae	50	100	0	0	0	0	0	100	0	95	100	0	0	100	100	35	0	100	100	0	50	0	0
Edwardsiella ictaluri	0	0	0	0	0	0	0	100	0	65	0	0	0	0	100	50	0	0	0	0	0	0	0
Edwardsiella tarda	99	100	0	1	100	0	0	100	0	100	98	0	0	0	100	100	0	0	0	0	0	0	0
Edwardsiella tarda bio-group 1	100	100	0	0	0	0	0	100	0	100	100	0	0	0	100	50	0	100	100	0	0	0	0
Enterobacter aerogenes	0	5	98	95	0	2	0	98	0	98	97	0	98	95	100	100	95	100	100	5	100	98	95
Enterobacter asburiae	0	100	2	100	0	60	0	0	21	95	0	0	97	3	100	95	75	100	100	0	100	0	0
Enterobacter cancero-genus (E. taylorae)	0	5	100	100	0	1	0	0	94	99	99	0	98	100	100	100	10	0	100	0	92	0	0
Enterobacter cloacae	0	5	100	100	0	65	0	0	97	96	95	0	98	75	100	100	93	97	100	15	75	25	15
Enterobacter dissolvens	0	0	100	100	0	100	0	0	100	100	0	0	100	100	100	100	0	100	100	0	100	0	0
Enterobacter hormaechei	0	57	100	96	0	87	4	0	78	91	52	0	100	100	100	83	9	100	100	87	44	0	0
Enterobacter kobei (Enteric Group 69)	0	0	100	100	0	0	0	0	100	100	100	0	100	100	100	100	100	25	100	100	100	0	0
Escherichia albertii	0	100	0	0	0	0	0	100	0	100	0	0	0	0	100	100	0	0	100	0	0	0	0

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100	100	33	100	100	100	100	100	0	0	0	33	0	100	100	100	33	0	0	100	0	100	0	100	0
100	100	0	100	100	100	100	100	0	0	0	0	0	0	100	100	0	0	0	100	0	100	0	100	0
100	100	0	100	100	100	100	100	0	0	17	100	0	83	100	100	83	0	0	100	0	100	0	100	0
100	100	0	100	100	100	100	0	0	0	0	0	0	100	100	100	100	0	0	100	0	100	0	100	0
100	100	10	100	95	100	100	45	0	0	5	10	5	90	100	100	65	0	0	85	0	90	0	100	0
0	100	99	100	100	100	100	100	96	0	100	100	0	15	1	1	96	0	0	99	0	100	98	100	0
0	13	0	0	100	0	100	0	0	0	0	0	0	65	0	0	0	0	0	100	0	0	0	100	0
0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
0	9	0	0	100	0	0	0	0	0	0	0	0	30	0	25	0	0	0	100	0	0	0	100	0
0	100	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
100	100	96	99	99	100	100	100	95	0	98	99	100	98	90	95	50	0	0	100	0	100	0	95	0
100	100	70	5	100	97	100	100	95	0	95	0	0	11	21	30	87	0	0	100	0	100	0	100	0
1	100	0	100	99	100	100	100	1	0	90	0	0	1	75	0	35	0	0	100	0	100	0	100	0
95	100	97	92	100	99	100	99	85	0	30	90	15	40	75	30	75	0	0	99	0	99	0	100	0
100	100	100	100	100	100	100	100	100	0	100	100	0	0	100	0	100	0	0	100	0	100	0	100	0
0	100	0	100	100	96	100	100	83	0	0	0	0	4	96	13	74	0	0	100	0	95	0	100	0
100	100	100	100	100	100	100	100	100	0	100	100	0	0	100	0	25	0	0	100	0	100	0	100	0
0	100	0	0	60	0	60	0	0	0	20	0	0	100	0	50	100	0	0	100	0	100	0	100	0

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Escherichia coli	98	99	0	1	1	1	0	90	17	65	95	0	3	0	100	95	95	50	98	60	40	5	1
Escherichia coli, inactive	80	95	0	1	1	1	0	40	3	20	5	0	1	0	100	5	25	15	93	40	10	3	1
Escherichia fergusonii	98	100	0	17	0	0	0	95	5	100	93	0	0	35	100	95	0	0	98	60	65	98	0
Escherichia hermannii	99	100	0	1	0	0	0	6	0	100	99	0	94	0	100	97	45	45	100	19	40	0	0
Escherichia vulneris	0	100	0	0	0	0	0	85	30	0	100	0	15	85	100	97	15	8	100	0	30	0	0
Ewingella americana	0	84	95	95	0	0	0	0	0	0	60	0	5	0	100	0	70	0	100	0	80	0	0
Hafnia alvei	0	40	85	10	0	4	0	100	6	98	85	0	95	50	100	98	5	10	99	0	13	0	0
Hafnia alvei biogroup 1	0	85	70	0	0	0	0	100	0	45	0	0	0	45	100	0	0	0	55	0	55	0	0
Klebsiella oxytoca	99	20	95	95	0	90	1	99	0	0	0	0	97	98	100	97	100	100	99	55	100	99	98
Klebsiella pneumoniae subsp. *ozaenae*	0	98	0	30	0	10	0	40	6	3	0	0	88	3	100	50	30	20	100	2	97	97	55
Klebsiella pneumoniae subsp. *pneumoniae*	0	10	98	98	0	95	0	98	0	0	0	0	98	93	100	97	98	100	99	30	99	90	95
Klebsiella pneumoniae subsp. *rhinoscleromatis*	0	100	0	0	0	0	0	0	0	0	0	0	80	95	100	0	0	75	100	0	98	100	95
Kluyvera ascorbata	92	100	0	96	0	0	0	97	0	100	98	0	92	96	100	93	98	98	100	25	100	0	0
Kluyvera cryocrescens	90	100	0	80	0	0	0	23	0	100	90	0	86	86	100	95	95	81	95	0	100	0	0
Kluyvera georgiana	100	100	0	100	0	0	0	100	0	100	100	0	83	50	100	17	83	100	100	33	100	0	0

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94	99	50	80	95	95	98	2	0	0	35	75	5	75	95	95	90	0	0	100	0	95	0	98	0
75	85	15	65	80	70	90	2	0	0	5	40	5	65	30	85	40	0	0	98	0	45	0	97	0
0	98	0	92	96	96	96	96	0	0	46	0	100	20	0	96	96	0	0	100	0	83	0	100	0
0	100	40	97	100	100	100	97	0	0	40	0	8	3	97	35	78	0	0	100	0	98	98	100	0
1	100	99	93	100	100	100	100	25	0	20	100	0	25	78	2	30	0	0	100	0	100	50	100	0
0	0	0	23	16	13	99	10	0	0	50	0	99	24	0	35	10	0	0	97	0	85	0	99	0
0	95	2	97	100	98	95	15	0	0	7	0	0	95	0	70	15	0	0	100	0	90	0	100	0
0	0	0	0	0	0	70	0	0	0	0	0	0	0	0	30	0	0	0	100	0	30	0	100	0
99	98	100	100	100	100	100	100	98	2	100	99	98	99	93	98	90	0	0	100	0	100	1	100	0
65	98	90	55	95	95	98	92	70	0	80	97	95	65	25	50	2	0	0	80	0	80	0	100	0
99	99	99	99	98	99	99	98	90	0	99	99	98	97	90	95	75	0	0	99	0	99	0	99	0
100	100	90	96	100	100	100	100	0	0	30	100	100	50	0	50	0	0	0	100	0	0	0	100	0
40	100	98	100	100	99	100	100	98	0	99	99	0	40	90	35	50	0	0	100	0	100	0	100	0
45	100	100	100	100	91	100	100	95	0	100	100	0	5	81	19	86	0	0	100	0	100	0	100	0
0	100	100	83	100	100	100	100	100	0	100	100	0	33	83	50	83	0	0	100	0	100	0	100	0

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Kluyvera (Entero-bacter) intermedia	0	100	100	65	0	0	0	0	0	89	89	0	65	100	100	100	100	65	100	100	100	0	0
Kosakonia (Entero-bacter) cowanii	0	NA	92	100	0	0	0	0	0	0	92	0	92	0	100	100	100	100	100	100	100	0	0
Leclercia adecarbox-ylata	100	100	0	0	0	48	0	0	0	0	79	0	97	93	100	97	93	66	100	86	100	93	0
Lelliottia (Entero-bacter) amnigena biogroup 1	0	7	100	70	0	0	0	0	9	55	92	0	100	91	100	100	70	100	100	0	91	0	0
Lelliottia (Entero-bacter) amnigena biogroup 2	0	65	100	100	0	0	0	0	35	100	100	0	100	100	100	100	35	0	100	0	100	0	0
Lelliottia (Entero-bacter) nimipres-suralis	0	100	100	0	0	0	0	0	0	100	0	0	100	100	100	100	0	0	100	0	100	0	0
Leminorella grimontii	0	100	0	100	100	0	0	0	0	0	0	0	0	0	100	33	0	0	0	83	0	0	0
Leminorella richardii	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0
Moellerella wiscon-sensis	0	100	0	80	0	0	0	0	0	0	0	0	70	0	100	0	100	100	60	0	0	100	0
Morganella morganii subsp. *morganii*	95	95	0	0	20	95	95	0	0	95	95	0	98	1	99	90	1	0	0	0	0	0	0
Morganella morganii subsp. *sibonii*	50	86	0	0	7	100	93	29	0	64	79	0	79	0	100	86	0	7	0	0	0	0	0
Morganella morganii biogroup 1	100	95	0	0	15	100	100	100	0	80	0	0	90	5	100	93	0	0	0	0	0	0	0

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100	100	100	100	100	100	100	100	100	0	100	100	0	100	100	100	0	0	0	100	0	100	0	100	0
100	0	100	100	100	100	100	100	0	0	100	100	0	100	91	100	100	0	0	100	0	100	66	100	NA
0	100	66	100	100	100	100	100	0	0	100	100	96	3	93	83	28	0	0	100	0	100	37	100	0
9	100	100	100	100	100	100	100	55	0	91	100	0	0	35	9	0	0	0	100	0	91	0	100	0
100	100	0	100	100	100	100	100	100	0	100	100	0	0	100	0	0	0	0	100	0	100	0	100	0
100	100	0	100	100	100	100	100	100	0	100	100	0	0	100	0	0	0	0	100	0	100	0	100	0
0	100	0	0	0	83	0	0	0	0	0	0	0	17	100	100	0	0	0	100	0	0	0	0	0
0	100	0	0	0	100	0	0	0	0	0	0	0	0	50	100	0	0	0	100	0	0	0	0	0
0	0	100	0	30	0	0	0	0	0	0	100	75	10	0	30	10	0	0	90	0	90	0	100	0
0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	95	0	0	0	90	0	10	0	98	100
0	0	0	0	0	0	100	0	0	0	0	0	0	7	7	100	0	0	0	100	0	0	0	100	100
0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	100	0	0	0	90	0	20	0	100	100

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Pantoea agglomerans complex	20	50	70	50	0	20	20	0	0	0	85	2	35	65	100	20	40	75	100	15	65	7	15
Pantoea dispersa	0	82	64	100	0	0	9	0	0	0	100	0	82	9	100	0	0	0	100	0	0	0	0
Photorhabdus luminescens (all tests at 25°C)	50	0	0	50	0	25	0	0	0	0	100	50	0	0	75	0	0	0	0	0	0	0	0
Photorhabdus asymbiotica	0	0	0	20	0	60	0	0	0	0	100	80	20	0	100	0	0	0	0	0	0	0	0
Plesiomonas shigelloides	100	90	0	0	0	0	3	100	98	100	95	0	0	0	100	0	80	0	0	0	0	0	95
Pluralibacter (Enterobacter) gergoviae	0	5	100	99	0	93	0	90	0	100	90	0	0	96	100	98	55	98	99	0	99	0	0
Pluralibacter (Enterobacter) pyrinus	0	29	86	0	0	86	0	100	0	100	43	0	0	86	100	100	14	100	100	0	100	0	100
Pragia fontium	0	100	0	89	89	0	22	0	0	0	100	0	0	0	100	0	0	0	0	0	78	0	0
Proteus hauseri	100	100	0	0	50	100	100	0	0	0	100	100	100	0	100	0	0	0	0	0	0	0	0
Proteus mirabilis	2	97	50	65	98	98	98	0	0	99	95	90	98	2	100	96	2	15	0	0	0	0	0
Proteus myxofaciens	0	100	100	50	0	100	100	0	0	0	100	100	100	0	100	100	0	100	0	0	0	0	0
Proteus penneri	0	100	0	0	30	100	99	0	0	0	85	50	99	0	100	45	1	100	0	0	0	0	0
Proteus vulgaris	98	95	0	15	95	95	99	0	0	0	95	91	99	0	100	85	2	97	0	0	50	0	0
Providencia alcalifaciens	99	99	0	98	0	0	98	0	0	1	96	0	100	0	100	85	0	15	2	0	1	98	1
Providencia heimbachae	0	85	0	0	0	0	100	0	0	0	46	0	8	0	100	0	0	0	0	0	0	92	46

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30	95	30	85	89	93	97	55	7	0	60	50	50	30	40	25	30	0	0	85	0	90	75	98	0
0	100	0	91	82	100	100	55	0	0	0	0	100	27	0	9	100	0	0	91	0	91	27	100	0
0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	50	0	0	0	0	0	0	50	100	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	20	0	0	0	0	0	60	100	0
0	0	0	0	95	0	100	0	0	0	0	70	0	35	0	50	8	0	0	100	100	90	0	10	0
0	99	97	99	100	99	100	99	2	0	97	97	97	100	2	97	93	0	0	99	0	97	0	100	0
0	100	0	100	100	0	100	100	0	0	100	0	0	0	0	0	0	0	0	100	0	100	0	100	0
0	0	0	0	0	0	0	0	0	0	78	0	0	0	0	0	0	0	0	100	0	0	0	0	0
0	0	0	0	100	100	0	0	50	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	100
0	0	1	1	0	98	98	1	0	0	0	0	0	70	0	87	20	92	50	95	0	0	0	0	100
0	0	0	0	100	0	100	0	100	0	0	0	0	100	0	100	0	100	50	100	0	0	0	0	100
0	0	1	0	100	100	55	0	80	0	0	0	0	55	0	85	5	45	40	90	0	1	0	0	100
0	0	1	5	97	95	30	0	60	1	50	0	0	60	0	80	25	80	80	98	0	1	0	0	100
1	1	1	0	1	1	2	0	0	0	0	0	0	15	0	90	40	0	0	100	0	1	0	100	100
0	0	0	100	54	8	0	0	0	0	0	0	92	0	0	69	0	0	0	100	0	0	0	100	100

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Providencia rettgeri	99	93	0	95	0	98	98	0	0	0	94	0	97	0	100	10	5	15	100	0	50	100	90
Providencia rustigianii	98	65	0	15	0	0	100	0	0	0	30	0	100	0	100	35	0	35	0	0	0	0	0
Providencia stuartii	98	100	0	93	0	30	95	0	0	0	85	0	100	0	100	0	2	50	10	0	2	5	95
Rahnella aquatilis	0	88	100	94	0	0	95	0	0	0	6	0	0	100	100	98	100	100	100	88	100	0	0
Raoultella ornithino-lytica	100	96	70	100	0	100	0	100	0	100	0	0	100	100	100	100	100	100	100	10	100	100	95
Raoultella planticola	20	100	98	100	0	98	0	100	0	0	0	0	100	100	100	100	100	100	100	15	100	100	100
Raoultella terrigena	0	60	100	40	0	0	0	100	0	20	0	0	100	100	100	80	100	100	100	20	100	100	80
Salmonella bongori	0	100	0	94	100	0	0	100	94	100	100	0	100	0	100	94	0	0	100	94	0	0	0
Salmonella enterica subsp. *arizonae*	1	100	0	99	99	0	0	99	70	99	99	0	1	95	100	99	15	1	100	0	0	0	0
Salmonella enterica subsp. *diarizonae*	2	100	0	98	99	0	0	99	70	99	99	0	1	95	100	99	85	5	100	1	0	0	0
Salmonella enterica subsp. *enterica*	1	100	0	95	95	1	0	98	70	97	95	0	0	0	100	96	1	1	100	96	0	0	35
Salmonella enterica subsp. *houtenae*	0	100	0	98	100	2	0	100	70	100	98	0	95	0	100	100	0	0	98	0	60	5	0
Salmonella enterica subsp. *indica*	0	100	0	89	100	0	0	100	67	100	100	0	0	0	100	100	22	0	100	67	0	0	0
Salmonella enterica subsp. *salamae*	2	100	0	100	100	0	0	100	90	100	98	2	0	95	100	100	1	1	100	90	5	0	5

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1	0	5	70	2	10	0	3	2	75	35	5	100	60	0	95	60	0	0	100	0	5	0	100	100
0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	50	25	0	0	100	0	0	0	100	100
1	1	7	0	1	7	98	5	0	0	0	0	0	50	0	90	75	0	10	100	0	10	0	100	100
94	100	94	94	94	94	100	100	0	0	100	100	0	13	30	6	6	0	0	100	0	100	0	100	0
100	100	100	100	100	100	100	100	100	0	100	100	100	100	96	100	95	0	0	100	0	100	0	100	0
92	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	62	0	0	100	0	100	1	100	0
100	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	20	0	0	100	0	100	0	100	0
100	94	0	88	100	100	100	0	0	0	0	94	0	0	88	0	100	0	0	100	0	94	0	100	0
99	99	1	99	98	100	99	1	1	0	1	95	1	10	90	0	90	0	2	100	0	100	0	100	0
99	99	1	99	98	100	99	1	1	0	1	95	1	10	30	0	75	0	2	100	0	92	0	100	0
95	99	2	95	97	97	99	5	2	0	5	95	0	5	90	90	90	0	2	100	0	2	0	100	0
100	100	0	98	100	100	100	50	0	0	0	100	5	0	0	0	70	0	0	100	0	0	0	100	0
0	100	0	100	100	100	100	0	0	0	0	89	0	33	89	0	89	0	0	100	0	44	0	100	0
100	100	0	100	100	100	100	0	8	0	15	8	0	25	96	0	95	0	0	100	0	15	0	95	0

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Salmonella serotype Choleraesuis	0	100	0	25	50	0	0	95	55	100	95	0	0	0	100	95	0	0	98	5	0	0	0
Salmonella serotype Gallinarum	0	100	0	0	100	0	0	90	10	1	0	0	0	0	100	0	0	0	100	90	0	0	0
Salmonella serotype Paratyphi A	0	100	0	0	10	0	0	0	15	95	95	0	0	0	100	99	0	0	100	90	0	0	0
Salmonella serotype Pullorum	0	90	0	0	90	0	0	100	10	95	0	0	0	0	100	90	0	0	100	0	0	0	0
Salmonella serotype Typhi	0	100	0	0	97	0	0	98	3	0	97	0	0	0	100	0	1	0	100	0	0	0	0
Serratia entomophila	0	20	100	100	0	0	0	0	0	0	100	100	100	0	100	0	0	100	100	0	100	0	0
Serratia ficaria	0	75	75	100	0	0	0	0	0	0	100	100	55	0	100	0	15	100	100	0	100	0	55
Serratia fonticola	0	100	9	91	0	13	0	100	0	97	91	0	70	88	100	79	97	21	100	91	100	100	30
Serratia liquefaciens complex	1	93	93	90	0	3	0	95	0	95	95	90	90	2	100	75	10	98	100	0	97	5	60
Serratia marcescens	1	20	98	98	0	15	0	99	0	99	97	90	95	3	100	55	2	99	99	0	95	40	75
Serratia marcescens biogroup 1	0	100	60	30	0	0	0	55	4	65	17	30	70	0	100	0	4	100	96	0	92	30	30
Serratia odorifera biogroup 1	60	100	50	100	0	5	0	100	0	100	100	95	60	0	100	0	70	100	100	0	98	50	100
Serratia odorifera biogroup 2	50	60	100	97	0	0	0	94	0	0	100	94	19	0	100	13	97	0	97	0	45	55	100
Serratia plymuthica	0	94	80	75	0	0	0	0	0	0	50	60	30	0	100	40	80	100	100	0	94	0	50
Serratia rubidaea	0	20	100	95	0	2	0	55	0	0	85	90	25	94	100	30	100	99	100	0	99	99	20

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90	0	1	100	95	98	0	0	0	1	0	45	1	0	0	85	1	0	0	98	0	0	0	95	0
1	80	10	10	90	70	50	10	0	1	0	0	0	0	50	100	0	0	10	100	0	0	0	100	0
95	100	0	100	95	0	100	5	0	0	0	95	0	10	0	0	0	0	0	100	0	0	0	100	0
10	100	1	100	5	90	90	5	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
99	2	0	0	97	82	100	0	0	0	0	100	0	20	0	100	0	0	0	100	0	0	0	100	0
0	0	0	0	100	40	100	0	0	0	100	0	60	0	0	100	80	20	100	100	0	100	0	100	0
100	100	70	35	100	100	100	100	8	0	100	40	100	0	0	17	40	77	100	92	8	100	0	100	0
100	100	100	76	97	85	100	6	91	0	100	98	100	88	0	58	15	0	0	100	0	100	0	100	0
95	98	85	15	98	100	100	5	5	0	97	75	0	95	0	75	40	85	85	100	0	93	0	100	0
99	0	2	0	96	7	99	5	0	1	95	0	0	95	0	75	50	98	98	98	0	95	0	99	0
92	0	0	0	70	0	100	4	0	0	96	0	0	92	0	50	4	75	82	83	0	75	0	100	0
100	100	100	95	100	100	100	100	0	0	95	100	0	40	5	100	60	35	100	100	0	100	0	100	0
100	100	7	94	100	100	100	100	0	7	40	96	0	50	0	100	65	65	100	100	0	100	0	100	0
65	100	94	0	94	94	100	88	70	0	81	93	0	50	0	100	55	70	100	100	0	70	0	100	0
1	100	99	1	99	99	100	94	1	0	94	99	85	20	0	70	80	99	99	100	0	100	0	100	0

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Shigella dysenteriae (Serogroup A)	45	99	0	0	0	0	0	0	2	0	0	0	0	0	100	0	0	0	100	0	0	0	0
Shigella flexneri (Serogroup B)	50	100	0	0	0	0	0	0	5	0	0	0	100	0	100	3	1	1	95	1	0	0	0
Shigella boydii (Serogroup C)	25	100	0	0	0	0	0	0	18	2	0	0	0	0	100	0	1	0	97	5	0	0	0
Shigella sonnei (Serogroup D)	0	100	0	0	0	0	0	0	2	98	0	0	0	0	100	0	2	1	99	0	0	0	0
Shimwellia (Escherichia) blattae	0	100	0	50	0	0	0	100	0	100	0	0	0	100	100	100	0	0	0	0	0	0	0
Shimwellia (Obesumbacterium) pseudoproteus	0	15	0	0	0	0	0	100	0	100	0	0	0	0	100	0	0	0	0	0	0	0	0
Tatumella ptyseos	0	0	5	2	0	0	90	0	0	0	0	0	0	0	100	0	0	98	0	0	55	0	0
Trabulsiella guamensis	40	100	0	88	100	0	0	100	50	100	100	0	100	0	100	100	0	0	100	0	13	0	0
Xenorhabdus nematophilus (all tests at 25°C)	40	0	0	0	0	0	0	0	0	0	100	80	0	0	80	0	0	0	0	0	0	0	0
Yersinia aldovae	0	80	0	0	0	60	0	0	0	40	0	0	0	0	100	0	0	20	80	0	0	0	0
Yersinia bercovieri	0	100	0	0	0	60	0	0	0	80	0	0	0	0	100	0	20	100	100	0	20	0	0
Yersinia enterocolitica	50	97	2	0	0	75	0	0	0	95	2	0	2	0	100	5	5	95	98	0	20	0	30
Yersinia frederiksenii	100	100	0	15	0	70	0	0	0	95	5	0	0	0	100	40	40	100	100	0	92	0	20
Yersinia intermedia	100	100	5	5	0	80	0	0	0	100	5	0	10	5	100	18	35	100	100	0	100	0	15

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30	45	0	30	15	4	90	0	0	0	0	0	0	10	0	75	0	0	0	99	0	30	0	100	0
29	60	40	5	30	2	65	0	0	0	0	55	1	10	0	30	8	0	0	99	0	1	0	100	0
43	94	0	1	20	11	85	0	0	0	0	15	0	50	0	50	0	0	0	100	0	10	0	100	0
2	95	3	75	90	2	100	5	0	0	0	25	0	15	10	90	0	0	0	100	0	90	0	100	0
0	100	0	100	100	100	75	0	0	0	0	0	0	100	50	50	0	0	0	100	0	0	0	100	0
0	0	0	15	50	15	85	0	0	0	0	0	0	0	0	15	0	0	0	100	0	0	0	85	0
0	0	11	0	0	9	93	0	0	0	0	25	0	7	0	0	0	0	0	98	0	0	0	100	0
100	100	0	100	100	100	100	100	0	0	40	0	0	0	100	50	88	0	0	100	0	100	0	100	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	0	20	20	0	0	60	80	0

60	60	0	0	0	40	80	0	0	0	0	0	0	0	0	100	0	0	0	100	0	0	0	100	0
100	100	0	0	100	100	100	100	0	0	20	0	0	0	0	100	0	0	0	100	0	80	0	100	0
99	98	5	1	75	70	98	75	0	0	25	1	40	90	0	85	15	55	5	98	0	95	0	100	0
100	100	30	99	100	100	100	100	0	0	85	0	100	85	5	55	15	55	0	100	0	100	0	100	0
100	100	45	100	100	100	100	96	77	0	100	80	45	60	6	88	18	12	0	94	0	90	0	100	0

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Yersinia kristensenii	30	92	0	0	0	77	0	0	0	92	5	0	0	0	100	23	8	0	100	0	15	0	15
Yersinia mollaretii	0	100	0	0	0	20	0	0	0	80	0	0	0	0	100	0	40	100	100	0	20	0	0
Yersinia pestis	0	80	0	0	0	5	0	0	0	0	0	0	0	0	100	0	0	0	97	0	70	0	0
Yersinia pseudotu-berculosis	0	100	0	0	0	95	0	0	0	0	0	0	0	0	100	0	0	0	100	0	25	0	0
Yersinia rohdei	0	62	0	0	0	62	0	0	0	25	0	0	0	0	100	0	0	100	100	0	0	0	0
Yersinia ruckeri	0	97	10	0	0	0	0	50	5	100	0	30	15	0	100	5	0	0	100	0	0	0	0
Yokenella regens-burgei (Koserella trabulsii)	0	100	0	92	0	0	0	100	8	100	100	0	92	0	100	100	0	0	100	0	8	0	0
Enteric Group 58 *(proposed Averyella dalhous-iensis)*	0	100	0	85	0	70	0	100	0	85	100	0	100	85	100	85	30	0	100	85	100	0	0
Enteric Group 59	10	100	0	100	0	0	30	0	60	0	100	0	80	90	100	100	80	0	100	0	100	0	0
Enteric Group 60	0	100	0	0	0	50	0	0	0	100	75	0	0	100	100	100	0	0	50	0	0	0	0
Enteric Group 68	0	100	50	0	0	0	0	0	0	0	0	0	100	0	100	0	0	100	100	0	50	0	0
Enteric Group 137	100	100	0	0	0	70	0	0	20	100	100	0	100	0	100	0	100	100	100	0	100	0	0

[a] Data provide by Brent Barrett using CDC Enterobacteriaceae Tables originally developed by J.J. Farmer, PhD, retired from CDC with revision by Caroline O'Hara, and updated with results published in the International Journal of Systemic Bacteriology added by A.P. Schreckenberger.
[b] Each number is the percentage of positive reactions after 2 days of incubation at 36°C unless otherwise indicated (gelatin hydrolysis and DNASE and all reactions of *Xenorhabdus* species, *P. luminescens* and *Y. ruckeri*). Reactions that become positive after 2 days are not considered. TSI, triple sugar iron agar; ONPG, o-nitrophenyl-β-D-galactopyrannoside; NA, not available.

species (*S. flexneri*) also can produce gas from the fermentation of glucose. The pathogenic spectrum of *E. coli* is much broader than that of *Shigella* species, and toxigenic strains of *E. coli* can produce dysentery-like diarrheal syndromes indistinguishable from shigellosis. Serologic testing may be required in some instances to differentiate certain closely related strains. The key characteristics of the Escherichieae are given in Table 6-6.

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D–Sorbitol fermentation	L–Arabinose fermentation	Raffinose fermentation	L–Rhamnose fermentation	Maltose fermentation	D–Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	alpha–methyl–D–glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D–Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate nitrite	Oxidase, Kovac's	ONPG test	Yellow pigment	D–Mannose fermentation	Tyrosine hydrolysis
100	77	0	0	100	85	100	100	0	0	0	0	45	70	0	40	8	0	0	100	0	70	0	100	0
100	100	0	0	60	60	100	100	0	0	0	0	0	20	0	100	0	0	0	100	0	20	0	100	0
50	100	0	1	80	90	100	0	0	0	50	20	0	50	0	0	0	0	0	85	0	50	0	100	0
0	50	15	70	95	100	100	0	0	0	95	70	0	50	0	50	0	0	0	95	0	70	0	100	0
100	100	62	0	0	38	100	25	0	0	0	50	0	38	0	100	0	0	0	88	0	50	0	100	0
50	5	5	0	95	0	95	5	0	0	0	0	0	30	0	30	0	30	0	75	0	50	0	100	0
0	100	25	100	100	100	100	100	0	0	67	92	0	0	0	0	25	0	0	100	0	100	0	100	0
100	100	0	100	100	100	100	100	55	0	0	0	0	30	0	60	45	0	0	100	0	100	0	100	0
0	100	0	100	100	100	100	100	10	0	100	0	10	10	60	50	50	0	0	100	0	100	25	100	0
0	25	0	75	0	0	100	0	0	0	0	0	0	75	0	75	0	0	0	100	0	100	0	100	0
0	0	0	0	50	0	100	0	0	0	0	0	0	50	0	0	0	0	100	100	0	0	0	100	0
100	100	100	100	100	100	100	100	80	0	100	100	0	100	100	50	100	0	0	100	0	100	0	100	0

Genus *Escherichia*. The genus *Escherichia* contains five species: *E. albertii*, *E. coli*, *E. fergusonii*, *E. hermannii*, and *E. vulneris*. A former member of the genus, *E. adecarboxylata*, has been assigned to a new genus as *Leclercia adecarboxylata* and is discussed elsewhere in this chapter.[622]

Another former member of the genus, *E. blattae,* has been transferred to a new genus, *Shimwellia,* as *S. blattae.*[541] It has been recovered from the intestinal tract of cockroaches, is indole-negative, does not ferment lactose, and has not been associated with human infections.[541] The different biochemical

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TABLE 6-8 Differentiation of Species Within the Genus *Escherichia*

Biochemical Test	*E. albertii*	*E. coli*	*E. fergusonii*	*E. hermannii*	*E. vulneris*
Indole	–	+	+	+	–
Methyl red	+	+	+	+	+
Voges–Proskauer	–	–	–	–	–
Citrate	–	–	V (17)	–	–
Lysine decarboxylase	+	+	+	–	V (85)
Arginine dihydrolase	–	V (17)	–	–	V (30)
Ornithine decarboxylase	+	V (65)	+	+	–
ONPG	+	+	V (83)	+	+
Fermentation of					
Lactose	–	+	–	V (45)	V (15)
Sorbitol	–	+[a]	–	–	–
Mannitol	+	+	+	+	+
Adonitol	–	–	+	–	–
Cellobiose	–	–	+	+	+
Yellow Pigment	–	–	–	+	V (50)

+, 90% or more strains positive; –, 90% or more strains are negative; V, 11%–89% of strains are positive.
[a]Strains of *E. coli* belonging to serotype 0157:H7 are sorbitol negative.

reactions for the recognized species of *Escherichia* are shown in Table 6-8.

E. coli is the bacterial species most commonly recovered in the clinical laboratories and has been incriminated in infectious diseases involving virtually every human tissue and organ system. *E. coli* is one of the common organisms involved in gram-negative sepsis and endotoxin-induced shock. Urinary tract and wound infections, pneumonia in immunosuppressed hospitalized patients, and meningitis in neonates are other common infections caused by *E. coli*. *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigens. More than 170 different O antigen serogroups are currently recognized.[457] The combination of O and H antigens defines a "serotype" of an isolate; for example, *E. coli* O157:H7 is a serotype of a virulent strain of *E. coli* associated with hemorrhagic colitis and hemolytic–uremic syndrome (HUS).

Carbon dioxide-dependent strains of *E. coli* have been reported from urine and empyema fluid specimens.[398,630] We have also observed such strains in our

laboratory. It is possible that the incidence of infections associated with capnophilic *E. coli* is higher than currently thought because culture plates from urine and wound specimens are not routinely incubated in CO_2.

E. coli *That Causes Gastroenteritis.* Certain strains of *E. coli* can cause enteritis or gastroenteritis by six distinct mechanisms, resulting in six different clinical syndromes. These include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC)[457] (Table 6-9). EPEC, EAEC, and DAEC isolates are characterized by their distinct patterns of adherence to epithelial cells in vitro. EPEC strains bind to host cells in a pattern called localized adherence, in which microcolonies form on the surfaces of the cells. EAEC isolates bind in an aggregative adherence pattern that is characterized by a stacked-brick-like arrangement on the surfaces of the cells. DAEC strains are defined by a pattern of diffuse adherence in which the bacteria uniformly cover the entire cell surface.[457] In addition to the six classes of diarrheagenic *E. coli* mentioned above, there are other potential classes, such as cytolethal distending toxin (CDT)-producing *E. coli* and cell-detaching *E. coli* (CDEC) that have yet to be fully characterized.[457]

Although assays to identify all categories of *E. coli* that cause gastroenteritis are available, in most cases it is not necessary to identify a specific *E. coli* pathogen in a particular patient. Most patients with gastroenteritis due to *E. coli* will resolve their diarrhea before seeking medical attention or their diarrhea resolves following treatment with empirical antibiotics given for other bacterial diarrheas. Therefore, with the exception of assays for the detection of EHEC (discussed below), phenotypic assays or serotyping of *E. coli* for diagnosis of diarrheagenic strains is not routinely performed in the clinical laboratory.

***Pathophysiology of Enterohemorrhagic* E. coli.** The clinical significance of EHEC was not known until 1982, when these organisms were associated with two conditions of previously unknown etiology: hemorrhagic colitis[564] and hemolytic–uremic syndrome.[339] (see Clinical Correlation Box 6-1.) *E. coli* O157:H7 was the first of several Shigatoxin-producing serotypes known to cause human illness. *E. coli* O157:H7 is so named because it expresses the 157th somatic (O) antigen identified and the 7th flagellar (H) antigen. Among the most important virulence characteristics of *E. coli* O157:H7 is its ability to produced one or more Shiga toxins (Stxs) (also called verocytotoxins, and formerly known as Shiga-like toxins). Stx1 is indistinguishable from Stx

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TABLE 6-9 Key Features of Diarrheagenic *E. coli*[191,457]

Term	Abbreviation	Pathogenic Phenotype	Signs and Symptoms
Enterotoxigenic *E. coli*	ETEC	Elaboration of secretory heat-labile (LT) and/or heat-stable (ST) enterotoxins that do not damage the mucosal epithelium. Toxin production is plasmid mediated and most commonly involves *E. coli* serogroups 06, 08, 015, 020, 025, 027, 063, 078, 080, 085, 092, 0115, 0128ac, 0139, 0148, 0153, 0159, and 0167.[547] From 1996 to 2003, 16 outbreaks of ETEC infections occurred in the US and on cruise ships. *E. coli* O169:H41 was identified in 10 outbreaks. This serotype was identified in 1 of 21 confirmed ETEC outbreaks before 1996.[41]	Associated with two major clinical syndromes: "weanling diarrhea" among children in the developing world and "Traveler's diarrhea." Typically abrupt in onset with short incubation period (14–50 hours). Profuse watery diarrhea is predominant symptom (similar to *Vibrio cholerae*), usually without blood, mucus, or pus. Often accompanied by mild abdominal cramps. Dehydration and vomiting occur in some cases.
Enteropathogenic *E. coli*	EPEC	Adhere to intestinal epithelial cells in localized microcolonies producing characteristic histopathologic lesions known as "attaching and effacing lesions" (A/E lesions). Serogroups 055, 086, 0111, 0119, 0126, 0127, 0128ab, and 0142 are most commonly involved.[547]	Usually occurs in infants. Characterized by low-grade fever, malaise, vomiting, and profuse, watery diarrhea, with a prominent amount of mucus, but with no gross blood. Fecal leukocytes are seen only occasionally.
Enteroinvasive *E. coli*	EIEC	Invade colonic epithelial cells. Pathogenesis virtually identical to *Shigella* species. As with *Shigella*, most strains are nonmotile, late– or non–lactose-fermenters, and anaerogenic. The most commonly involved serogroups are 028ac, 029, 0112ac, 0124, 0136, 0143, 0144, 0152, and 0164.[547]	Infection presents most commonly as watery diarrhea, indistinguishable from ETEC. Some patients present with a dysentery syndrome; hallmarks are fever and colitis. Symptoms are urgency and tenesmus; blood, mucus, and many leukocytes in stool.
Enterohemorrhagic *E. coli*	EHEC	Elaboration of Stxs, an enterohemolysin, and like EPEC produce A/E lesions. Most commonly involved serogroup is O157:H7.	Bloody diarrhea without WBCs. Often no fever. Abdominal pain is common. May progress to HUS.
Enteroaggregative *E. coli*	EAEC	Adhere to epithelial cells in a pattern resembling a pile of stacked bricks and produce an ST-like toxin (EAST1), an LT toxin, and fimbrial colonization factors called "AAFs" (aggregative adherence fimbriae).	Watery, mucoid, secretory diarrheal illness with low-grade fever and little or no vomiting. Stools usually without gross blood or fecal leukocytes. Recovered principally from children with chronic diarrhea.[458,499]
Diffusely adherent *E. coli*	DAEC	Adhere to epithelial cells in a diffuse pattern and carry a gene encoding surface fimbria designated *F1845*.	Majority of patients have watery diarrhea without blood or fecal leukocytes.

CLINICAL CORRELATION BOX 6-1 Hemorrhagic Colitis and Hemolytic–Uremic Syndrome

Hemolytic–uremic syndrome (HUS) is a thrombotic disease of the kidney microvasculature in which the endothelial cell is the primary site of damage. HUS is defined by a triad of features: (1) the appearance of fragmented red cells (microangiopathic hemolytic anemia), (2) reduced platelet count (thrombocytopenia), and (3) acute renal failure, evidenced by a reduced glomerular filtration rate and low urine output. HUS is the leading cause of acute renal failure in children. In its most common form, this syndrome is preceded by a diarrheal illness that presents with severe abdominal pain, vomiting, and watery diarrhea. The diarrhea subsequently becomes blood-streaked or grossly bloody, and despite evidence of colitis, the children typically have little or no fever. This constellation of signs and symptoms is called **hemorrhagic colitis.** In the most severe infections, ischemic colitis and perforation can occur. In some patients, colonic stenosis can develop following

ischemic colitis. EHEC organisms colonize the large intestine via the formation of distinctive attaching and effacing lesions that provide a tight junction between the EHEC and the surface of the intestinal epithelial cells. Development of attaching and effacing lesions (A/E lesions) requires numerous genes that are located on a 35-kb chromosome pathogenicity island termed the locus on enterocyte effacement (LEE). Following colonization, EHEC produce Stxs that translocate into the circulation, probably facilitated by the influx (transmigration) of neutrophils. Once in the circulation, Stx travels to the kidneys, where it is transferred and bound via the B subunit of the toxin to neutral glycolipid globotriaosylceramide (Gb_3) receptors on target cells (i.e., glomerular endothelial and tubular epithelial cells). The toxin is then internalized and routed to the endoplasmic reticulum, where the single A subunit enzymatically inactivates the ribosomes, causing inhibition of protein synthesis and cell damage. This leads to glomerular endothelial cell swelling and detachment from the underlying basement membrane with secondary activation of both platelets and the coagulation cascade.

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This sequence of events results in the classic signs of Stx-mediated HUS. The coagulation cascade results in fibrin deposition, causing narrowing of the capillaries and shearing of red cells as they are pushed through the damaged vessels. The shearing and tearing of red blood cells result in the fragmented erythrocytes typical of microangiopathic hemolytic anemia. The consumption of platelets leads to thrombocytopenia, and the anemia in addition to restricted blood flow to the kidneys, which consequently leads to renal failure.[352,594,675] There are no treatments of proven value other than supportive care for patients with HUS. Antibiotic treatment of children with *E. coli* O157:H7 infection increases the risk of HUS and therefore should be avoided.[509] Research is currently underway to develop specific Stx antibodies to block the binding of Stx to endothelial cells as well as synthetic Gb3 receptor analogues that can be given orally to entrap Stx in the gut and prevent the entry of the toxin into the circulation.[338,657]

produced by *S. dysenteriae* type 1. Stx2 is a more divergent molecule that has multiple variants (Stx2, Stx2c, Stx2d, Stx2e, Stx2f) that are closely related to each other but more distantly related to Stx1. Some *E. coli* O157 strains produce only Stx1, some only Stx2, and others produce both. The toxins do not carry equivalent risks of causing HUS. Strains that produce only Stx2 have the highest risk; strains that produce only Stx1 have the lowest risk, and strains that make both toxins carry an intermediate

risk. Both toxins are composed of five B subunits and a single A subunit. The B subunit binds to globotriaosylceramide (Gb3), a glycolipid found in varying degrees in membranes of eukaryotic cells. The A subunit is a potent cytotoxin and is responsible for cell injury. Production of Stx is not in itself sufficient to cause disease. Other virulence factors identified in *E. coli* O157 include a 60-MDa-virulence plasmid (pO157) and the locus of enterocyte effacement (LEE). The 60-MDa plasmid encodes an enterohemolysin (designated EHEC-Hly) that may allow *E. coli* O157 to use hemoglobin released by the action of EHEC-Hly as a source of iron, thereby stimulating the growth of *E. coli* O157 in the gut.[509] The LEE contains genes for an adhesion molecule (intimin) and for other factors important for the production of attaching–effacing lesions. The clinical manifestations of *E. coli* O157 infection range from asymptomatic carriage to nonbloody diarrhea, hemorrhagic colitis, HUS, and death. The incubation period varies from 1 to 8 days, with an average interval of 3 days between exposure and illness. Most patients with hemorrhagic colitis recover within 7 days. Illness typically begins with abdominal cramps and nonbloody diarrhea. Stool may become bloody after the first 1 to 2 days, with the amount of blood varying from a few small streaks to stools that are almost entirely blood. Vomiting occurs in 30% to 60% of cases, and fever is usually low-grade or absent.[426] HUS following urinary tract infection with EHEC has been reported[436,608]; however, because of the low prevalence, routine testing of sorbitol-negative *E. coli* urinary isolates for the presence of Stx does not seem warranted.[634,671] Currently, antibiotics are not recommended for treatment of *E. coli* O157 infections because of the concern that antibiotic use will increase the production of Stxs. Stx genes are bacteriophageencoded, and antibiotics that cause bacteriophage induction (e.g., fluoroquinolones, trimethoprim–sulfamethoxazole, and furazolidone) may increase Stx expression. In view of these issues, it is recommended that clinical laboratories do not report antimicrobial susceptibility results for O157 or other Stx *E. coli* isolates.[352,675]

***Epidemiology of enterohemorrhagic* E. coli.** In early 1993, the largest *E. coli* food-poisoning outbreak to date caused by *E. coli* O157:H7 occurred in the states of Washington, Idaho, California, and Nevada. Altogether, 582 culture-confirmed cases were reported, causing 171 hospitalizations, 41 cases of HUS, and 4 deaths. Hamburgers from a single fast-food restaurant chain were implicated. The source of most cases of *E. coli*-related illnesses in the United States is considered to be ground beef. Hamburgers, in particular, have been the cause of multiple outbreaks of disease. This is because of the high prevalence of *E. coli* O157:H7 colonization in cows. For a summary of risk factors during all the steps of industrial scale beef production from farm to fork see the review by Meyer–Broseta and colleagues.[433] According to recent market-basket surveys, *E. coli* O157:H7 is present in 1.0% to 2.5% of meat and poultry samples. It also turns up less frequently in supermarket samples of lamb and pork, although such isolates could arise from secondary contamination in the

butchering area. In the United States, cases have tended to occur in the last two-thirds of the year and in states bordering Canada. Infection persists in animal reservoirs in part because the organism survives in soil for long periods. Other animals, including pigs, sheep, deer, and rabbits, can also carry EHEC, and many other foods and water can be contaminated with the feces of infected animals.[245,473] The result has been outbreaks involving municipal water supplies,[491] venison,[546] uncooked vegetables, cheese curd,[119] apple cider,[113] alfalfa sprouts.[75,442] spinach,[265] and raw prepackaged cookie dough.[460] In addition, because incredibly low numbers of these organisms can cause illness, personto-person spread has occurred within families and in daycare centers, among children during visits to dairy farms,[120] and petting zoos,[285] and among persons exposed to sawdust and other surfaces in county fair buildings used for showing animals.[645] Although O157:H7 is the prototypic serotype of EHEC in the United States, several other EHEC serotypes, such as O26:H11, O48:H21, O103:H2, O111:NM (nonmotile), and O145:NM, have been recognized in other countries.[204, 331,399] Although non-O157 serotypes of EHEC are rare in the United States, one outbreak of hemorrhagic colitis caused by EHEC serotype O104:H21 was reported among 11 patients in Helena, Montana, in 1994.[112] Another outbreak occurred among teenage campers in Texas in June 1999, caused by EHEC serotype O111:H8, in which 55 campers became ill and HUS developed in two.[77,118] *E. coli* O111 is the second most common non-O157 Shiga toxin-producing *E. coli* (STEC) reported in the United States,[78] and it is among the most common reported in Europe.[86] A large outbreak of HUS caused by STEC O104:H4 occurred in Germany in May 2011. There were 3,469 confirmed cases of STEC and 852 cases of HUS. A unique feature of this outbreak was that 90% of affected cases were adults and more than two-thirds of

case subjects with HUS were female. The outbreak was traced to contaminated fenugreek sprouts served at several restaurants.[82] To learn more about infections caused by EHEC, the reviews by Kaper and O'Brien,[335] Mead and Griffin,[426] Nataro and Kaper,[457] Paton and Paton,[509] and Tarr[625] may be consulted.

***Detection of Enterohemorrhagic* E. coli.** Isolation of *E. coli* O157:H7 is possible only during the acute phase of illness, and the organisms may not be detectable 5 to 7 days after onset. Because the majority of EHEC infections are caused by *E. coli* serotype O157:H7, the current laboratory approaches to detection are based on either detection of Shiga-toxin-producing strains or detection of the O157:H7 serotype. The currently accepted methods for detection can be summarized as follows: (1) assays for the detection of the O157 serotype[406,503,607] or Stxs[341,406,468] directly from stool; (2)

direct plating on sorbitol–MacConkey agar (SMAC),[414] cefixime–SMAC,[135] SMAC supplemented with cefixime and tellurite (CT-SMAC),[686] or media containing either 5-bromo-5-chloro-3-indoxyl-β-D-glucuronide[490] or 4-methylumbelliferyl-β-D-glucuronide[633]; and (3) immunomagnetic separation (IMS) using O157-specific, antibodycoated beads, followed by bacteriologic culture.[136,337] Many laboratories in the United States and elsewhere use SMAC agar to identify the slow sorbitol fermentation phenotype (i.e., sorbitol-negative at 24 hour) of O157:H7. SMAC agar contains 1% D-sorbitol rather than lactose to differentiate sorbitol-negative *E. coli* strains (colonies appear colorless, similar to lactose-negative colonies on regular MacConkey agar). Suspected isolates are then confirmed with specific O157:H7 antisera. This medium, however, does not detect other, sorbitol-positive EHEC serotypes. Other detection methods such as chromogenic medium and enzyme-linked immunosorbent assay (ELISA) methods for detection of Stx are more sensitive and specific, but are considerably more expensive than SMAC.[468,503] Incorporation of this testing into the routine microbiology laboratory comes at a significant additional cost, and laboratories may want to limit testing to patients at highest risk. One of the recommendations published by the CDC for diagnosis of STEC infections by clinical laboratories is that all stools submitted for routine testing from patients with acute community-acquired diarrhea (regardless of patient age, season of the year, or presence or absence of blood in the stool) be simultaneously cultured for *E. coli* O157:H7 and tested with an assay that detects Stxs to detect non-O157 STEC.[127] A mini-review on the role of the laboratory in the diagnosis of EHEC infections has been provided by Kehl.[342]

***Species Other Than* E. coli.** Strains designated in the CDC classification as *E. coli*-inactive are anaerogenic (non–gasproducing), lactose-negative (or delayed), and nonmotile. These strains were previously known as the Alkalescens–Dispar serotype.

Escherichia albertii is a newly described species of indole-, D-sorbitol-, and lactose-negative enterobacterial isolates recovered from diarrheal stools of children.[306] Because these strains are not included in the databases of commercial systems at present, they are most often identified as *Hafnia*, *Salmonella*, *E. coli*, or *Yersinia ruckeri*.[5] *E. albertii* most closely resemble inactive *E. coli*, although they do not resemble the Alkalescens–Dispar group because of their ability to produce gas from D-glucose. They can be separated from *H. alvei* based on acetate assimilation, negative VP test, and failure to grow in potassium cyanide (KCN). In addition, *E. albertii* show weak-to-moderate L-prolineaminopeptidase (PYR) reactions, whereas *H. alvei* express strong PYR activity.[5] Laboratory workers should be advised that isolates recovered from stool specimens that give an unacceptable first-choice identification of *H. alvei* for an isolate that is L-rhamnose-, D-xylose-, and VP-negative should be further tested

to rule out *E. albertii*.

E. albertii has recently been identified as the cause of two food-borne outbreaks of gastroenteritis in Japan.[361,496] The clinical significance of *E. albertii* has been studied by Ooka and colleagues who examined 278 *eae*-positive strains that were originally identified by routine diagnostic protocols as EPEC or EHEC. They were isolated from humans, animals, and the environment in Japan, Belgium, Brazil, and Germany during 1993 to 2009. From this group, 26 strains (14 humans, 11 birds, and 1 cat) were identified as *E. albertii*.[495] Because discriminating *E. albertii* from other *Enterobacteriaceae* is difficult and because *E. albertii* possesses the *eae* gene, many strains identified as enterohemorrhagic or enteropathogenic *E. coli* might in fact be strains of *E. albertii*.[495]

E. fergusonii (formerly CDC enteric group 10) has been recovered from blood, gallbladder, urine, and feces; however, its clinical significance has not been establi shed.[220,221,239,240,371,372,560] It is differentiated from *E. coli* by being sorbitoland lactose-negative, but adonitoland cellobiose-positive. One case of cystitis caused by an ESBL-producing strain of *E. fergusonii* has been reported.[371]

E. hermannii (formerly CDC enteric group 11) has been most commonly found in human wounds, sputum, and feces.[525] Of eight strains recovered at the University of Illinois Medical Center, five have been from wound specimens, including four leg wounds in patients with cellulitis and one finger wound, and one strain each has been recovered from urine, maxillary sinus, and blood. The isolates recovered from wounds were mixed with other pathogenic species; however, the blood, urine, and sinus isolates were present as the only isolates. Ginsberg and Daum[254] reported recovery of *E. hermannii* from blood, cerebrospinal fluid (CSF), and peritoneal fluid of a septic neonate with bowel perforation; however, other organisms were recovered from the blood, and the pathogenic role in this patient was uncertain. *E. hermannii* has been reported as the sole pathogen in a catheter-related bloodstream infection,[334] and from a patient with acute purulent conjunctivitis after a minor ocular injury.[538] The only documented case of invasive disease caused by *E. hermannii* is from a neonate with bacterial infection of a cephalohematoma with meningitis documented by culture from multiple CSF specimens and fluid aspirated from an enlarging cephalohematoma.[161] *E. hermannii* strains are yellow pigmented, indole-positive, and sorbitol-negative.[66] Because *E. hermannii* are sorbitol-negative, they appear biochemically similar to the O157 serotype of *E. coli*.

E. vulneris (formerly CDC enteric group 1) has a high propensity for causing human wound infections, particularly of the arms and legs,[70,525] which may lead to osteomyelitis.[383] In addition, there have been reports of urosepsis, peritonitis in patients receiving continuous ambulatory peritoneal dialysis (CAPD), intravenous

catheter-related bacteremia, and meningitis caused by *E. vulneris*.[22,27,299,441,589,603] One case of *E. vulneris* bacteremia in a 40-day-old infant and another case of *E. vulneris* bacteremia traced to an infected

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right chest wall permacath have been seen in the author's laboratory (P. Schreckenberger, Personal communication). *E. vulneris* bacteremia secondary to a subcutaneous abscess in a patient with chronic lymphocytic leukemia has also been reported.[348] Over half of the strains of *E. vulneris* are yellow pigmented, and they are both indoleand sorbitol-negative.

Genus *Shigella*. *Shigella* species can be suspected in cultures because they are non–lactose-fermenters and tend to be biochemically inert. They typically do not produce gas from carbohydrates, with the exception of certain biogroups of *S. flexneri* that are aerogenic. Rare strains of *S. sonnei* can slowly ferment lactose (2%) and sucrose (1%), and most strains can decarboxylate ornithine—characteristics not shared by other *Shigella* species.

There are 4 major subgroups and 43 recognized serotypes of *Shigella*, as shown in Box 6-9.[100] The CDC classification combines *S. dysenteriae* (group A), *S. flexneri* (group B), and *S. boydii* (group C) as "*Shigella* serogroups A, B, and C" because of their biochemical similarities. The presence of ornithine decarboxylase activity and β-galactosidase make most strains of *S. sonnei* biochemically distinct from the other *Shigella* species. The inability to ferment mannitol and a negative catalase reaction distinguish *S. dysenteriae*. *S. dysenteriae* types 1, 3, 4, 6, 9, 11, and 12 and *S. boydii* type 12 are catalase-negative, whereas other species of *Shigella* as well as EIEC and STEC are catalase-positive.[336] The differential characteristics for the four species of *Shigella* are included in Table 6-10. Isolates recovered from stool specimens from patients with diarrheal disease that are suspected of being *Shigella* species should be biochemically categorized and the species confirmed by serologic testing. In the near future, it may be possible to detect *Shigella* species and enteroinvasive strains of *E. coli* using DNA probes selected to detect the virulence plasmids responsible for coding the gene products that initiate intracellular penetration and bowel wall invasion.[55]

BOX 6-9

Subgroups, Serotypes, and Subtypes of *Shigella*

Subgroup	Serotypes and Subtypes

Group A: *Shigella*	15 serotypes (type 1 pro- *dysenteriae* duces Stx)
Group B: *Shigella flexneri*	8 serotypes and 9 subtypes
Group C: *Shigella boydii*	19 serotypes
Group D: *Shigella sonnei*	1 serotype

Pathogenicity. All species of *Shigella* cause acute bloody diarrhea by invading and causing patchy destruction of the colonic epithelium. This leads to the formation of microulcers and inflammatory exudates, and causes inflammatory cells (polymorphonuclear leukocytes, PMNs) and blood to appear in the stool. The diarrheal stool contains 10^6 to 10^8 shigellae per gram. Once excreted, the organism is very sensitive to environmental conditions and dies rapidly, especially when dried or exposed to direct sunlight.[678] For this reason, stool specimens submitted for culture should be processed rapidly (within 2 hours) or placed in a stool preservative such as Cary–Blair Transport Medium.

Incidence and Sources of* Shigella *Infections. Shigellosis is the most communicable of the bacterial diarrheas, and worldwide are responsible for greater than 80 million cases of bloody diarrhea and 700,000 deaths each year.[678] Humans serve as the natural host, and disease is transmitted by the fecal–oral route, with as few as 200 viable organisms being able to cause disease (see Clinical Correlation Box 6-2). Between 20,000 and 30,000 cases of shigellosis are reported annually in the United States.[125] *S. sonnei* is the serotype most commonly associated with diarrheal disease in the United States, accounting for 77% of the *Shigella* serogroups reported to the CDC in 2001.[100] Symptoms associated with *S. sonnei* infection tend to be mild, and some patients may be asymptomatic. *S. dysenteriae* is the least commonly recovered species in

TABLE 6-10 Differentiation of Species Within the Genus *Shigella*

Biochemical Test	*S. dysenteriae*	*S. flexneri*	*S. boydii*	*S. sonnei*
Serogroup	A	B	C	D
ONPG	–	–	–	+
Ornithine decarboxylase	–	–	–	+
Fermentation of:				
Lactose	–	–	–	–
Mannitol	–	+	+	+
Raffinose	–	d	–	–
Sucrose	–	–	–	–
Xylose	–	–	d	–
Indole production	d	d	d	–

+, 90% or more strains positive; –, 90% or more strains negative; d, different strains positive/negative.

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CLINICAL CORRELATION BOX 6-2 *Shigella* Infection (Bacillary Dysentery)

The term "dysentery" was used by Hippocrates to indicate a condition characterized by frequent passage of stool containing blood and mucus accompanied by straining and painful defecation.[193] Humans are the only natural host for *Shigella*, and infection occurs by ingestion. Bacillary dysentery is the most communicable of the bacterial diarrheas, with an infective dose as low as 10 to 100 bacteria producing disease in healthy adults.[194] Fever, watery diarrhea with cramping abdominal pain, and generalized myalgias are the most common early symptoms suggesting shigellosis.[91] Fluid and electrolyte losses may also be noted early in the illness, owing to the action of enterotoxin on the intestinal epithelial cells. After 2 or 3 days, bowel movements become less frequent, and the quantity of stool decreases, but the presence of bright red blood and mucus in the feces and the onset of tenesmus (a distressing urge to defecate) indicate the dysenteric phase of illness, suggesting that bacterial penetration of the bowel has probably occurred. *Shigella* infections should be suspected in community-wide outbreaks of diarrheal illness that disproportionately affect young children. Outbreaks can occur at any time of the year, but are most common in summer.

the United States, but it is the most virulent serotype and the most common serotype isolated in developing countries. The belief that *Shigella* species remain confined to

the bowel and neither invade the bowel lymphatics nor extend to other organs may no longer pertain. Drow and associates[188] reported the recovery of *S. flexneri* from a splenic abscess in a patient with diabetes, indicating that extraintestinal sites of infection may be encountered. Specimens other than stool from which *Shigella* spp. have been recovered include liver, mesenteric lymph nodes, CSF, synovial fluid, vaginal lesions, lungs, conjunctival sacs, corneal scrapings, blood, cutaneous lesions of the penile shaft, and urine.[192,502] Urinary tract infections due to *Shigella* spp. in adults are extremely rare. *Shigella* vaginitis with or without accompanying UTI has been reported in prepubertal girls.[33] Some patients experience a bloody vaginal discharge that may be confused with gonorrhea.[600] Most cases of *Shigella* vulvovaginitis are caused by *S. flexneri*.[451]

Treatment. The World Health Organization has recommended ciprofloxacin as a first-line antimicrobial drug for shigellosis treatment, and third-generation cephalosporins are considered as alternative drug treatmens.[678] Unfortunately, resistance of *Shigella* to ampicillin, trimethoprimsulfamethoxazole, and nalidixic acid has become widespread, and these are no longer recommended. Strains of *S. flexneri* that are resistant to ciprofloxacin and cefotaxime have been reported, thus raising serious questions about the effective treatment of shigellosis in the future.[545]

Tribe *Edwardsielleae*. The Edwardsielleae were initially called the Asakusa group by Sakazaki and Murata in 1963,[573] and the Bartholomew group by King and Adler in 1964.[353] Ewing and associates suggested the name *Edwardsielleae* in 1965,[213] in honor of the prominent American microbiologist, P.R. Edwards. The *Edwardsielleae* consists of one genus, *Edwardsiella*.

Genus *Edwardsiella*. The genus *Edwardsiella* has three species, but only one species, *E. tarda*, is of medical importance. The chief reservoirs in nature are reptiles (especially snakes, toads, and turtles) and freshwater fish. The key characteristics that suggest *E. tarda* are listed in Table 6-6.

A key feature of *E. tarda* is the production of abundant amounts of hydrogen sulfide. Except for this feature, the biochemical properties of the bacterium are similar to those of *E. coli*. The organism also resembles some *Citrobacter* and *Salmonella* species by its production of hydrogen sulfide in TSI agar and its failure to use lactose. This failure to ferment lactose and many other carbohydrates is the basis for the species name *tarda.* A species similar to *E. tarda,* which is hydrogen sulfide-negative, but mannitol-, sucrose-, and arabinose-positive, has been designated "*E. tarda* biogroup 1."[224] This biotype is rarely encountered in laboratory practice and does not yet appear to have clinical significance.

E. tarda has been cited as the cause of a variety of extraintestinal infections.[147,598] The most common are wound infections resulting from trauma, often related to

aquatic accidents. Also common are abscesses that may lead to bacteremia or myonecrosis.[598] Seven patients in one study had typhoidal illnesses, an important differential consideration because *E. tarda* can simulate *S. typhi* in culture.[147] Most reports of enteric illness describe a mild gastroenteritis that improves without therapy in 2 to 3 days. However, Vandepitte and associates[642] reported one case of protracted diarrhea in a 2-month-old infant in whom *E. tarda* (of the same biogroup isolated from tropical aquarium fish in the home of the patient) was the only potential pathogen recovered. Marsh and Gorbach[415] reported the isolation of *E. tarda* from the stool of a patient with bloody diarrhea, and sigmoidoscopic findings of multiple colonic ulcers and mucosal thickening consistent with Crohn's disease. The patient became asymptomatic after 2 days of antibiotic therapy. Iron availability has been thought to regulate the seriousness of *E. tarda* infection.[318,319] Iron overload, caused by such conditions as red cell sickling, leukemia, and cirrhosis, is associated with *E. tarda* septicemia.[318, 672,679] Infection with *E. tarda* related to vascular prostheses has also been reported.[152,462] Clusters of asymptomatic *E. tarda* infections are believed to occur in humans, and at least one such cluster has been reported among seven children and a teacher in a Florida day-care center.[179]

Two other species in the genus *Edwardsiella* have been described. Grimont and associates[269] described *E. hoshinae*, initially recovered from birds, reptiles, and water. This species has also been recovered from human feces; however, it is not known to cause diarrhea. Hawke and coworkers[281] described *E. ictaluri*, an organism that has been recovered only from fish and, at present, has no clinical significance. The biochemical characterization of *Edwardsiella* species is shown in Table 6-11.

Tribe *Salmonelleae*. The *Salmonelleae* contain a single genus, *Salmonella*, and are named after the American microbiologist, D.E. Salmon.

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TABLE 6-11 Differentiation of Species Within the Genus *Edwardsiella*

Biochemical Test	*E. tarda*	*E. tarda* biogroup 1	*E. hoshinae*	*E. ictaluri*
Indole	+	+	V (50)	–
Hydrogen sulfide	+	–	–	–
Motility	+	+	+	–
Fermentation of:				
Mannitol	–	+	+	–
Sucrose	–	+	+	–
Arabinose	–	+	V (13)	–
Trehalose	–	–	+	–

+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive.

Genus *Salmonella*. Salmonellae have somatic (O) antigens that are lipopolysaccharides, and flagellar (H) antigens that are proteins. *S. typhi* also has a capsular or virulence (Vi) antigen. Biochemically, they are usually both lactoseand sucrose-negative. The key characteristics by which the genus *Salmonella* can be suspected are presented in Table 6-6.

***Classification of* Salmonellae.** From the time of the first isolation of microorganisms of the *Salmonella* group, reported in 1884 by Gaffky (*Bacterium typhosum*) and in 1886 by Salmon and Smith (*Salmonella choleraesuis*), the development of *Salmonella* nomenclature has been very complex and a matter of dispute (Box 6-10). The salmonellae are the

BOX 6-10

Salmonella Taxonomy and Nomenclature

The antigenic formulae of *Salmonella* serotypes are defined and maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France, and new serotypes are listed in annual updates of the Kauffmann–White scheme.[534,535] In this scheme, the salmonellae are grouped (A, B, C, etc.) on the basis of somatic O antigens and are subdivided into serotypes (1, 2, 3, etc.) by their flagellar H antigens, resulting in serogroups designated A, B, C_1, C_2, C_3, etc. Each unique serotype is given a name. The first salmonellae were given names that indicated the disease and/or the animal from which the organism was isolated (e.g., *S.* ser. Typhi, *S.* ser. Choleraesuis, etc.). New serotypes are now given the name of the geographic location where they were first isolated (*Salmonella* ser. Canada, *Salmonella* ser. Cleveland, etc.). Initially, each serotype was considered a separate species (for example, *S. canada*, *S. cleveland*); however, today this would result in over 2400 species of Salmonella. DNA relatedness studies have demonstrated that all *Salmonella* strains and all serotypes of

"*Arizona*" form a single DNA hybridization group with seven subgroups, with the exception of *S. bongori* (subgroup V) that has been shown by DNA–DNA hybridization to form a distinct species.[156,381,382,551] Since *S. choleraesuis* was already recognized as the type species of *Salmonella*, it had priority as the species designation for the six subgroups considered to comprise a single hybridization group. The name "choleraesuis," however, can lead to confusion because the name refers to both a species and a serotype. So, in 1986, the Subcommittee on *Enterobacteriaceae* of the International Committee on Systematic Bacteriology at the XIV International Congress on Microbiology unanimously recommended that the type species for *Salmonella* be changed to *S. enterica*, a name that had not been used previously as a serovar.[514] In 1987, Le Minor and Popoff[380] made a proposal as a "Request for an Opinion" to the Judicial Commission of the International Committee of Systematic Bacteriology that the genus *Salmonella* consist of only one species "*Salmonella enterica*" to include seven subspecies. *S. typhi*, *S. typhimurium,* and *S. enteritidis* were included in *S. enterica*. This recommendation was adopted by the CDC and many other laboratories. However, after a long delay, the Judicial Commission denied the request based on the fact that the status of *Salmonella* ser. Typhi was not adequately addressed in the Le Minor and Popoff proposal.[661,662] They were concerned that if *S. enterica* were adopted, then *Salmonella* ser. Typhi would be referred to as *S. enterica* subsp. *enterica* ser. Typhi and might be missed or overlooked by physicians.[74] The Judicial Committee therefore ruled that *S. choleraesuis* be retained as the legitimate type species pending an amended request for an opinion.[661,662] In 1987, Le Minor and Popoff[380] also proposed that the seven subgenera of *Salmonella* be referred to as subspecies (subspecies I, II, IIIa, IIIb, IV, V, and VI). Most recently, Euzéby[211] proposed that the genus *Salmonella* consists of three species named *S. bongori*, *S. enterica*, and *S. typhi*. He further proposed that *S. enterica* be divided into six subspecies: *S. enterica* subsp. *enterica, S. enterica* subsp. *arizonae,* S. *enterica* subsp. *diarizonae, S. enterica* subsp. *houtenae, S. enterica* subsp. *indica*, and *S. enterica* subsp. *salamae*. As of this writing, the Judicial Committee has not acted on this latest proposal.

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most complex of all the *Enterobacteriaceae*, with more than 2,400 serotypes described in the current Kauffmann–White scheme.[534] Prior to July 1, 1983, three species of *Salmonella* were used to report positive results: *S. choleraesuis, S. typhi*, and *S. enteritidis*, with most of the serotypes belonging to the last species, *S. enteritidis*. Presently, all former species and subgroups of *Salmonella* and *Arizona* are considered to be the same species, but can be separated into seven taxa representing

six distinct subgroups. The only exception is *Salmonella bongori*, previously known as subgenera V, which by DNA–DNA hybridization is a distinct species.[551] Thus, there are two species and six subspecies of *Salmonella enterica* in the current system used by the CDC (Box 6-11). The differential characteristics of *Salmonella* species and subspecies are given in Table 6-12.

BOX 6-11

Classification of *Salmonella* Species and Subspecies

There are two species of *Salmonella*: *S. enterica,* which encompasses six subspecies, and *S. bongori*

S. enterica subsp. *enterica* (I): includes most serotypes
S. enterica subsp. *salamae* (II)
S. enterica subsp. *arizonae* (IIIa)
S. enterica subsp. *diarizonae* (IIIb)
S. enterica subsp. *houtenae* (IV)
S. enterica subsp. *indica* (VI)
Salmonella bongori (formerly subspecies V)

Salmonellae *Nomenclature.* Beginning in 1966, the World Health Organization (WHO) began naming serotypes only in subspecies I and dropped all existing serotype names in the other subspecies. The CDC follows this practice and uses names for serotypes in subspecies I and uses antigenic formulas for unnamed serotypes described after 1966 in subspecies II, IV, and VI, and *S. bongori*. For named serotypes, to emphasize that they are not separate species, the serotype name is not italicized, and the first letter is capitalized. At the first citation of a serotype, the genus name is given followed by the word "serotype" or the abbreviation "ser." and then the serotype name (*Salmonella* serotype or ser. Typhimurium). Subsequently, the name may be written with the genus followed directly by the serotype name *Salmonella* Typhimurium or *S.* Typhimurium (Box 6-12).[74]

In day-to-day practice, unknown isolates from clinical specimens that are biochemically suggestive of *Salmonella* species are confirmed using polyclonal antisera containing antibodies to all the major subgroups. Subcultures of confirmed isolates are forwarded to public health laboratories, where serotype designations (e.g., *S.* serotype Typhimurium) are made on the basis of serologic reactions to O and H determinants.

***Identification of* Salmonella typhi.** Although most *Salmonella* serotypes cannot be

distinguished by biochemical reactions, one serotype, namely *S. typhi*, does possess some unique biochemical characteristics that will allow it to be differentiated from other serotypes. First and foremost is the observation that strains of *S. typhi* produce only a trace amount of hydrogen sulfide, which is usually observed as a crescent-shaped wedge of black precipitate forming at the interface of the slant and butt in KIA or TSI media (Image Plate 6-4B). In addition, *S. typhi* strains are noted to be less active biochemically than

TABLE 6-12 Differential Characteristics of *Salmonella* Species and Subspecies

Species	*S. enterica*						
Subspecies	I *enterica*	II *salamae*	IIIa *arizonae*	IIIb *diarizonae*	IV *houtenae*	VI *indica*	*S. bongori*
Biochemical Test							
Dulcitol	+	+	–	–	–	d	+
ONPG (2 hours)	–	–	+	+	–	d	+
Malonate	–	+	+	+	–	–	–
Gelatinase	–	+	+	+	+	+	–
Sorbitol	+	+	+	+	–/+	–	+
KCN	–	–	–	–	+	–	+
D-tartrate	+	–	–	–	–	–	–
Galacturonate	–	+	–	+	+	+	+
β-glucuronidase (MUG)	d	d	–	+	–	d	–
Mucate	+	+	+	– (70%)	–	+	+
Salicin	–	–	–	–	+	–	–
Lactose	–	–	– (75%)	+ (75%)	–	d	–

+, 90% or more strains are positive; –, 90% or more strains are negative; d, different reactions given by different serovars.
Modified from reference 212.

the more common serotypes and specifically are negative in the following reactions: Simmon's citrate; ornithine decarboxylase; gas from glucose; fermentation of dulcitol, arabinose, and rhamnose; and mucate and acetate utilization. Consequently, the authors believe that it is within the capabilities of most clinical laboratories to make a preliminary report of *S. typhi* or *Salmonella* species, not *S. typhi,* while the laboratory awaits specific serotype confirmation from their local public health laboratory.

BOX 6-12

Salmonella Nomenclature in Use at CDC[74]

Taxonomic Current	Nomenclature Position
Genus (italics)	*Salmonella*
Species (italics)	*enterica*, which includes subspecies I, II, IIIa, IIIb, IV, and VI *bongori* (formerly subspecies V)
Serotype (capitalized, not italicized)	—The first time a serotype is mentioned in the text, the name should be preceded by the word "serotype" or "ser."
	—Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II, III, IV, VI, and *S. bongori* (e.g., *Salmonella* serotype (ser.) Typhimurium, *Salmonella* II 50:b:z_6, *Salmonella* IIIb 60:k:z)
	—Members of subspecies II, IV, VI, and *S. bongori* retain their names if named before 1966 (e.g., *Salmonella* ser. Marina [IV 48: g, z_{51}])

Incidence and Sources of Salmonelloses. Salmonellosis is a major cause of bacterial enteric illness in both humans and animals (see Clinical Correlation Box 6-3). Each year, an estimated 1.2 million cases of salmonellosis occur among humans in the United States, resulting in 23,000 hospitalizations, and nearly 450 deaths.[128] Approximately 35,000 of these cases are serotyped by public health laboratories, and the results are electronically transmitted to the CDC. Human infections with salmonellae are most commonly caused by ingestion of food, water, or milk contaminated by human or animal excreta. Salmonellae are primary pathogens of lower animals (e.g., poultry, cows, pigs, pets, birds, sheep, seals, donkeys, lizards, and snakes), which are the principal source of nontyphoidal salmonellosis in humans. Interestingly, humans are the only known reservoir for *S. typhi*. Although the incidence of typhoid fever has declined in developed countries, sporadic outbreaks continue to occur. About 400 cases are reported annually in the United States.[99] About half the salmonellosis epidemics are the result of contaminated poultry and poultry products. Salmonellae in the feces of hens contaminate the surface of eggs or penetrate internally through hairline cracks. In hens with ovarian infection, the organisms may gain access to the yolk. The Egg Products Inspection Act of 1970 requires pasteurization of all bulk egg products and federally supervised inspection of shell eggs for cracks.

CLINICAL CORRELATION BOX 6-3 *Salmonella* Infection

Five clinical types of *Salmonella* infection may be distinguished[596]: (1) gastroenteritis, the most frequent manifestation, ranging from mild to fulminant diarrhea, accompanied by low-grade fever and varying degrees of nausea and vomiting; (2) bacteremia or septicemia without major gastrointestinal symptoms (*S. choleraesuis* is particularly invasive) characterized by high, spiking fever and positive blood cultures; (3) enteric fever, potentially caused by any strain of *Salmonella* species, usually manifested as mild fever and diarrhea, except for classical cases of typhoid fever (*S.* Typhi), in which the disease progresses through a bimodal course, characterized by an early period (lasting 1 to 2 weeks) of fever and constipation, during which blood cultures are positive and stool cultures remain negative, followed by a second (diarrheic) phase during which blood cultures become negative and stool cultures positive; (4) a carrier state in which persons with previous infection, especially with *S.* Typhi, may continue to excrete the organism in their feces for up to 1 year following remission of symptoms; and (5) focal infections, e.g., osteomyelitis, meningitis, brain abscess, endocarditis. Of some concern are reports of lactose-positive strains of *S. virchow* causing bacteremia and meningitis.[569] Although detection of lactose-positive strains in the blood or cerebrospinal fluid would not be difficult, finding such strains in stool would pose a problem for most laboratories owing to the similarity in appearance to other lactosepositive coliforms present in stool specimens.

Historically, *S.* Typhimurium has been the most frequently reported serotype, accounting for slightly more than 20% of isolates reported to the CDC annually. The three most common serotypes of *Salmonella* in 2001 were Typhimurium (22%), Enteritidis (18%), and Newport (10%), which accounted for 50% of all isolates. The largest single source outbreak of salmonellosis in U.S. history (16,000 culture-confirmed cases with epidemiologic data indicating that 150,000 to 200,000 persons were actually infected) occurred in 1985 in Illinois and surrounding states and was traced to a faulty valve in a major commercial milk supply firm.[104] Several *S.* Enteritidis outbreaks

have occurred in the United States since 1990 that were associated with shell eggs.[105,107,109,117] An estimated 0.01% of all shell eggs contain *S.* Enteritidis.

Consequently, foods containing raw or undercooked eggs (e.g., homemade eggnog or ice cream, hollandaise sauce, Caesar salad dressing, homemade mayonnaise, and runny omelets) pose a slight risk of infection with *S.* Enteritidis.[107,109] In 1994, an outbreak of *S.* Enteritidis infection was linked to a nationally distributed ice cream brand. Illnesses were documented in 41 states, and more than 200,000 persons were estimated to have been ill.[110] The incidence of *S.* Enteritidis illness and the number of such outbreaks in the United States have decreased by almost 50% between the mid-1990s and 1999.[510] To meet the challenge of further reducing the incidence of such infections, the President's Council on Food Safety announced an Egg Safety Action Plan on December 10, 1999, with the interim goal of reducing egg-associated *S.* Enteritidis illnesses by half by 2005 and eliminating them by 2010.[540] These outbreaks serve as a constant reminder that modern technology cannot always protect against the ravages of infectious diseases that may occur in explosive and widespread epidemics.

Salmonellosis has also been associated with direct or indirect contact with reptiles (i.e., lizards, snakes, turtles). Reptiles are commonly colonized with *Salmonella* and shed the organism intermittently in their feces.[116] During the early 1970s, small pet turtles were an important source of *Salmonella* infection in the United States. In 1975, the Food and Drug Administration (FDA) prohibited the distribution and sale of small turtles, resulting in the prevention of 100,000 cases of salmonellosis annually.[150] However, since 1986, the popularity of iguanas and other reptiles that can transmit infection to humans has given rise to an increased incidence of *Salmonella* infections caused by reptileassociated serotypes.[111,116,124] Increasing evidence now suggests that amphibians (e.g., frogs, toads, newts, and salamanders) can also pose risks for salmonellosis in humans.[430] Overall, reptile and amphibian contacts are estimated to account for 74,000 (6%) of the *Salmonella* infections that occur each year in the United States.[430] Because young children are at increased risk for reptileand amphibian-associated salmonellosis with potentially severe complications (e.g., septicemia and meningitis), reducing exposure of infants or children younger than 5 years of age to reptiles is particularly important. For this reason, reptiles and amphibians should be kept out of households that include children <5 years old or immunocompromised persons, and should not be allowed in child-care centers.[124] For a review of recent *Salmonella* outbreaks, go to: http://www.cdc.gov/salmonella/outbreaks.html.

***Treatment and Emergence of Multidrug-Resistant* Salmonellae.** *Salmonella* gastrointestinal infections usually resolve in 5 to 7 days, and most do not require treatment other than oral fluids. Persons with severe diarrhea may require rehydration with intravenous fluids. Antibiotic therapy can prolong the duration of excretion of nontyphoidal *Salmonella* and is recommended only for patients with severe illness (e.g., those with severe diarrhea, high fever, bloodstream infection, or who need

hospitalization) or those at risk of severe disease or complications, including young infants, older adults (over 65 years old), and immunocompromised persons. Choices for antibiotic therapy for severe infections include fluoroquinolones, third-generation cephalosporins, and ampicillin (for susceptible infections).[129]

A multidrug-resistant (MDR) strain of *S.* Typhimurium definitive type 104 (DT104) that is resistant to five antibiotics (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) emerged across the United States during the 1990s.[257] In a national survey conducted by the CDC in 2000, 50% of the *S.* Typhimurium isolates were resistant to one or more drugs, and 28% had a five-drug resistance pattern characteristic of the phage type DT104.[99] Similarly, since 1998, *S.* Newport has emerged as a major MDR pathogen.[273] In 2013, CDC reported that about 3% of nontyphoidal *Salmonella* are resistant to ceftriaxone, with another 3% showing some level of resistance to ciprofloxacin. Five percent of nontyphoidal *Salmonella* tested by CDC are resistant to five or more types of drugs.[129]

***Infections due to* Salmonella enterica *Subspecies* arizonae.** *S. enterica* subsp. *arizonae* resembles salmonellae antigenically, clinically, and epidemiologically. It was first recovered in 1939 from diseased reptiles in Arizona, and was initially called "*Salmonella* sp. Dar-es-salaam type, variety from Arizona."[83] It was later distinguished from *Salmonella* and placed in the new genus *Arizona*, with a single species *A. hinshawii*. However, in 1983, it was again reclassified as a serotype of the genus *Salmonella* based on DNA homology studies. Although most *Salmonella* serotypes cannot be distinguished by biochemical reactions, *S. enterica* subsp. *arizonae* can be easily differentiated on the basis of having positive malonate and negative dulcitol reactions. In addition, some strains ferment lactose, and all strains are ONPG-positive. As a result of these unique biochemical reactions, a correct serotype designation can be easily made by most of the commercially available identification systems. For clinical significance, see Clinical Correlation Box 6-4.

***New Methods for Recovery and Characterization of* Salmonellae.** In addition to the classic media discussed previously (see Tables 6-1 through 6-3), new media have been described that are intended to improve the isolation of *Salmonella* species from stool samples.

Selective and Differential Media. These include novobiocin–brilliant green–glucose agar (NBG),[181] novobiocin–brilliant green–glycerol–lactose agar (NBGL),[533,568] xylose–lysine–Tergitol 4 (XLT4) medium,[440] and modified semisolid Rappaport Vassiliadis medium (MSRV).[26,180,259] The formulations and differential properties of these media are displayed in Table 6-13.

Chromogenic Media. Chromogenic enzyme substrates are compounds that act as the substrate for specific enzymes and change color due to the action of the enzyme on the chromogenic substrate (Fig. 6-6). The first medium of this type was Rambach

agar. It uses a chromogenic substrate for β-galactosidase (X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in conjunction with propylene glycol, which is fermented by *Salmonella* spp. to generate acid.[548] Rambach agar is highly specific; however, it does not detect *S.* Typhi and *S.* Paratyphi A.[195,196,526] SM-ID agar is similar to Rambach except that it incorporates two chromogenic substrates, X-Gal, for β-galactosidase, and X-GLU, for β-glucosidase. SM-ID medium detects both Typhi and Paratyphi A serotypes.[195,196,444,515,526,568] *Salmonella* chromogenic

CLINICAL CORRELATION BOX 6-4 *Salmonella arizonae*

Reptiles, particularly snakes, are the main natural reservoir of *S. arizonae*, but humans, poultry, and other animals have also contracted disease from this organism. Human infection should trigger inquiries of a possible connection with reptiles as well as poultry and egg products. *S. arizonae* infection in cancer patients and HIV-positive patients following consumption of snake powder capsules as a folk remedy have been reported by several investigators.[28,102,216,658] A fatal infection of *S. arizonae* gastroenteritis has been reported in a child with microcephaly born to a family of snake charmers in New Delhi, India.[409]

The clinical spectrum of disease varies from benign gastroenteritis to enteric fever and septicemia with localized infection, and is similar to that caused by other *Salmonella* serotypes. Keren et al. reported that gastroenteritis may be seen in any age group and is characterized by cramping abdominal pain, diarrhea (often watery), nausea, vomiting, and low-grade fever.[344] Diarrhea is usually self-limited and lasts from 1 to 5 days. Septicemia may follow, but has also been reported in the absence of gastroenteritis. Localized infection in diverse organ sites, such as brain, bone, liver, lung, joints, and gallbladder, is presumed, although not always documented, to occur following bacteremia. Instances of osteomyelitis caused by this organism have been well-documented.[344] The prevalence of human infections caused by *S. arizonae* is probably underreported because the gastrointestinal symptoms are usually mild. However, this pathogen should be considered in the differential diagnoses of patients with sepsis and severe gastroenteritis who have a history of contact with reptiles, especially snakes. Ownership of reptiles should be discouraged, especially in households with children under 5 years of age.[409]

TABLE 6-13 New Media for Recovery of *Salmonella* Species From Stool

Medium	Formulation	Principle and Interpretation
Novobiocin–brilliant green–glucose agar (NBG)	Tryptic soy agar, 40 g Ferric ammonium citrate, 1.5 g Sodium thiosulfate pentahydrate, 5 g Phenol red (sodium salt), 80 mg Glucose, 1 g Brilliant green, 7 mg Novobiocin, 10 mg Distilled water to1 L Final pH 7.3	*Salmonella* colonies appear smooth and entire with medium- to large-sized, dark black, nucleated centers owing to H_2S production. In addition, reddening and a visible zone of clearing occur in the medium around each colony. Coliforms are either inhibited or fail to produce black-centered colonies. Some *Citrobacter freundii* strains produce colonies indistinguishable from *Salmonella* species.
Novobiocin–brilliant green–glycerol lactose agar (NBGL)	Trypticase soy agar, 40 g Ferric ammonium citrate, 1.5 g Sodium thiosulfate, 5 g Lactose, 10 g Glycerol, 10 mL Brilliant green, 7 mg Novobiocin, 10 mg Distilled water to 1 L	The detection of *Salmonella* spp. is based on the production of H_2S resulting in black colonies. Sufficient H_2S formation is achieved only by colonies that do not produce acid from glycerol or lactose, because a low pH interferes with H_2S formation. This results in colorless colonies for most *Proteus* and *Citrobacter* species.
Rambach agar	Propylene glycol, 10 g Peptone, 5 g Yeast extract, 2 g Sodium desoxycholate, 1 g Neutral red, 0.03 g 5-Bromo-4-chloro-3-indolyl-β-D-Galactopyranoside, 0.1 g Agar, 15 g Distilled water to 1 L	Detects ability of *Salmonella* spp. to metabolize propylene glycol. Suspect colonies on this medium are usually bright red. Contains moderate amount of bile salts to inhibit coliforms.
SM–ID agar	Beef extract, 3 g Bio-Polytone, 6 g Yeast extract, 2 g Bile salts, 4 g Neutral red, 0.025 g Tris buffer, 0.65 g Brilliant green, 0.3 mg Chromogen substrate 1 (galactopyranoside), 0.17 g Sodium glucuronate, 12 g Chromogen substrate 2 (glucopyranoside), 0.026 g Sorbitol, 8 g Agar, 3.5 g Distilled water to 1 L Final pH 7.6 ± 0.2	Detection of *Salmonella* spp. is based on the formation of acid from the glucuronate and on the absence of β-galactosidase. *Salmonella* serotypes produce pinkish red colonies (sometimes with a colorless rim), whereas coliforms form other colors (green, blue, or violet) if they are positive for β-galactosidase, or remain colorless. Contains moderate amount of bile salts to inhibit coliforms.

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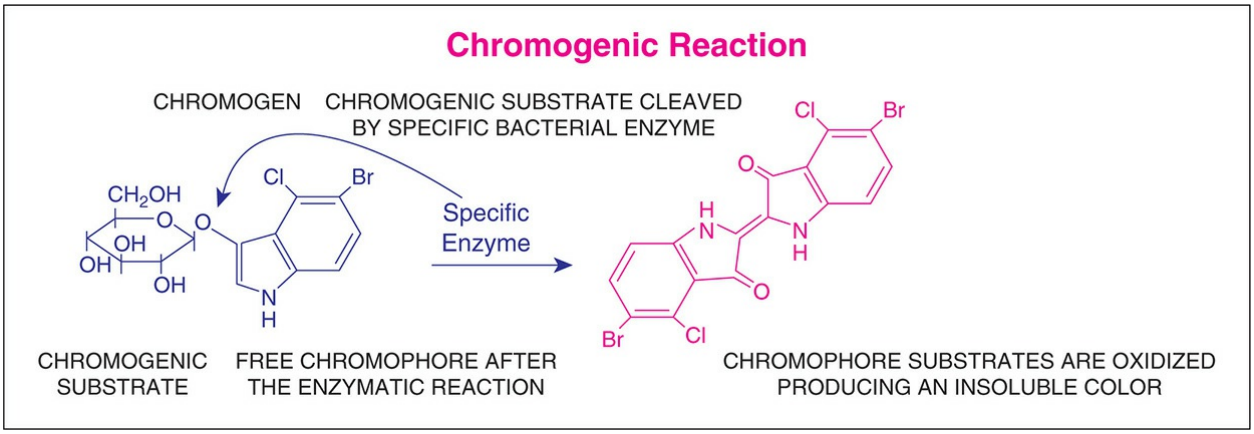
Modified Semisolid Rappaport–Vassiliadis Medium (MSRV)	Tryptose, 4.59 g Casein hydrolysate acid, 4.59 g Sodium chloride, 7.34 g Potassium dihydrogen phosphate, 1.47 g Magnesium chloride (anhydrous), 10.93 g Malachite green oxalate, 0.037 g Agar, 2.7 g Distilled water to1 L Novobiocin (2% solution) added after sterilization, 1 mL Final pH 5.2 ± 0.2	Based on the swarming phenomenon of motile bacteria (*Salmonella* spp. and others) at reduced agar concentrations. After incubation, the plates are checked for motile bacteria that appear as a halo of growth spreading out from the original inoculation point. Subcultures are taken from the edge of migration to check for purity and for further biochemical and serologic tests. Coliforms are inhibited by a combination of increased osmotic pressure, malachite green, and incubation at 41°C–43°C.
Xylose–Lysine–Tergitol 4 (XLT4)	Bacto proteose peptone No. 3, 1.6 g Bacto yeast extract, 3.0 g L-lysine, 5.0 g Bacto xylose, 3.75 g Bacto lactose, 7.5 g Bacto saccharose, 7.5 g Ferric ammonium citrate, 0.8 g Sodium thiosulfate, 6.8 g Sodium chloride, 5 g Bacto agar, 18 g Bacto phenol red, 0.08 g Distilled water to 1 L Final pH 7.4 ± 0.2	This is a highly selective medium that substitutes the anionic surfactant Tergitol 4 for sodium desoxycholate found in XLD agar. The XLT4 agar completely inhibits the growth of all gram-positive bacteria and fungi, and either completely or strongly inhibits the growth of numerous gram-negative bacteria including *Proteus*, *Providence*, and *Pseudomonas*. In addition, *Citrobacter* species are somewhat inhibited and very rarely produce colonies with black centers after overnight incubation. *Salmonella* colonies (H_2S-positive) appear black or black centered with a yellow periphery after 18–24 hours' incubation. After continued incubation, the colonies become entirely black or pink to red with black centers. Rare strains of *Salmonella* that produce no H_2S display pink to pinkish yellow colonies that can be differentiated from bright yellow non–salmonellae colonies.
Modified ABC medium (αβ-chromogenic medium with alafosfalin)	Bacteriologic agar, 10 g L-arginine, 0.1 g L-aspartic acid, 0.1 g L-cysteine, 0.005g Glycine, 0.1g L-histidine, 0.1g L-isoleucine, 0.1g L-leucine, 0.1g L-lysine, 0.1g L-methionine, 0.005g L-phenylalanine, 0.1g L-proline, 0.1g L-serine, 0.1g L-threonine, 0.1g L-tryptophan, 0.1g L-tyrosine, 0.1g L-valine, 0 .1g Guanine, 0.01g Uracil, 0.01g Cytosine, 0.01g Adenine, 0.01g Sodium citrate, 6.5g Magnesium sulfate, 0.1g Ammonium sulfate, 1g Yeast extract, 0.1g Dipotassium hydrogen phosphate, 7g Potassium dihydrogen phosphate, 2g Chromogen Mix: Ferric ammonium citrate, 0.5g X-α-Gal, 0.08g CHE-Gal, 0.3g IPTG, 0.03g Distilled water to 1L Ingredients added after sterilization: Sodium deoxycholate, 0.5g Alafosfalin final concentration range in agar of 32–0.125 mg/L (Perry et al.[518])	*Salmonella* yield blue colonies; other *Enterobacteriaceae* yield black colonies. Alafosfalin is taken up by bacteria at different rates via a stereospecific permease and may be cleaved intracellularly by an aminopeptidase to yield fosfalin. This released compound interferes with bacterial metabolism via interaction with the alanine racemase enzyme responsible for generating D-alanine. This interaction may result in growth inhibition.[518]

BBL CHROMagar Salmonella (sold by BD under a licensing agreement with CHROMagar, Paris, France)	Chromopeptone, 22.0 g Chromogen mix, 0.34 g Inhibitory Agents, 0.02 g Agar, 15.0 g Distilled water to 1 L	*Salmonella* spp. appear as light mauve to mauve-colored colonies. *Citrobacter freundii* and other coliforms appear as light blue-green to blue-green colored colonies. Some organisms that do not hydrolyze any of the chromogenic compounds may appear as colorless colonies (Image Plates 6-5E through H).

Note: Rambach, XLT4, MSRV, NBG, and NBGL are not suitable for use in the isolation of Typhoid *Salmonella* serotypes.



■ **FIGURE 6-6** Chromogenic reaction: artificial substrates (chromogens) release differently colored compounds upon degradation by specific bacterial enzymes.

medium (SCM; Oxoid, Basingstoke, United Kingdom) contains two chromogenic substrates. The first is Magenta-cap (5-bromo-6-chloro-3-indolylcaprylate), which is hydrolyzed by *Salmonella* species to yield magenta colonies. The second substrate is X-Gal, which causes β-D-galactosidaseproducing organisms to turn blue. Other colonies that do not utilize the chromogens grow as colorless colonies.[97] ABC medium (α, β-chromogenic medium) uses two chromogenic substrates. The first substrate, 3,4-cyclohexenoesculetin-β-D-galactoside (CHE-GAL), is used to detect β-galactosidaseproducing organisms that appear as black colonies in the presence of iron. The second substrate, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal), is hydrolyzed by *Salmonella* spp., which appear as green colonies.[472,515,517] Perry and colleagues subsequently modified ABC medium with the incorporation of alafosfalin (L-alanyl-1-aminoethylphosphonic acid), a "suicide substrate," that aids in the recovery of *Salmonella* spp. by inhibiting the growth of a wide range of gram-negative bacteria.[518] COMPASS Salmonella agar (Biokar Diagnostics, Beauvais, France) utilizes two chromogenic substrates—5-bromo-6-chloro-3-indolylcaprylate, which detects esterase activity from *Salmonella* spp. yielding magenta colonies, and 5-bromo-4-chloro-3-indolyl-glucopyranoside, which detects β-glucosidase activity from other *Enterobacteriaceae* yielding blue colonies.[515] CHROMagar Salmonella (CHROMagar Microbiology, Paris, France; also sold by BD under the name BBL

CHROMagar Salmonella, BD Diagnostics Sparks, MD) is a newer selective chromogenic medium that uses a chromogen mixture (patented substrates) that detects esterase activity of *Salmonella* spp. and β-galactosidase activity of other *Enterobacteriaceae*.[203,244,407,515] The formulas, principles, and interpretations of the chromogenic agars are given in Table 6-13. Pictures of colonies growing on CHROMagar can be found in Image Plates 6-5E through H)

Evaluations. In a study by Ruiz and associates[568] comparing five plating media for isolation of *Salmonella*, NBGL media had the highest sensitivity (78.4%) and positive predictive value (61%) for direct recovery of *Salmonella* from stool. These authors recommend the use of SM-ID for recovery of *S.* Typhi, which is not detected on NBGL agar. Monnery and colleagues found SM-ID and Rambach agars to be considerably more specific than SS agar and Hektoen agar.[444] Dusch and Altwegg compared six media (HE agar, Rambach agar, SM-ID medium, XLT4 agar, NBGL agar, and MSRV medium) and concluded that MSRV was the most sensitive medium tested for the isolation of nontyphoid salmonellae from stool; however, these authors noted that the semisolid nature of the medium was a disadvantage and requires careful handling in the laboratory. They noted that XLT4 had sensitivity comparable with HE and nearly 100% specificity and can be considered an alternative for the isolation of salmonellae from stools.[196] Perez and colleagues[515] compared four chromogenic media (ABC medium, COMPASS Salmonella agar, CHROMagar Salmonella agar, and SM-ID agar) with conventional Hektoen agar on 916 stool samples from

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inpatients at three hospitals. After 48 hours of incubation, the sensitivity before and after enrichment in selenite broth was 62.5% and 89.1% with ABC medium, 77.1% and 93.8% with COMPASS agar, 66.7% and 89.1% with CHROMagar, 68.8% and 85.9% with SM-ID agar, and 85.4% and 98.4% with Hektoen agar, respectively. Without enrichment, after 24 hours of incubation, between 45.8% and 62.5% of all the *Salmonella* isolates were detected, according to the medium used. Hektoen was significantly more sensitive than the four chromogenic media, whereas the sensitivities of the four chromogenic media were not significantly different from each other. With enrichment and a 48-hour incubation period, Hektoen agar again had the highest sensitivity (98.4%), whereas the sensitivities of COMPASS, ABC, CHROMagar, and SM-ID were 93.8%, 89.1%, 89.1%, and 85.9%, respectively.[515]

Other Rapid Detection Methods. The 4-methylumbelliferyl caprylate test (MUCAP test; Biolife, Milan, Italy) is a fluorescence test for rapid identification of *Salmonella* strains directly from agar plates. The test consists of an eight-carbon-atom ester conjugated with methylumbelliferone (MEU). This substrate interacts with the *Salmonella* C8 esterase, leading to release of umbelliferone, which is strongly

fluorescent at 365 nm. The test is performed by applying a drop of the reagent directly onto suspect colonies on the agar surface and then observing for the appearance of blue fluorescence of the colony under a Wood's lamp within 5 minutes. Several studies have shown this test to provide nearly 100% sensitivity and specificity in detecting *Salmonella* strains and to offer a useful and rapid adjunct to routine biochemical characterization of *Salmonella* strains.[12,493,570,571] The Oxoid Biochemical Identification System (O.B.I.S.) Salmonella Test (Oxoid) is a rapid colorimetric spot test for the determination of pyroglutamyl aminopeptidase (PYRase) and nitrophenylalanine deaminase (NPA) activity. A sample is taken from a colony growing on an agar plate and applied to the PYR and NPA test areas on the card. A drop of buffer solution is added to both test areas, and after 5 minutes, one drop of PYR developing solution is added to the PYR test area, and one drop of NPA developing solution is added to the NPA test area. The PYRase area on the Test Card is impregnated with L-pyroglutamic acid 7-amino-4-methylcourmarin (7AMC). The enzymatic hydrolysis of the substrate produces a purple color on addition of the PYR developing solution (dimethylaminocinnamaldehyde). The NPA area on the Test Card is impregnated with nitrophenylalanine. Deamination of the reagent is shown by an orange–brown color when the NPA developing solution (0.25M sodium hydroxide) is added. The lack of PYRase and NPA activity in *Salmonella* spp. can be used to differentiate them from *Citrobacter* spp., which possess PYRase activity,[131,307,448] and *Proteus*, *Morganella,* and *Providencia* spp., which have NPA activity.[252]

Molecular Detection Methods. Molecular methods performed directly on stool specimens collected in stool transport media can increase sensitivity and specificity for detection of *Salmonella* and other enteric pathogens compared to stool culture.[271,497,527] Several real-time polymerase chain reaction (PCR) assays have been evaluated and reported to offer reliable detection of enteric pathogens at concentrations 1-to 2-$\log_{10}$ lower than those needed for culture detection.[19,80,159,391,392]

Other new techniques are being introduced that may significantly alter the future identification of *Salmonella* species, both in the clinical laboratory and in epidemiologic field studies. As an example of new applications, Olsvik and associates[494] traced the transmission of *S.* Typhimurium strains from diseased cattle in four separate herds in Norway to human farm workers, by demonstrating identical cryptic plasmid profiles for the various isolates by using restriction endonuclease digestion techniques. As these authors point out, conventional serotyping and biotyping techniques are often not sufficiently specific to determine definitively that two or more isolates from different sources are, in fact, identical. Matrix-assisted laser desorption/ionization time-offlight (MALDI-TOF)–based intact cell mass spectrometry (ICMS) has been used to identify salmonellae at the species and subspecies level[183]

and for rapid discrimination of *S.* Typhi from other *Salmonella* serovars when performed on isolates recovered from blood cultures.[369] Techniques such as MALDI-TOF, restriction endonuclease analysis, or genetic probes will make epidemiologic and diagnostic work in microbiology much more exacting in the future. These techniques are discussed in more detail in Chapter 4.

Tribe *Citrobactereae.* The *Citrobactereae* contains a single genus—*Citrobacter*—and 11 species.

Genus *Citrobacter.* The genus *Citrobacter* and the species *C. freundii* were designated in 1932 by Werkman and Gillen. In 1970, Frederiksen described a new species that he named *C. koseri*. In 1971, Young and coworkers proposed the name *Levinea malonatica* for a similar group of organisms, and in 1972, Ewing and Davis described *Citrobacter diversus*. Frederiksen examined all three strains and determined that they were phenotypically alike, and proposed that the name *C. koseri* be restored as the valid name for this taxon.[236] In 1993, Brenner and colleagues, using DNA relatedness studies, showed that organisms identified as *C. freundii* consisted of a heterogeneous group representing several genetic species.[67] This work led to the establishment of 11 genomospecies within the genus *Citrobacter*, as shown in Table 6-14.

The characteristics that suggest that an isolate may belong to the genus *Citrobacter* are listed in Table 6-6. The key characteristics that differentiate *C. freundii* and other H_2S-positive Citrobacters from salmonellae are growth in KCN (*Salmonella* species are negative), absence of lysine decarboxylase activity (*Salmonella* species are positive), and the hydrolysis of ONPG (*Salmonella* species are negative). The biochemical differentiation among the *Citrobacter* species is shown in Table 6-15. Human isolates of all genomospecies except *C. koseri* have been obtained predominantly from stools.[67] Farmer and coworkers,[220] who reviewed strains referred to the CDC, cited *C. freundii* as a possible cause of diarrhea (although most fecal isolates do not appear to be associated with disease) and as a cause of isolated cases of extraintestinal infections. They also cite a possible association between *C. koseri* and outbreaks of meningitis and brain abscesses in neonates, and report the recovery of *C. amalonaticus* from a few blood cultures. Janda and colleagues at the Microbial Diseases Laboratory in Berkeley, California, reported that *C. freundii* was the most common species identified from all body sites except feces. In gastrointestinal specimens, *C. freundii* ranked fourth behind *C. youngae, C. braakii*, and *C. werkmanii*.[324]

TABLE 6-14 Former and Current Species Within the Genus *Citrobacter*

Former Species Designation	Genomospecies	Current Species
C. freundii complex	1	*C. freundii*
	5	*C. youngae*
	6	*C. braakii*
	7	*C. werkmanii*
	8	*C. sedlakii*
	9	*C. rodentium*
	10	*C. gillenii*
	11	*C. murliniae*
C. diversus	2	*C. koseri*
C. amalonaticus	3	*C. amalonaticus*
C. amalonaticus biogroup 1	4	*C. farmeri*

TABLE 6-15 Differentiation of Species Within the Genus *Citrobacter*[a]

	Citrobacter										
Biochemical Test	***koseri***	***werkmanii***	***sedlakii***	***rodentium***	***gillenii***	***amalon-aticus***	***farmeri***	***braakii***	***freundii***	***murliniae***	***youn-gae***
Adonitol	+	–	–	–	–	–	–	–	–	–	–
Malonate	+	+	+	+	+	–	–	–	–	–	–
Ornithine	+	–	+	+	–	+	+	+	–	–	–
Melibiose	–	–	+	–	V (67)	–	+	V (78)	+	V (33)	–
Sucrose	V (44)	–	–	–	V (33)	V (13)	+	–	+	V (33)	V (19)
Indole	+	–	+	–	–	+	+	V (33)	V (38)	+	V (14)
Dulcitol	V (38)	–	+	–	–	–	–	V (33)	V (13)	+	V (86)
H_2S	–	+	–	–	V (67)	V (13)	–	V (60)	V (75)	V (67)	V (67)

[a]Data obtained from reference 67.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; numbers in parentheses are percentage of strains giving positive reaction.

C. freundii (complex) has been reported as a cause of gastrointestinal illness

associated with imported Brie cheese,[103] and isolation of a *C. freundii* strain that carries the *E. coli* O157 antigen has been reported.[46] *C. koseri* has been isolated most often from urine and respiratory tract specimens.[291,400] *C. koseri* has also been reported with increasing frequency as a cause of sporadic and epidemic meningitis in neonates and young infants.[261,358,359,506,649] Brain abscesses are found in 75% of infants with *C. koseri* meningitis, a prevalence far higher than that reported for other bacteria that cause meningitis.[262,358] One-third of infants with *C. koseri* meningitis die, and at least 75% of survivors have severe neurologic impairment.[262] Other reports corroborate the tendency for *C. koseri* to cause meningitis and brain abscesses, particularly in association with the anaerobic gram-negative bacillus *Prevotella melaninogenica*.[23,160,384] *C. sedlakii* was isolated from the blood and cerebrospinal fluid of a 5-day-old premature infant seen with sepsis, meningitis, and a brain abscess at the University of Illinois Medical Center.[197] The child was treated with ampicillin, piperacillin, and cefotaxime. By day 14, the infant was clinically stable, and the therapy was changed to intravenous cefotaxime and oral cotrimoxazole. The child was discharged after 8 weeks to complete 2 more weeks of oral cotrimoxazole therapy at home. At 5½ months of age, a computed tomographic scan of the brain showed resolution of all abscess cavities. Additional isolates of *C. sedlakii* at the University of Illinois have been from urine and an infected arteriovenous graft of the leg. Of 13 *C. braakii* identified at the University of Illinois Medical Center, 5 were recovered from urines, 4 from peritoneal fluids, 1 from an abdominal wound, 2 from endotracheal specimens, and 1 from a bronchial specimen. *C. farmeri* isolates at the University of Illinois have been from stool, urine, abdominal wall tissue, and aqueous fluid. Bruckner and colleagues reported a case of *C. farmeri* bacteremia in a child with short-bowel syndrome in whom a septic episode developed shortly after commencement of a total parenteral nutrition (TPN) infusion.[79] Only three *C. gillenii* isolates have been recovered at

the University of Illinois from three separate sources: urine, peritoneal fluid, and a central venous catheter tip. Most *C. youngae* isolates have been recovered from urine. *C. rodentium* has been isolated only from rodents and causes a disease in laboratory mice known as transmissible murine colonic hyperplasia.[401,581]

Identification of *Citrobacter* species is hampered because the new species are not yet included in the databases of most commercial identification systems. To assist laboratories in speciating the new *Citrobacter* species, O'Hara and colleagues[481] have published a dichotomous key using conventional biochemical tests. The susceptibility pattern of isolates also offers an aid to identification. *C. koseri* has an antibiotic

susceptibility pattern similar to *Klebsiella* (i.e., resistant to ampicillin and ticarcillin), whereas *C. freundii* has a pattern more typical of *Enterobacter* species (i.e., resistant to ampicillin and first-generation cephalosporins).

Tribe *Klebsielleae*. The tribe *Klebsielleae* includes four major genera—*Klebsiella, Enterobacter, Hafnia, and Serratia*—each of which includes several species that are overt and opportunistic pathogens in humans. A new, fifth genus, *Pantoea*, has been added to accommodate the reclassification of the organism formerly named *Enterobacter agglomerans* biotype XIII and now called *Pantoea agglomerans*.[248] The key characteristics suggesting that an unknown isolate belongs to the *Klebsielleae* are listed in Table 6-6. The biochemical differences between the major genera and species within the tribe are presented in Table 6-16.

Genus *Klebsiella*. The genus *Klebsiella* was named after Edwin Klebs, a late–19th-century German microbiologist. The bacillus, now known as *Klebsiella,* was also described by Carl Friedlander, and for many years the "Friedlander bacillus" was well known as a cause of severe, often fatal, pneumonia. The genus includes six species: *K. pneumoniae, K. oxytoca, K. granulomatis, K. variicola, K. singaporensis*, and *K. alba*. *K. pneumoniae* is the type species of this genus.

***Taxonomic Changes in the Genus* Klebsiella.** In 2001, Drancourt and colleagues[187] performed a comparative analysis of the sequences of the 16S rRNA and *rpoB* genes (encoding the bacterial RNA polymerase β-subunit) of the type stains of nine *Klebsiella* species. Their findings confirmed that *Klebsiella* is heterogeneous and composed of species that form three phyletic clusters. Cluster I comprises *K. pneumoniae* subspecies *pneumoniae*, *rhinoscleromatis*, and *ozaenae*, and *K. granulomatis*; cluster II contains *K. ornithinolytica*, *K. planticola*, *K. trevisanii*, and *K. terrigena*; and cluster III contains *K. oxytoca*.[187] On the basis of this evidence, they proposed that the genus *Klebsiella* be divided into two genera, *Klebsiella* and *Raoultella* (named after the French bacteriologist Didier Raoult), and that *K. oxytoca* should be left as a monophyletic taxon. Species included in cluster I are retained in the genus *Klebsiella*, whereas species included in cluster II are transferred to the new genus *Raoultella*.[187] Granier et al.[263] have further shown that the *K. oxytoca* taxon is divided into two clades, which correspond to two genetic groups, called oxy-1 and oxy-2.[264] In 1999, *Calymmatobacterium granulomatis*, the presumed causative agent of donovanosis, was reclassified as *K. granulomatis* on the basis of phylogenetic data.[95,346] Finally, three novel environmental species have been added to the genus, namely, *K. variicola,*[566] *K. singaporensis,*[385]and *K. alba*[680] that have been isolated from plants and soil samples.

Klebsiella and *Raoultella* species are widely distributed in nature and in the gastrointestinal tracts of humans and animals. A *Klebsiella* species should be

suspected when

TABLE 6-16 Differentiation of the Major Genera and Species Within the Tribe *Klebsielleae*

Biochemical Test	*Klebsiella*		*Enterobacter*		*Pantoea*	*Hafnia*	*Serratia*	
	K. pneumoniae	*K. oxytoca*	*E. aerogenes*	*E. cloacae*	*P. agglomerans*	*H. alvei*	*S. marcescens*	*S. liquefaciens*
Indole	−	+	−	−	V (20)	−	−	−
Motility	−	−	+	+	V (85)	V (85)	+	+
Lysine	+	+	+	−	−	+	+	+
Arginine	−	−	−	+	−	−	−	−
Ornithine	−	−	+	+	−	+	+	+
DNase (25°C)	−	−	−	−	−	−	+	V (85)
Gelatinase (22°C)	−	−	−	−	−	−	+	+
Fermentation of:								
Lactose	+	+	+	+	V (40)	−	−	−
Sucrose	+	+	+	+	V (75)	−	+	+
Sorbitol	+	+	+	+	V (30)	−	+	+
Adonitol	+	+	+	V (25)	−	−	V (40)	−
Arabinose	+	+	+	+	+	+	−	+

+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive.

large colonies with a mucoid consistency are recovered on primary isolation plates. On MacConkey agar, the colonies typically appear large, mucoid, and purple, indicating fermentation of lactose and acid production (see Image Plate 6-1E). Not all strains, however, are mucoid, and certain species of *Enterobacter* can closely simulate the *Klebsiella* species in screening tests. All *Klebsiella* and *Raoultella* species are nonmotile, and most do not decarboxylate ornithine (*R. ornithinolytica* is ornithine-positive)—characteristics that are positive for most *Enterobacter* species. Many strains of *Klebsiella* and *Raoultella* hydrolyze urea slowly, producing a lightpink color in the slant of Christensen's urea agar. Production of indole from tryptophan can be used to separate the two principal species. *K. pneumoniae* is indole-negative, and *K. oxytoca* is indole-positive. Certain strains do not produce these classic reactions, which led to the naming of several additional species. See Table 6-17 for a listing of the characteristics by which these various species can be differentiated.

K. pneumoniae is most frequently recovered from clinical specimens and can cause a classic form of primary pneumonia. It is infrequently found in the oropharynx of normal persons (1% to 6% carrier rate)[557]; however, a prevalence as high as 20% may

occur in hospitalized patients. This colonization may prove to be the source of lung infections that generally occur in patients with debilitating conditions such as alcoholism, diabetes mellitus, and chronic obstructive pulmonary disease.[557] The pneumonia tends to be destructive, with extensive necrosis and hemorrhage, resulting in the production of sputum that may be thick, mucoid, and brickred, or thin and "currant jelly-like" in appearance. Lung abscesses, chronic cavitary disease, internal hemorrhage, and hemoptysis may be seen in severe cases. Pleuritis is commonly present, which explains why pleuritic pain is found in about 80% of patients. *K. pneumoniae* can also cause a variety of extrapulmonary infections, including enteritis and meningitis (in infants), urinary tract infections (in children and adults), and septicemia.

A new, hypervirulent clinical variant of *K. pneumonia* (hvKP) has emerged over the last two decades. Initial reports were from countries in the Asian Pacific Rim (e.g., Taiwan, Korea, Vietnam, and Japan) describing a unique clinical syndrome of community-acquired *K. pneumonia* infections presenting as pyogenic liver abscesses with a propensity for metastatic spread to distant sites.[139,393,655] Subsequently, an increasing number of cases are now being reported in North America,[235,345,377,421,454] South America,[647] the Carribbean,[185] and other continents around the world.[175,208,272,601,637,685] A combination of clinical and bacterial phenotypic features defined this new hvKP strain: (1) its ability to cause infection in ambulatory, healthy patients, (2) unusual sites of infection (e.g., liver, eye, CSF), (3) ability for metastatic spread, and (4) production of hypermucoviscous colonies when grown on agar plates.[593] The hypermucoviscous colonies are demonstrated by a positive "string test." The string test is positive when a bacteriology inoculation loop or needle is able to generate a viscous string >5 mm in length by touching the bacterial colony and raising the loop above the rim of the agar plate, creating a string of bacteria (see Image Plate 6-2H). Because of the unique and severe clinical features of these hvKP strains, laboratories should consider reporting *K. pneumoniae* strains that demonstrate a positive string test as "hypervirulent *K. pneumoniae*." For more on this topic, see review by Shon and colleagues.[593]

K. pneumoniae *Carbapenemase.* *K. pneumoniae* that produce *K. pneumoniae* carbapenemase (KPC) have rapidly spread across hospitals worldwide in the past decade.[61,466,677] KPC-producing *K. pneumoniae* isolates are typically resistant to carbapenems (ertapenem, doripenem, imipenem,

TABLE 6-17 Differentiation of Species Within the Genera *Klebsiella* and *Raoultella*[a]

Biochemical Test	*K. pneumoniae* subsp. *pneumoniae*	*K. pneumoniae* subsp. *ozaenae*	*K. pneumoniae* subsp. *rhinoscleromatis*	*K. oxytoca*	*R. ornithinolytica*	*R. planticola*	*R. terrigena*
Indole	–	–	–	+	+	V (20)	–
Methyl red	–	+	+	V (20)	+	+	V (60)
Voges–Proskauer	+	–	–	+	V (70)	+	+
Urease	+	–	–	+	+	+	–
Lysine	+	V (40)	–	+	+	+	+
Ornithine	–	–	–	–	+	–	V (20)
ONPG	+	V (80)	–	+	+	+	+
Malonate	+	–	+	+	+	+	+
Growth at[a]							
5°C	–	–	–	–	+	+	+
10°C	–	–	–	+	+	+	+
41°C	+	NA	NA	+	+	+	–

[a]Data from reference 220.
+, 90% or more strains are positive; −, 90% or more strains are negative; V, 11%–89% of strains are positive; NA, results not available.

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meropenem) as well as penicillins, cephalosporins, fluoroquinolones, and frequently also aminoglycosides. Because carbapenemase resistance can occur in species of *Enterobacteriaceae* other than *K. pneumoniae*, the phrase "Carbapenem-Resistant *Enterobacteriaceae* (CRE)" has been coined as the preferred term when referring to these multidrugresistant strains. Detection of CRE-producing bacteria may be difficult based on routine antibiotic susceptibility testing. It is therefore crucial to implement efficient infection control measures to limit the spread of these pathogens.[130] Surveillance cultures have been applied effectively as part of the multifaceted nationwide interventional strategy to decrease the spread of CREs using either selective broth or agar plates containing carbapenems or carbapenem disks[9,375,396] or selective chromogenic media such as CHROMagar KPC-Colorex KPC (CHROMagar, Paris, France),[9,255,574,669] Brilliance CRE (Oxoid, Thermofisher Scientific, Lenexa KS),[171,255] chrom ID CARBA (bioMerieux, Marcy l'Etoile, FR),[651,669] and SUPRA-CARBA.[255,467] For more on CREs, refer to Chapter 17.

K. ozaenae and *K. rhinoscleromatis* are infrequent isolates that are now considered to be subspecies of *K. pneumoniae*; however, each is associated with a unique spectrum of disease. *K. ozaenae* is associated with atrophic rhinitis, a condition called ozena, and purulent infections of the nasal mucous membranes. Janda and colleagues[326] have also reported a case of corneal abscess caused by *K. ozaenae*. Reports of the isolation of *K. ozaenae* from blood, urine, and soft tissue suggest that the spectrum of disease caused by this organism is more extensive than had been

thought previously.[258] *K. rhinoscleromatis* causes the granulomatous disease rhinoscleroma, an infection of the respiratory mucosa, oropharynx, nose, and paranasal sinuses. Clinical correlations should be made when these species are recovered in cultures to determine their medical significance in individual cases. Even though these two species are no longer considered to be true species but, rather, biochemically inactive strains of *K. pneumoniae*,[64] we believe that there is medical relevance in reporting the names *K. ozaenae* and *K. rhinoscleromatis* because of the specific disease association of these two strains.

Nearly half of the isolates of *K. oxytoca* submitted to the CDC have been from feces, with the next most common source being blood.[220] *K. oxytoca* has now been shown to be the causative agent of antibiotic-associated hemorrhagic colitis (AAHC).[294] AAHC is a distinct form of antibioticassociated diarrhea, in which *C. difficile* is absent and patients experience a sudden onset of bloody diarrhea often in combination with severe abdominal cramps. AAHC is most commonly associated with the use of penicillins, quinolones, and cephalosporins.[294,522] Colonoscopy usually reveals extensive hemorrhage and inflammation in the lamina propria, with lack of pseudomembranes. Spontaneous resolution usually occurs shortly after cessation of the antibiotic.[522] For unknown reasons, *K. oxytoca* AAHC has been associated with concurrent use of nonsteroidal anti-inflammatory drugs.[294,522]

Because of the role of *K. oxytoca* in the pathogenesis of AAHC, laboratories should report *K. oxytoca* from stool cultures when present in significant amounts. For the majority of patients with AAHC, stool testing reveals *K. oxytoca* in significant amounts ($>10^6$ CFU/mL)[689] In our experience, patients with this condition present with nearly pure cultures of *K. oxytoca* in their stool. When reporting *K. oxytoca* from stool cultures, laboratories may want to consider adding a comment to the laboratory report as follows: "Toxigenic strains of *K. oxytoca* have been reported to cause AAHC. Patients usually respond to withdrawal of antimicrobial agents." *K. oxytoca* is not a cause of nonhemorrhagic colitis.[689] Therefore, testing for *K. oxytoca* is warranted only for patients experiencing bloody diarrhea during antibiotic therapy with significant quantities of *K. oxytoca* in their stool culture.

Genus *Raoultella*. In 2001, based on 16S rRNA gene and rpoB sequence analysis, the new genus *Raoultella* was created to accommodate three members of the genus *Klebsiella* Cluster II that were renamed: *Raoultella ornithinolytica, Raoultella terrigena,* and *Raoultella planticola*.[187]

R. ornithinolytica is found in aquatic environments, fish, and ticks[445] and is a rare human pathogen; however, several cases of *R. ornithinolytica* bacteremia have been reported.[275,279,417,445,576] The more recently named species, *R. terrigena*[311] and *R. planticola*,[32] reflect their sources in nature. *R. terrigena* closely resembles *K.*

pneumoniae and has been isolated mainly from soil and water. Human isolates have been recovered from the feces of healthy humans[530] and the respiratory tract[528]; however, their capacity to cause human infection has not been shown. *R. planticola* (synonym *K. trevisanii*)[227,247] has been isolated primarily from botanical and aquatic environments. Early reports of human isolates were from the respiratory tract, urine, cerebrospinal fluid, and blood,[237,238] with the majority of isolates representing colonization, rather than infection. More recent reports suggest that *R. planticola* may be the cause of severe pancreatitis,[17] cholangitis with septic shock,[682] bacteremia,[303] urinary tract infection,[492] acute prostatitis,[366] chronic conjunctivitis,[690] and may be regarded as an emerging pathogen.[210]

Studies from France and Germany suggest that up to 19% of *Klebsiella/Raoultella* species are actually *R. planticola*[446,529,531]; however, the occurrence of *R. planticola* in the United States appears to be less frequent.[668] One problem might be the separation of *R. planticola* from *R. ornithinolytica,* which are closely related species that can be distinguished from each other by very few biochemical tests. *R. planticola* are ornithine decarboxylase (ODC) negative and indole variable, whereas *R. ornithinolytica* are positive for ODC and indole-negative. ODC-negative strains of *R. ornithinolytica* have been described, and these would likely be identified as *R. planticola* or *K. oxytoca* by phenotypic methods.[504,653] In a comparison of three phenotypic identification systems, Park et al.[504] reported that 27/27 *R. ornithinolytica* clinical isolates were identified as *R. ornithinolytica* by Vitek 2; however, MicroScan identified 25/27 (92.6%) as *K. oxytoca,* and API 20E identified 24/27 (88.9%) as *K. oxytoca* using sequence-specific primer PCR as the reference identification method. Similarly, de Jong et al.[177] reported difficulty in separating *Raoultella* species by MALDI-TOF mass spectrometry (MALDI-TOF MS) because mass spectra from *R. planticola* and *R. ornithinolytica* are highly similar and resemble those from *K. oxytoca*.

The higher incidence of infections due to *Klebsiella* species during the past decade probably reflects both an increase in nosocomial infections in debilitated or immunosuppressed patients and a trend toward greater antibiotic resistance. In

the United States, *Klebsiella* accounts for 3% to 7% of all nosocomial bacterial infections, placing them among the eight most important infectious pathogens in hospitals.[532] *Klebsielleae* have a tendency to harbor antibiotic-resistant plasmids; thus, infections with multiple antibiotic-resistant strains can be anticipated. Virtually all clinical strains are resistant to ampicillin, carbenicillin, and ticarcillin. Of particular

concern is the recent appearance of *Klebsiella* strains that possess plasmids that mediate resistance to extended-spectrum β-lactam drugs. This form of resistance is due to the production of unique β-lactamase enzymes, referred to as ES-BLs.[327,394] These enzymes have been seen mostly in strains of *K. pneumoniae* and *E. coli,* and cause them to be resistant to most β-lactam drugs, including the third-generation cephalosporins. A unique feature of ESBLs is their ability to escape detection with most of the commonly used susceptibility tests and the resultant concern that organisms possessing ESBLs are reported to be susceptible to antibiotics to which they are, in fact, resistant.[316,432] In addition, carbapenem resistance among *K. pneumoniae* strains has become widespread in the United States and throughout the world due to the dissemination of strains that carry bla_{KPC} plasmid.[61,677,466] This subject is discussed earlier in this chapter and in more detail in Chapter 17.

K. granulomatis (formerly *C. granulomatis*)[95] is a fastidious gram-negative encapsulated bacillus found intracytoplasmically within macrophages. It is the etiologic agent of granuloma inguinale (also known as Donovanosis), a sexually transmitted disease involving the genitalia and surrounding sites.[561] It is found in specific geographic foci, namely, New Guinea, northwest Australia, southeast India, the Caribbean, parts of South America, parts of central Africa, and the Kwa-Zulu/Natal region of South Africa.[347] *K. granulomatis* cannot be cultured on conventional agar media, although it has been successfully grown in embryonated eggs and in a monocyte coculture system.[347] Currently, the laboratory diagnosis of granuloma inguinale relies on the observation of "Donovan bodies" in tissue smears or biopsy specimens examined by Giemsa and Wright stains. To increase the specificity of the diagnosis, Dieterle's, Warthin–Starry,[266] and rapid Giemsa (Diff Quick)[475] stains have been used. A PCR for detection of donovanosis in genital specimens has been reported.[94,96] Azithromycin has emerged as the drug of choice and should be used if the diagnosis is confirmed or suspected.[474]

Genus *Enterobacter*. Because large amounts of gas are produced by many strains of the genus *Enterobacter*, for many years the type species was called *Aerobacter aerogenes*. The genus designation was changed to *Enterobacter* by Hormaeche and Edwards in 1960.[300]

The taxonomy of Enterobacter has a complicated history, with several species transferred to other genera. There were 29 species and subspecies originally included in the genus *Enterobacter*. However, recent polyphasic taxonomic analysis has determined that the nucleotide sequences of some species are not aligned with the genus *Enterobacter,* and as a result, several species have been transferred to 8 different new genera (Table 6-18). As a genus, *Enterobacter* has the general characteristics of the *Klebsielleae*, but can be differentiated from most *Klebsiella* species because they are motile and ornithine-positive. The biochemical characteristics

by which the medically important species can be differentiated are included in Table 6-19.

TABLE 6-18 Taxonomy of *Enterobacter* Species—29 Species Including Subspecies

Previous Designation	Current Name	Synonym	Reference
E. aerogenes	Same	*Klebsiella mobilis Aerobacter aerogenes*	
E. agglomerans	*Pantoea agglomerans*	*Erwinia herbicola* *Erwinia milletiae*	Gavini et al.[248]
E. amnigenus	*Lelliottia amnigena*		Brady et al.[62]
E. arachidis	*Kosakonia arachidis*		Brady et al.[62]
E. asburiae	Same		
E. cancerogenus	Same	*Erwinia cancerogena* *Enterobacter taylorae*	
E. cloacae	This species has been divided into subspecies (see below)		
	E. cloacae subsp. *cloacae*	*Aerobacter cloacae* *Bacillus cloacae*	Hoffmann et al.[293]
	E. cloacae subsp. *Dissolvens*	*Enterobacter dissolvens* *Erwinia dissolvens*	Hoffmann et al.[293]
E. cowanii	*Kosakonia cowanii*		Brady et al.[62]
E. dissolvens	*E. cloacae* subsp. *dissolvens*	*Enterobacter dissolvens* *Erwinia dissolvens*	Hoffmann et al.[293]
E. gergoviae	*Pluralibacter gergoviae*		Brady et al.[62]
E. helveticus	*Franconibacter helveticus*	*Cronobacter helveticus*	Brady et al.[62], Stephan et al.[612]
E. hormaechei	Same	CDC Enteric Group 75	
E. intermedius	*Kluyvera intermedia*	*Kluyvera cochleae Enterobacter intermedium*	Pavan et al.[511]
E. kobei	Same	NIH group 21	

E. ludwigii	Same		
E. massiliensis	Same		
E. mori	Same		
E. nimipressuralis	*Lelliottia nimipressuralis*	*Erwinia nimipressuralis*	Brady et al.[62]
E. oryzae	*Kosakonia oryzae*		Brady et al.[62]
E. pulveris	*Franconibacter pulveris*	*Cronobacter pulveris*	Brady et al.[62], Stephan et al.[612]
E. pyrinus	*Pluralibacter pyrinus*		Brady et al.[62]
E. radicincitans	*Kosakonia radicincitans*		Brady et al.[62]
E. sacchari	*Kosakonia sacchari*		Gu et al.[270]
E. siamensis	Same		
E. sakazakii	*Cronobacter sakazakii*	"yellow-pigmented *Enterobacter cloacae*"	Iversen et al.[310]
E. soli	Same		
E. taylorae	*Enterobacter cancerogenus*	CDC Enteric Group 19	Schønheyder et al.[585]
E. turicensis	*Siccibacter turicensis*	*Cronobacter zurichensis*	Brady et al.[62], Stephan et al.[612]
E. xiangfangensis	Same		Gu et al.[270]

TABLE 6-19 Differentiation of Clinically Important Species Within the Genera

Cronobacter, Enterobacter, Kluyvera, Kosakonia, Lelliottia, and *Pluralibacter*[a]

Test	*E. aerogenes*	*L. amnigenus* biogroup 1	*L. amingenus* biogroup 2	*E. asburiae*	*E. cancerogenus*	*E. cloacae*	*K. cowanii*	*P. gergoviae*	*E. hormaechei*	*K. intermedius*	*E. kobei*	*C. sakazakii*
Methyl red	–	–	V (65)	+	–	–	NA	–	V (57)	+	–	–
Voges–Proskauer	+	+	+	–	+	+	+	+	+	+	+	+
Lysine	+	–	–	–	–	–	–	+	–	–	–	–
Arginine	–	–	V (35)	V (21)	+	+	–	–	V (78)	–	+	+
Ornithine	+	V (55)	+	+	+	+	–	+	V (91)	V (89)	+	+
Urease	–	–	–	V (60)	–	V (65)	–	+	V (87)	–	–	–
Motility	+	+	+	–	+	+	+	+	V (52)	V (89)	+	+
Fermentation of												
Lactose	+	V (70)	V (35)	V (75)	–	+	+	V (55)	–	+	+	+
Sucrose	+	+	–	+	–	+	+	+	+	V (65)	V (25)	+
Adonitol	+	–	–	–	–	V (25)	–	–	–	–	–	–
Sorbitol	+	–	+	+	–	+	+	–	–	+	+	–
Raffinose	+	+	–	V (70)	–	+	+	+	–	+	+	+
Rhamnose	+	+	+	–	+	+	+	+	+	+	+	+
Melibiose	+	+	+	–	–	+	+	+	–	+	+	+
Yellow Pigment	–	–	–	–	–	–	V (66)	–	–	–	–	+

[a]Table includes only those *Enterobacter* species that have been isolated from human clinical specimens. Isolates that are triple decarboxylase negative may be *Pantoea* species, and isolates that are both lactose- and sucrose-negative but are lysine-positive may be *Hafnia alvei* (Table 6-16).
+, 90% or more strains are positive; −, 90% or more strains are negative; V, 11%–89% of strains are positive; NA, not available.

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Enterobacter aerogenes and *Enterobacter cloacae* are the species most commonly encountered in clinical specimens. They are widely distributed in water, in sewage, in soil, and on vegetables. They are part of the commensal enteric flora and are not believed to cause diarrhea, although a Shiga-like toxin-producing strain of *E. cloacae* has been isolated from the feces of an infant with hemolytic–uremic syndrome.[508] They are also associated with a variety of opportunistic infections involving the urinary tract, respiratory tract, and cutaneous wounds, and, on occasion, cause septicemia and meningitis.[505] *E. cloacae* is closely aligned with five other *Enterobacter* species namely: *E. asburiae, E. hormaechei, E. kobei, E. ludwigii*, and *Lelliottia [Enterobacter] nimipressuralis.* These six species are collectively referred to as the *E. cloacae* complex. Furthermore, *E. cloacae* is divided into two subspecies: *E. cloacae* subsp. *cloacae* and *E. cloacae* subsp. *dissolvens* (formerly *E. dissolvens*).[293]

Separation of these species and subspecies by phenotypic testing is challenging, and separation using MALDI-TOF MS is not yet possible. As a result, laboratories may wish to report the identification of these species as the "*E. cloacae* complex." The clinical significance of the species in the *E. cloacae* complex has been reviewed by Mezzatesta and colleagues.[434]

E. asburiae (formerly called CDC enteric group 17, or atypical *Citrobacter*) is biochemically similar to *E. cloacae;* however, it is unique among the *Enterobacter* species by being nonmotile and VP-negative. It has been reported from a variety of human sources, including blood, urine, wounds, respiratory tract, and feces.[69] One case of community-acquired pneumonia caused by *E. asburiae* has been reported.[614] We have found considerable variation in the ability of commercial identification systems to correctly identify this species.

E. cancerogenus (formerly called *Erwinia cancerogena*, *Enterobacter taylorae*, and CDC enteric group 19)[221,585] has been reported to cause a variety of clinical infections, including osteomyelitis after an open fracture,[666] wound infection,[552] especially after severe trauma or crush injuries,[2] urinary tract infection,[555,567] and bacteremia and pneumonia.[567] The key biochemical features are lysine-negative, arginine-positive, and ornithine-positive and -negative reactions in adonitol, inositol, sorbitol, raffinose, and melibiose. They are lactosenegative but ONPG-positive, and we have noted that colonies growing on MacConkey agar will develop purple centers after extended incubation.

E. hormaechei was a new species of *Enterobacter* named in 1989 after Estenio Hormaeche, a Uruguayan microbiologist who (with P.R. Edwards) proposed and defined the genus *Enterobacter*.[484] Formerly known as enteric group 75, it is biochemically closest to *E. taylorae* (now *E. cancerogenus*) except it is urea-positive, sucrose-positive, and esculinnegative. Isolates have been reported from blood, sputum, wounds, ear, gallbladder, and stool. In March 1993, CDC investigators identified a nosocomial outbreak of *E. hormaechei* septicemia in a neonatal intensive care unit. Five infants had positive blood cultures with *E. hormaechei*, and another infant had *E. hormaechei* tracheitis. Four additional infants were identified with *E. hormaechei* colonization. No deaths were reported. Environmental cultures showed the organism to be present on three isolettes and one doorknob.[101,664] An outbreak of *E. hormaechei* bloodstream infections were reported in three neonatal intensive care units in Rio de Janeiro. Chart review for previous procedures revealed parenteral nutrition as the only common procedure.[169] A case of recurrent bacteremia caused by *E. hormaechei* has been seen at the University of Illinois Medical Center in a 2-year-old patient with a neuroblastoma involving a left supraclavicular lymph node, an adrenal gland, and a perinavel lymph node.

In 1981, CDC enteric group 69 was defined for a group of yellow-pigmented strains

that resembled *E. cloacae* biochemically. In 1996, *Enterobacter kobei* was described as a new species of *Enterobacteriaceae* resembling *Enterobacter cloacae*,[364] and similar to enteric group 69, but which were negative in tests for VP, fermentation of adonitol, and yellow pigment production. DNA hybridization studies performed at the CDC and the National Institute of Health (NIH), Tokyo, have led to the conclusion that enteric group 69 species are yellow-pigmented biochemical variants of *E. kobei*.[364,482] The majority of strains of *E. kobei* were isolated from clinical specimens including blood, sputum, throat, and urine.[364,602] A case of nosocomial urosepsis has also been reported in a patient with a history of urinary bladder operation.[292]

Enterobacter species together with certain other members of the family *Enterobacteriaceae* (namely, *C. freundii, Serratia* species, *Morganella morganii*, *Providence* species, and *Y. enterocolitica*) carry a gene for chromosomally encoded β-lactamase that can be induced by certain antibiotics, amino acids, or body fluids.[315,394] Unlike plasmid-mediated β-lactamases, these enzymes are not normally expressed. Only under the influence of an inducer or following mutation does the gene become activated and the enzyme expressed. It is a concern, therefore, that organisms harboring genes for inducible β-lactamases may show false susceptibility if tested in the uninduced state. Recently, methods for the detection of resistance owing to inducible β-lactamases have been described.[304] This topic is discussed further in Chapter 17.

Genus *Cronobacter*. *Cronobacter [Enterobacter] sakazakii* was defined as a novel species by Farmer et al.[218] in 1980; however, the existence of divergent biogroups suggested that these organisms represented multiple species. Using a polyphasic taxonomic approach, Iversen et al.[309] showed that these organisms comprise at least five genomospecies and proposed that these species should be moved to a novel genus, *Cronobacter* gen. nov. within the *Enterobacteriaceae*, initially comprising four named species. The taxonomy was expanded to five named species in 2008,[310] seven named species in 2011, nine species in 2012,[333] and ten named species in 2013.[62] In 2014, *C. helveticus* and *C. pulveris* were reclassified into the genus *Franconibacter* and *C. zurichensis* was reclassified into the new genus *Siccibacter* and renamed *S. turicensis*.[612] (Box 6-13, Table 6-18).

C. sakazakii, known as yellow-pigmented *E. cloacae*,[218] has been found in several cases of neonatal meningitis and sepsis.[47,145,332,357,452,638] Fatality rates as high as 75% have been reported,[373,459] indicating that this organism can be highly virulent. Recent reports have linked *C. sakazakii* infection in neonates with contaminated powdered-milk formulas.[121,126,641] The bright yellow pigment (particularly intense if cultures are incubated at 25°C) and "tough" nature of the colonies are the initial clues that this organism is present (*Pantoea agglomerans* also produces a yellow pigment,

usually less intense, and often only after delayed incubation at room temperature). The decarboxylase pattern of *C. sakazakii* (lysine-negative, arginine-positive, and ornithine-positive) helps to differentiate it from *E. aerogenes* (lysine-positive, arginine-negative, and ornithine-positive) and *P. agglomerans* (lysine-, arginine-,

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BOX 6-13

Currently Named Species of *Cronobacter*

C. condimenti
C. dublinensis subsp. *dublinensis*
C. dublinensis subsp. *lactaridi*
C. dublinensis subsp. *lausannensis*
C. helveticus (syn. *Franconibacter helveticus*)
C. malonaticus
C. muytjensii
C. pulveris (syn. *Franconibacter pulveris*)
C. sakazakii
C. turicensis
C. universalis
C. zurichensis (syn. *Siccibacter turicensis*)

All *Cronobacter* species, except *C. condimenti*, have been linked to clinical cases of infection in either adults or infants.

and ornithine-negative); and *E. sakazakii* does not ferment sorbitol, in contrast to *E. cloacae*, which does. *C. sakazakii* are naturally resistant to all macrolides, lincomycin, clindamycin, streptogramins, rifampicin, fusidic acid, and fosfomycin, but are susceptible to tetracyclines, aminoglycosides, numerous β-lactams, chloramphenicol, antifolates, and quinolones.[618]

Genus *Hafnia*. Members of the genus *Hafnia* are commonly isolated from the gastrointestinal tract of humans and animals and from foods including meat and dairy products.[321] *Hafnia alvei*, formerly *Enterobacter hafnia*, was originally thought to be the only species in the genus *Hafnia.* However, DNA–DNA hybridization studies conducted at the CDC in the 1970s revealed that *H. alvei* is genetically heterogeneous and consists of at least two DNA hybridization groups (HGs) designated HG 1 and HG

2. Huys et al.,[305] using molecular techniques and phenotypic data, confirmed that *H. alvei* comprises at least two taxa at the species level, of which HG 1 corresponds to *H. alvei* and HG 2 represents a novel species, for which the name *Hafnia paralvei* was proposed.

The biochemical characteristics of *Hafnia* species are similar to those of *Enterobacter* species except that *Hafnia species* do not produce acids from the following carbohydrates: lactose, sucrose, melibiose, raffinose, adonitol, sorbitol, dulcitol, and inositol (see Table 6-16). *Hafnia* species can be distinguished from *Serratia* species because they do not produce lipase, gelatinase, or deoxyribonuclease. We have also noted that, unlike other species of *Enterobacteriaceae*, *Hafnia* species give off a strong scent of human feces. Abbott and colleagues reported that the two *Hafnia* species could be separated unambiguously based upon a battery of four tests. *H. alvei* strains are typically malonate-, salicin-, and β-glucosidase-positive and D-arabinose-negative, whereas *H. paralvei* have the opposite reactions.[4]

The clinical significance of *Hafnia* species is not well defined. Both species have been recovered from clinical samples with almost identical distribution,[323] but little is known regarding their relative frequency or disease distribution. *Hafnia* species have been recovered from human feces in the absence of symptoms, although isolated cases of infection have been reported from persons in whom *Hafnia* species have been recovered from wounds, abscesses, sputum, urine, blood, and other sites. *Hafnia* species have been reported in cases of hemolytic–uremic syndrome,[154] graft-versus-host disease,[578] and sepsis following stem-cell and tissue transplantation.[42,84] There is also evidence to suggest that *Hafnia* species may be an emerging cause of acute bacterial gastroenteritis.[15,549,550,554,563,667] However, Janda et al. have subsequently shown that the prototypal diarrheagenic stains of *Hafnia alvei* that were reported to contain the *eae* gene of enteropathogenic *E. coli* are in fact a category of diarrheagenic isolates belonging to the genus *Escherichia* that were misidentified as *Hafnia alvei* by the API 20E system.[306,322] These strains have been designated as the new species *E. albertii* discussed previously.[306]

Hafnia strains are reported to be uniformly susceptible to amikacin, gentamicin, ciprofloxacin, gatifloxacin, ofloxacin, trimethoprim–sulfamethoxazole, imipenem, meropenem, and cefepime. Resistance is often noted with β-lactams, β-lactam inhibitor combinations, and cephalosporins owing to the presence of chromosomal AmpC β-lactamases.[4]

Genus *Kosakonia*. Five former *Enterobacter* species are included in the genus *Kosakonia*: *K. arachidis*, *K. cowanii*, *K. oryzae*, *K. radicincitans,* and *K. sacchari*.[270]

K. [*Enterobacter] cowanii* was originally proposed for a group of organisms referred to as NIH Group 42.[308] *K. cowanii* strains are negative for lysine and

ornithine decarboxylases and arginine dihydrolase; therefore, phenotypically, they most closely resemble *Pantoea agglomerans*. Tests useful for differentiation from *P. agglomerans* are negative malonate utilization and fermentation of dulcitol and sorbitol (both negative for *P. agglomerans*). Of the nine strains studied, eight were isolated from clinical specimens: urine (four), sputum (two), blood (one), and pus (one), but their clinical significance is unknown.[308]

Genus *Lelliottia*. The genus *Lelliottia* contains two species, *Lelliottia [Enterobacter] amnigena* and *Lelliottia [Enterobacter] nimipressuralis*.[62]

L. amnigena is primarily a water organism that has been isolated from human specimens.[313] A case of posttransfusion septicemia caused by *L. amnigena* has been reported in France.[317]

L. amnigena has been reported to have caused sepsis following blood transfusion[317] and heart transplantation.[56] Other site infections (cholecystitis, cystitis, lymphadenitis, osteomyelitis) have also been reported to be caused by this microorganism.[85]

L. nimipressuralis has been reported as a cause of pseudobacteremia due to contaminated salined cotton used before applying iodine to collect blood for culture.[351]

Genus *Pantoea*. The genus *Pantoea* was created in 1989 with the type species being *Pantoea agglomerans*.[248] This taxon includes the former type strains of *Enterobacter agglomerans, Erwinia herbicola*, and *Erwinia milletiae*. *Pantoea* is derived from a Greek word meaning "of all sorts and sources," thus describing these bacteria that come from diverse geographic and ecologic sources. There are currently 22 named species in the genus *Pantoea*, most of which are plant pathogens and rarely associated with human disease. Only seven species have been reported to cause human infection, with *P. agglomerans* being the most common. In the early 1970s, *P. agglomerans* (then called *Enterobacter agglomerans*) was responsible for a nationwide outbreak of septicemia caused by contaminated intravenous fluids.[405,410] Subsequently,

multiple cases of *P. agglomerans* sepsis have been reported, usually in neonates with significant prematurity, comorbidity, or indwelling catheters,[45,158,374] but also in adult patients with comorbidity associated with immune suppression.[138,386] *P. agglomerans* has also been associated with cases of septic arthritis or synovitis,[368] osteitis,[376] colelithiasis,[232] occupational respiratory infections and skin allergy,[437] peritonitis,[387] and an outbreak secondary to contaminated parental nutrition.[274] *P. ananatis* (originally named *P. ananas*) has been isolated from a corneal infiltrate following a rice husk injury[412] and from the blood of an elderly man following a colonoscopy for anal

hemorrhage.[172] *P. dispersa* has been isolated from plant surfaces, seeds, humans, and the environment. Two cases of neonatal sepsis due to *P. dispersa* have been reported.[427] Four newly named *Pantoea* species have also been recovered from human clinical specimens: *P. septica* from blood and skin; *P. eucrina* from trachea, cyst, urine, and CSF; *P. brenneri* from urethra and sputum; and *P. conspicua* from blood; however, the clinical significance of these isolates was not reported.[63] *P. gaviniae* and *P. calida* were isolated from infant formula and an infant formula production environment, but no human infections have been reported.[536] *Pantoea* species are tripledecarboxylase-negative (lysine-negative, arginine-negative, and ornithine-negative), and often yellow-pigmented.[248] See Tables 6-7 and 6-16 for additional phenotypic characteristics.

Genus *Pluralibacter*. The genus *Pluralibacter* contains two genera, *Pluralibacter [Enterobacter] gergoviae* and *Pluralibacter [Enterobacter] pyrinus*.[62]

P. gergoviae causes urinary tract infections, and additional isolates have been recovered from the respiratory tract and blood.[72] Biochemically, it is closest to *E. aerogenes* (lysine-positive, arginine-negative, ornithine-positive), but is strongly urease-positive. It can be further differentiated by negative reactions in adonitol, inositol, and sorbitol, whereas *E. aerogenes* is positive for all three reactions.

Genus *Serratia*. *Serratia* species are unique among the *Enterobacteriaceae* in producing three hydrolytic enzymes: lipase, gelatinase, and DNase. Resistance to colistin and cephalothin are additional distinguishing features. There are currently 10 species recognized, 7 of which have been recovered from human clinical specimens. The biochemical differentiation of *Serratia* species of clinical importance is shown in Table 6-20.

S. marcescens is the most important member of the genus *Serratia* and is often associated with a variety of human infections, particularly pneumonia and septicemia in patients with reticuloendothelial malignancies who are receiving chemotherapeutic agents. At one time, the organism was used as a harmless commensal to trace environmental contamination, primarily because the characteristic red pigmentation of some strains was easy to spot in culture media (see Image Plate 6-2G). However, the organism is now recognized as an important pathogen with invasive properties and a tendency to resist many commonly used antibiotics. *S. marcescens* can be a significant nosocomial opportunist, as evidenced by a recent case of childhood meningitis following the use of contaminated benzalkonium chloride disinfectant solution.[577] The species referred to as *S. liquefaciens* is now known to be not a single species but a collection of several DNA HGs, including species named *S. proteamaculans* and

TABLE 6-20 Differentiation of Clinically Important Species Within the Genus *Serratia*[a]

Biochemical Test	*S. marcescens*	*S. liquefaciens*	*S. rubidaea*	*S. plymuthica*	*S. ficaria*	*S. fonticola*	S. odorifera Biogroup 1	S. odorifera Biogroup 2
DNase (25°C)	+	V (85)	+	+	+	–	+	+
Lipase (corn oil)	+	V (85)	+	V (70)	V (77)	–	V (35)	V (65)
Gelatinase (22°C)	+	+	+	V (60)	+	–	+	+
Lysine (Moeller's)	+	+	V (55)	–	–	+	+	+
Ornithine (Moeller's)	+	+	–	–	–	+	+	–
Odor of potatoes	–	–	V	–	+	–	+	+
Red, pink, or orange pigment	V	–	V	V	–	–	–	–
Fermentation of:								
L-Arabinose	–	+	+	+	+	+	+	+
D-Arabitol	–	–	V (85)	–	+	+	–	–
D-Sorbitol	+	+	–	V (65)	+	+	+	+
Sucrose	+	+	+	+	+	V (21)	+	–
Raffinose	–	V (85)	+	+	V (70)	+	+	–
Malonate utilization	–	–	+	–	–	+	–	–

+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive.
Table includes only those *Serratia* species that have been isolated from human clinical specimens.
[a]Data obtained from reference 253 and Table 6-7.

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S. grimesii. They are found on the surfaces of plants and belong to the "*Serratia liquefaciens–proteamaculans–grimesii*" complex. Strains of these species produce plant-growthpromoting chemicals, have antifungal properties, encourage the establishment of nitrogen-fixing symbionts, and act as insect pathogens.[24] Because the species that make up this HG cannot be separated by currently used biochemical tests, it is suggested that members of this species be reported as "*Serratia liquefaciens* group." This group is differentiated from *S. marcescens* by virtue of its ability to ferment L-arabinose.

S. rubidaea, as its name would imply, produces colonies that are red-pigmented but is rarely isolated from human clinical specimens. Ursua and associates[640] reported a case of *S. rubidaea* isolated from the bile and blood of a patient with a bile tract carcinoma who underwent invasive procedures. *S. rubidaea* bacteremia has also been reported in a patient with an arterial catheter.[588] *S. odorifera* produces a dirty, musty odor, similar to unpeeled potatoes. Two biogroups are described. Biogroup 1 is ornithine-, sucrose-, and raffinose-positive and is isolated predominantly from sputum; however, it has been reported to cause severe sepsis in elderly, compromised patients,[141,429] and catheter-associated sepsis in an adolescent patient with thalassemia

major who had been splenectomized.[256] Biogroup 2 is negative for these three reactions and has been recovered from blood and cerebrospinal fluid.[220] Strains of *S. plymuthica* may be redpigmented and have been isolated from soil, water, and human sputum specimens. Although *S. plymuthica* is generally not considered to be a cause of serious human infections, recent reports have shown that it can be a significant pathogen causing chronic osteomyelitis,[687] wound infections,[92,146] and community-acquired[553] and nosocomial bacteremia[92,184,301] and has been isolated from the peritoneal fluid of a patient with cholecystitis.[92] *S. ficaria* has a natural habitat in figs and the fig wasp.[253] Isolation of this species from human specimens is extremely rare and is usually accompanied by a history of ingestion of figs.[18,29,168] There is a single report of the isolation of *S. ficaria* from blood and wound following a dog bite.[166] *S. entomophila* is an insect pathogen, and no human isolates have been reported. "*S.*" *fonticola* is not really a species of *Serratia* and is likely to be reclassified. It is a water organism that has been isolated rarely from human clinical specimens, mostly wounds.[57,220,521] *S. fonticola* has been recovered from the blood of a patient with a digestive neoplasm at the University of Illinois Medical Center. The natural antimicrobial susceptibilities of strains of the less commonly isolated *Serratia* species: *S. ficaria, S. fonticola, S. odorifera, S. plymuthica,* and *S. rubidaea* have been reported by Stock and colleagues.[615]

Tribe *Proteeae*. The *Proteeae* comprise three genera: *Proteus, Morganella, and Providencia*. The characteristics suggesting that an organism belongs to this tribe are given in Table 6-6. The classification, identification, and clinical significance of the Proteeae has been reviewed by O'Hara et al.[477]

Genus *Proteus*. DNA relatedness studies have clarified the classification of organisms within the *Proteeae*. The genus *Proteus* now includes five named species: *P. vulgaris*, *P. mirabilis*, *P. myxofaciens*, *P. penneri*, and *P. hauseri*, and three unnamed genomospecies that were formerly identified as members of biogroup 3 of *P. vulgaris*.[68] Strains of *P. vulgaris* have traditionally been placed into three biogroups as follows:

Proteus biogroup 1: indole-, salicin-, esculin-negative; chloramphenicol-resistant
Proteus biogroup 2: indole-, salicin-, esculin-positive
Proteus biogroup 3: indole-positive, salicinand esculin-negative

Proteus biogroup 1 is a single genetic species and is now known as *P. penneri*.[286] *Proteus* biogroup 2 is a single genetic species and will retain the name *P. vulgaris*. *Proteus* biogroup 3 consists of four separate genetic species that are designated DNA groups (genomovars) 3, 4, 5, and 6. DNA group 3 can be distinguished from *Proteus*

DNA groups 4, 5, and 6 because it is negative for Jordan's tartrate, lipase, and DNase (Table 6-21). Because DNA group 3 can be phenotypically separated from the other *Proteus* genomospecies, it has been officially named *Proteus hauseri* to honor Gustav Hauser, the

TABLE 6-21 Differentiation of Species Within the Members of the Genus *Proteus*[a]

						Proteus vulgaris biogroup 3		
TEST	***P. mirabilis***	***P. myxofaciens***	***P. penneri***	***P. vulgaris***	***P. hauseri***	**DNA Group 4**	**DNA Group 5**	**DNA Group 6**
Ornithine	+	–	–	–	–	–	–	–
Indole	–	–	–	+	+	+	+	+
Esculin	–	–	–	+	–	–	–	V (9)
Salicin	–	–	–	+	–	–	–	V (9)
Lipase	+	+	V (35)	V (14)	–	+	+	V (90)
Tartrate	V (87)	+	V (89)	V (14)	–	+	+	+
Rhamnose	–	–	–	–	–	+	V (17)	–
DNase 25°C	V (50)	V (50)	V (12)	+	–	+	+	V (55)
Acetate	V (20)	–	V (12)	V (14)	–	–	V (12)	V (18)

[a]Data obtained from reference 478.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; numbers in parentheses are the percentages of strains giving positive reactions.

German microbiologist, who proposed the genus *Proteus* in 1885.[478] *Proteus* genomospecies 4, 5, and 6 will remain unnamed until better phenotypic differentiation can be accomplished. Biochemical separation of the *Proteus* species and DNA groups is given in Table 6-21.

The genus *Proteus* is found in soil, water, and fecally contaminated materials. *Proteus* species exhibit the characteristic feature of swarming motility, which is observed on noninhibitory agar (e.g., blood agar plate) as a wavelike spreading of the organism across the entire surface of the agar (see Image Plate 6-1D). Whenever swarming is observed, *Proteus* species should be suspected. *P. mirabilis* is the species most frequently recovered from humans, particularly as the causative agent of both urinary tract and wound infections. *P. vulgaris* is more commonly recovered from infected sites in immunosuppressed hosts, particularly those receiving prolonged regimens of antibiotics. As noted in Table 6-21, *P. vulgaris* is indole-positive, whereas *P. mirabilis* is indolenegative. Thus, by performing a rapid spot indole test on a characteristic swarming colony, a rapid presumptive identification of *P. mirabilis* or *P. vulgaris* can be made. The species *P. penneri*[286] and *P. myxofaciens* are also indole-negative but are rarely encountered in clinical laboratories (the latter is a pathogen of

gypsy moth larvae and has not been recovered from human specimens). Therefore, for practical purposes, the recovery of an indole-negative *Proteus* species can be presumptively identified as *P. mirabilis*. Virtually all strains of *P. mirabilis* are sensitive to ampicillin and cephalosporins, whereas *P. vulgaris* is resistant; therefore, most patients with clinical infection, from whom an indole-negative *Proteus* species is recovered, can be treated with one of the broadspectrum penicillins or cephalosporins.

P. penneri closely resembles *P. vulgaris* but differs from *P. vulgaris* by being indole-, salicin-, and esculin-negative and by failing to produce hydrogen sulfide in TSI. When *P. penneri* is suspected, a chloramphenicol susceptibility test should be performed for identification purposes. *P. penneri* is chloramphenicol-resistant, whereas other indole-negative *Proteus* species are chloramphenicol-susceptible (see Table 6-21).[286] Documented human infections with *P. penneri* have been limited mainly to the urinary tract and wounds of the abdomen, groin, neck, and ankle.[286,367] In one report, a patient with leukemia was described, in whom a *P. penneri* bacteremia developed with a concomitant subcutaneous thigh abscess, demonstrating the invasive potential of this bacterium.[209] Microbiologists are advised to be suspicious of any *P. vulgaris* isolates that are indole-negative and hydrogen sulfide-negative because these may possibly be isolates of *P. penneri*.

Genus *Morganella*. On the basis of genetic studies performed by Brenner and colleagues in 1978, the organism previously designated *Proteus morganii* was reassigned to the new genus *Morganella* as *M. morganii*.[64] Studies by Jensen and colleagues have shown that *M. morganii* can be further separated into three DNA relatedness groups and seven biogroups.[328] DNA relatedness group 1 contains biogroups A through D. DNA relatedness group 2 contains biogroups E and F and two-thirds of biogroup G (termed biogroup G-2). DNA relatedness group 3 contains the remaining one-third of biogroup G (termed biogroup G-1). Because G-1 and G-2 are phenotypically indistinguishable, Jensen and associates[328] have proposed dividing *M. morganii* into just two subspecies based on trehalose fermentation. *M. morganii* that are unable to ferment trehalose are designated *M. morganii* subsp. *morganii*, and those that are able to utilize trehalose are designated *M. morganii* subsp. *sibonii*.

M. morganii is a cause of both urinary tract and wound infections and has been implicated as a cause of diarrhea. Serious infections reportedly caused by *M. morganii* include a case of meningitis in a patient with AIDS[416] and a case of meningitis and brain abscess in an 8-day-old infant.[646] *M. psychrotolerans* is a psychrotolerant strain of a *Morganella* capable of growing at temperatures of 2°C to 35°C. It has been isolated from cold-smoked tuna involved in an outbreak of histamine poisoning in Denmark; however, no human infections with this organism have been reported to date.[207] As shown in Table 6-6, the pattern of Simmon's citrate–negative, hydrogen sulfide-negative, and ornithine decarboxylase-positive is characteristic of this genus.

The biochemical differentiation of the subspecies and biogroups is given in Table 6-22.

Genus *Providencia*. *Providencia* of clinical importance are: *Providencia alcalifaciens*, *Providencia stuartii*, *Providencia rettgeri*, and the newly described species *Providencia rustigianii*[287] and *Providencia heimbachae*.[450] All species of the genus *Providencia* deaminate phenylalanine, but only *P. rettgeri* consistently hydrolyzes urea. The biochemical differences of the species are shown in Table 6-23.

TABLE 6-22 Differentiation of Species Within the Genus *Morganella*[a]

	M. Morganii subsp. *morganii* Biogroups				*M. morganii* subsp. *sibonii* Biogroups		
Biochemical Test	***A***	***B***	***C***	***D***	***E***	***F***	***G***
Lysine	–	+	–	+	+	d+	–
Ornithine	+	+	–	–	+	–	+
Trehalose	–	–	–	–	+	+	+
Tetracycline (% susceptible)	100[b]	100	14	100	0	0	21
Motility	+	–	d+	–	+	+	+

[a]Data obtained from reference 328.
[b]Strains with a zone of ≥28 mm around tetracycline were considered susceptible (minimal inhibitory concentration [MIC] correlate, ≤2 μg/mL), and those with a zone diameter ≤15 mm were considered tetracycline-resistant (MIC correlate, ≥32 μg/mL).
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; d+, delayed reaction, 50%–89% positive within 48 hour.

TABLE 6-23 Differentiation of Species Within the Genus *Providencia*[a]

Biochemical Test	***P. alcalifaciens***	***P. rustigianii***	***P. heinbachae***	***P. stuartii***	***P. rettgeri***
Urea hydrolysis	–	–	–	V (30)	+
Citrate utilization	+	–	–	+	+
Fermentation of:					
Inositol	–	–	V (46)	+	1
Adonitol	+	–	+	–	+
Arabitol	–	–	+	–	+
Trehalose	–	–	–	+	–
Galactose	–	+	+	+	+

+, 90% or more strains are positive; −, 90% or more strains are negative.
[a]Data obtained from reference 450 and other sources.

Except for causing urinary tract infections, for which Penner has cited several nosocomial outbreaks,[513] infections with *Providencia* species are uncommon and are limited to isolated case reports. All species may be recovered from feces; however, only *P. alcalifaciens* may be associated with diarrheal illness, usually in children.[325]

Hickman–Brenner and associates[287] designated *P. rustigianii* to what was previously known as *P. alcalifaciens* biogroup 3. This organism has also been recovered from feces; however, its role in diarrheal disease is still questionable. A new species, *P. heimbachae*, has been reported from penguin feces and an aborted cow fetus.[450] There has been only one report of human *P. heimbachae* infection, from the stool of a 23-year-old woman with idiopathic diarrhea.[483] Chamberland and colleagues have reported a case of *Providencia stuartii* from a renal abscess that was misidentified using the Vitek 2 GN ID card (bioMerieux) as *Pasteurella canis* (99% probability). The same isolate was identified on the API 20 E system (bioM-erieux) to the genus level of *Pasteurella multocida* (86.1%) versus *Pasteurella pneumotropica/Mannheimia haemolytica* (13.5%). The isolate was identified by MALDI-TOF MS as *Providencia stuartii* using the Biotyper system (software version 3.0; Bruker–Daltonics, Billerica, MA) and Vitek MS (database version 2.0 bioMerieux). Following this, 16S rRNA gene sequencing yielded a definitive result for *Providencia stuartii*.[132]

Tribe *Yersinieae*. Three species of Pasteurella, including the causative agent of human plague, Pasteurella pestis, were formally assigned to a new genus, Yersinia, in the eighth edition of Bergey's Manual and placed in the Enterobacteriaceae. The name for the genus Yersinia was derived from the French bacteriologist Alexander Yersin, who in 1894, first identified the organism now called Yersinia pestis. The key characteristics of the Yersinieae are given in Table 6-6.

Although *Yersinia* species qualify biochemically for inclusion in the *Enterobacteriaceae*, the cells appear small and coccobacillary in Gram-stained smears, and may be small and pinpoint on MacConkey agar, particularly for certain strains of *Y. pestis* and *Y. pseudotuberculosis*. Optimal growth occurs from 25°C to 32°C. Colonies tend to be pinpoint in size after 24 hours of incubation on sheep blood agar. If incubation is continued at room temperature, after 48 hours, gray–white, convex colonies measuring 1 to 2 mm in diameter may be observed.

Genus *Yersinia*. *Yersinia* is the only genus in the *Yersinieae*. Three species, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, were included when the genus was transferred to the *Enterobacteriaceae*. In 1980, three new species were proposed for strains that were former subgroups of *Y. enterocolitica*:[44,65,73,639] *Y. frederiksenii* is the name given to the rhamnose-positive biogroup[639]; *Y. intermedia* is the designation for those atypical strains that ferment rhamnose, raffinose, and melibiose[65]; and *Y. kristensenii* is the name for the previous sucrose-negative, trehalose-positive biogroup of *Y. enterocolitica*.[44] Presently, 17 species are included in the genus *Yersinia*; however, only three (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) have been unquestionably shown to be human pathogens. One species,

Y. ruckeri, a fish pathogen not known to cause human infection, will probably be moved to a new genus.[214] The differential characteristics for these various species of clinical importance are included in Table 6-24.

***Plague*—Y. pestis.** Plague is an infectious disease of antiquity that persists in modern times. *Y. pestis*, which undergoes an obligate flea–rodent–flea life cycle, causes bubonic plague, a rapid and highly fatal zoonotic disease that was responsible for at least three pandemics occurring in the 5th–6th, 8th–14th, and 19th–21st centuries (Box 6-14).[519]

Epidemiology. *Y. pestis* is endemic in various rodents, including rats, ground squirrels, prairie dogs, mice, and rabbits. Two epidemic forms of disease occur: urban plague, which is maintained in the urban rat population, and sylvatic plague, which is endemic in 17 Western states (Fig. 6-7) and is carried by prairie dogs, mice, rabbits, and rats. The organism is transferred from rodent to rodent or from rodent to human by fleas. Human cases have been concentrated in two principal regions in the United States: (1) a

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TABLE 6-24 Differentiation of Species Within the Genus *Yersinia*[a]

Biochemical Test	*Y. pestis*	*Y. pseudotuberculosis*	*Y. enterocolitica*	*Y. frederiksenii*	*Y. intermedia*	*Y. kristensenii*	*Y. aldovae*	*Y. bercovieri*	*Y. mollaretii*	*Y. rohdei*
Indole	–	–	V (50)	+	+	V (30)	–	–	–	–
Ornithine	–	–	+	+	+	+	V (40)	V (80)	V (80)	V (25)
Motility 25–28°C	–	+	+	+	+	+	+	+	+	NA
Fermentation of:										
Sucrose	–	–	+	+	+	–	V (20)	+	+	+
Rhamnose	–	V (70)	–	+	+	–	–	–	–	–
Cellobiose	–	–	V (75)	+	+	+	–	+	+	V (25)
Sorbitol	V(50)	–	+	+	+	+	V (60)	+	+	+
Melibiose	V(50)	V (70)	–	–	V (80)	–	–	–	–	V (50)

[a]Data obtained from references 43, 660, and other sources. All tests were done at 25°C–28°C.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; NA, results not available.

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BOX 6-14

History of Plague

Three major plague pandemics have occurred at intervals of 600 years, each occurring at the end of a major historical epoch: Justinian Plague at the end of Antiquity, Black Death at the end of the Middle Ages, and the Modern Pandemic at the end of the

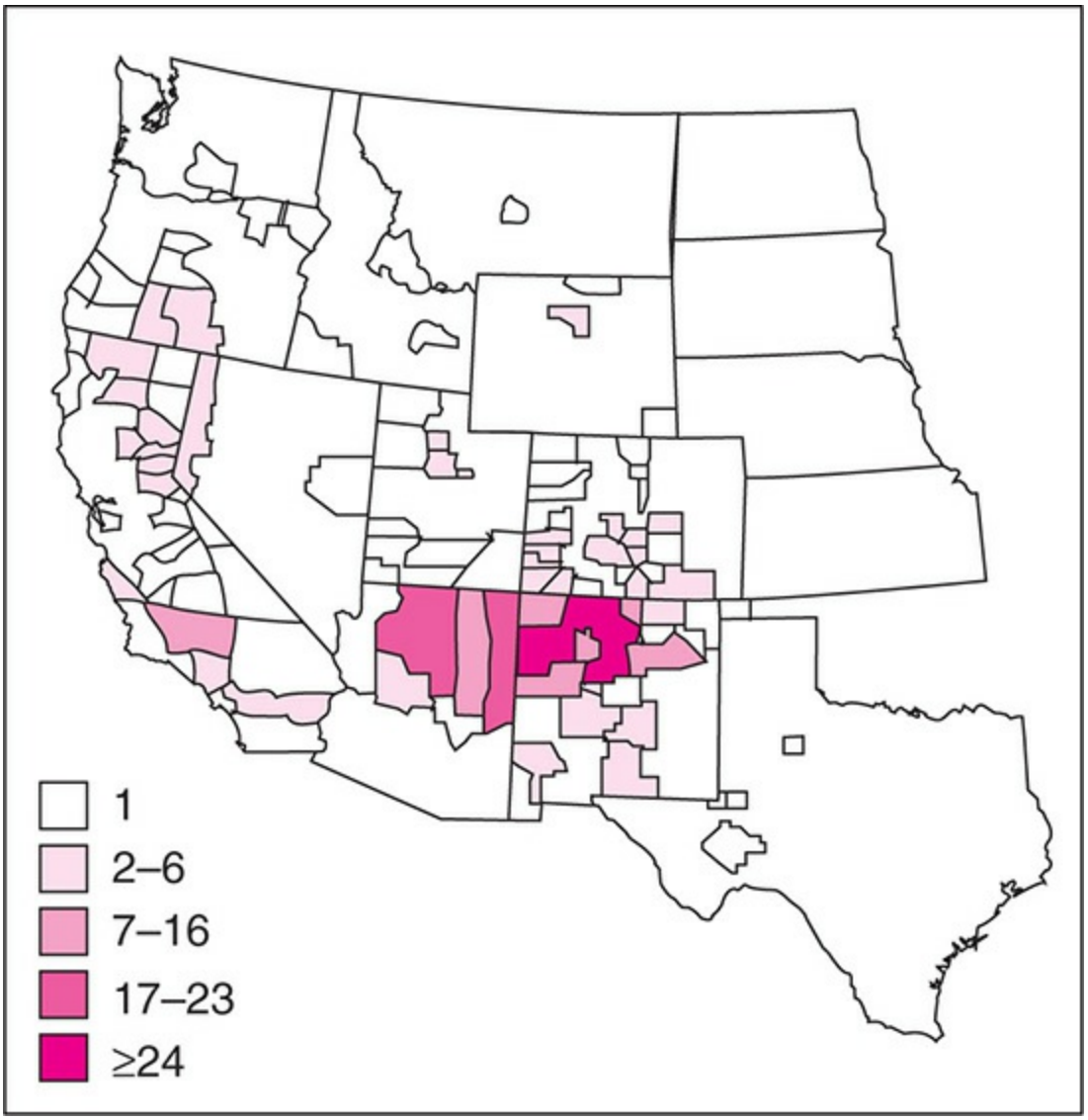
current era.

First Pandemic—The Justinian Plague: (A.D. 541–544) named after the Roman emperor of the 6th century, affected the Mediterranean countries from 532 to 595 and resulted in 100+ million deaths. It began in Egypt and spread through the Middle East and Mediterranean Europe. After that, the 2nd–11th epidemics (A.D. 558–654) occurred in 8- to 12-year cycles, eventually affecting all the "known world."

Second Pandemic—The Black Death: (A.D. 1347–1351) originated in the area of the Black Sea, spread to Sicily, and eventually affected the entire area of Europe up to Great Britain, Scandinavia, and Western Russia. About 17 to 28 million Europeans died from the disease, which represented 30% to 40% of the European population. Epidemics continued in 2- to 5-year cycles from 1361 to 1480, and in less frequent cycles into the 17th century. The second pandemic again encompassed all the "known world."

Third Pandemic—The Modern Pandemic: started in 1855 in Yunnan, a Southwestern province of China. Hong Kong was affected in May 1894; Bombay in 1896; Madagascar in 1898; Egypt, Portugal, Japan, Paraguay, and Eastern Africa in 1899; and Manila, Glasgow, Sydney, and San Francisco in 1900. The outbreaks in India affected more than 10+ million victims between 1896 and 1918, causing the majority of the deaths that occurred during this pandemic. Local epidemics subsequently broke out all over the world until the 1950s, when the pandemic ended.

Plague in the United States—After plague appeared in Hong Kong in 1894, it spread rapidly, reaching the port of San Francisco by boat in 1900. At approximately the same time, it also appeared in Brazil, New Orleans, and New York. Reluctance of California health officials to admit that plague existed in Chinatown resulted in initiation of sylvatic infection. Through sylvatic infection (primarily in the ground squirrel), the infection eventually covered much of the western US (17 States), with the majority of cases occurring in Arizona, California, Colorado, New Mexico, and Oregon. Between 1947 and 1996, 390 cases of plague occurred in the US, resulting in 60 (15.4%) deaths. The most common form was bubonic plague: 327 (84%) cases resulting in 44 (14%) deaths, followed by primary septicemic plague: 49 (13%) cases and 11 (22%) deaths, and primary pneumonic plague: 7 (2%) cases and 4 (57%) deaths. Seven cases and one death were unclassified.[115]



■ **FIGURE 6-7** Number of plague cases, by county—western United States, 1970 to 2002. (Centers for Disease Control and Prevention. Imported plague—New York City, 2002, MMWR Morb Mortal Wkly Rep 2003;52:725–728.)

southwestern area that includes New Mexico, northeastern Arizona, southern Colorado, and southern Utah; and (2) a Pacific Coast region located in California, Oregon, and western Nevada (Fig. 6-7).[108] During 1988 to 2002, a total of 112 human cases of plague were reported from 11 Western states. The majority (97) were exposed in four states (New Mexico, Colorado, Arizona, and California).[122] Approximately 80% of these exposures occurred in peridomestic environments, particularly those that provided abundant food and harbor for flea-infested, plague-susceptible rodents. Household infections occur when people, domestic animals (especially cats), and peridomestic rodents bring fleas into the house, exposing more

persons. Domestic cats permitted to roam freely in areas where plague occurs in rodents are at increased risk for infection and, therefore, increase the risk for peridomestic transmission to humans. Mouth abscesses develop in infected cats, by which they pass the plague directly to people through licking, scratching, or biting.[537] Between 1977 and 1998, 23 human cases of cat-transmitted plague were reported in Western states, and five of those who were ill died.[243]

Travelers can acquire plague in one area and become ill in another area where plague is not endemic (i.e., peripatetic plague). Although rare, peripatetic plague is more likely to

result in fatal outcomes because of delays in seeking treatment or misdiagnosis in areas where healthcare providers might be less familiar with the disease.[122] In the current state of heightened awareness of possible terrorism, peripatetic cases also might be confused with those arising from an intentional release of plague bacteria (Box 6-15).

Clinical Syndromes. Three clinical forms of plague are recognized: bubonic, septicemic, and pneumonic. The septicemic and pneumonic forms are usually secondary to the bubonic form, and the bubonic form is the most common in the United States (see Box 6-14). Bubonic plague is characterized by swelling of cervical, axillary, and inguinal lymph nodes, depending on the location of the portal of entry of the bacteria. Hematogenous dissemination of the bacteria to other organs and tissues may cause intravascular coagulation and endotoxic shock, producing dark discoloration in the extremities (Image Plate 6-5A; Clinical Correlation Box 6-5).

Laboratory Diagnosis. Laboratory confirmation of all forms of plague is performed either by microbiologic methods or by serologic demonstration of antigen or antibody titers. Our colleagues at the Colorado Department of Health have noted that blood cultures are positive in approximately 80% of patients with bubonic plague and 100% of patients with septicemic plague. Gram's stains (Image Plate 6-5B) of bubo aspirates show gram-negative rods in about two-thirds of cases, and Wright–Giemsa stains (Image Plate 6-5C) of peripheral blood smears often reveal the characteristic bipolar staining typical of *Yersinia*. The colonies are slow-growing on ordinary media and are said to have the appearance of beaten copper when viewed under the stereoscope (Image Plate 6-5D). The reaction observed on TSI agar in 24 hours is similar to that seen with *Pasteurella* species (i.e., weak acid production on the slant, with little or no change in the butt). A rapid diagnostic test has been developed that uses a simple dipstick test using monoclonal antibodies to detect the F1 antigen, a protein that is specific to *Y. pestis*. The test produces reliable results within 15 minutes and could help control the disease in developing countries and perhaps speed detection and treatment of the infection during a bioterrorist attack.[134]

BOX 6-15

Epidemiology, Diagnosis, Treatment, and Prevention and Reporting of Plague (*Yersinia pestis*)

Epidemiology

- Plague is usually transmitted to humans by the bite of an infected rodent flea
- Incubation period is 1–7 days for bubonic plague and 1–4 days for pneumonic plague
- Case-fatality rate for untreated bubonic plague is ≥50%
- Domestic pets (i.e., cats and dogs) can carry plague-infected fleas
- Risks include hunting, trapping, cat ownership, and rural residence in areas where plague is endemic
- Person-to-person transmission can occur after contact with a suppurating lesion (bubonic plague) or via respiratory droplets (pneumonic plague)
- Naturally acquired plague typically begins as bubonic plague; intentional release (i.e., terrorism) would manifest chiefly as pneumonic plague

Clinical Findings

- Signs and symptoms include fever, chills, malaise, sore throat, and headache
- A lymphadenitis (bubo) commonly develops; inguinal lymph nodes are affected in 90% of cases
- Infection can progress to shock (septicemic plague) and pneumonia (pneumonic plague)

Laboratory Testing

- Biopolar staining, "safety pin" ovoid, gram-negative organisms are suggestive of plague infection
- Direct fluorescent antibody testing or antigen capture enzyme-linked immunosorbent assay are specific tests
- Confirmatory testing includes culture or a fourfold or greater change in antibody titer

Recommended Treatment

- Primary therapy: streptomycin; alternatively use gentamicin, tetracyclines, or chloramphenicol
- Mortality from bubonic plague is reduced markedly by appropriate therapy
- Patients with primary pneumonic plague are not likely to survive if they do not receive adequate therapy within 18 hours after onset of respiratory symptoms

Prevention and Reporting

- Educate the public about plague symptoms, mode of transmission, and prevention

methods
- Use insect repellents
- Rodent-proof buildings
- Avoid handling rodents or camping near rodent burrows
- Treat dogs and cats in rural areas where plague is endemic with insecticides
- Report plague cases and sick or dead animals to health authorities

See Image Plates 6-5A–D
From reference 122.

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CLINICAL CORRELATION BOX 6-5 Human Plague

When plague bacilli are introduced into a human host, the microorganisms replicate at the initial site of infection, which may be the site of the flea bite, the bloodstream, or the lung, and consequently, three clinical forms of human plague may occur:

1. **Bubonic:** Incubation period of bubonic plague is 7 days or less after a bite from an infected flea. The infecting bacteria spread to regional lymph nodes, usually in the groin (most common), axilla, or neck. An early sign of lymph node infection is the appearance of large, painful swellings called buboes. There is often a sudden onset of fever, chills, weakness, and headache. Within hours, patients may notice intense pain in the anatomic regions with buboes. Patients become prostrate and lethargic and may exhibit agitation, particularly when the bubo is disturbed. Bubonic plague is the mildest form of plague; however, the fatality rate in untreated cases is approximately 75%.
2. **Pneumonic:** This form is usually secondary to the bubonic process, although it may also result from direct exposure to respiratory droplets from another patient with pneumonic plague or from an infected cat.[202,665] There is a shorter (2 to 3 days) incubation period with pneumonic plague. Patients initially have fever and malaise, and then pulmonary signs develop within 1 day. In such patients, cough, chest pain, and hemoptysis are seen, with the sputum usually purulent and containing plague bacilli (Image Plate 6-5B, 6-5C). Pneumonic plague is quickly followed by sepsis and death unless antimicrobial therapy is initiated within the first day. The fatality rate exceeds 90% if the patient is not treated. Persons suspected of having pneumonic plague should be placed in respiratory isolation and reported immediately to public health authorities so that rapid diagnosis, environmental assessments, and control measures can be initiated.

3. **Septicemic:** Direct infection of the bloodstream by the flea bite, or spread of the plague bacillus from lymph nodes to the bloodstream can result in septicemic plague. These patients have positive blood cultures uniformly. Disease is characterized by high fever, delirium, seizures in children, septic shock, and disseminated intravascular coagulation (DIC). Black hemorrhagic splotches (Image Plate 6-5A) develop that gave plague the name "Black Death."

Diagnosis: Plague should be suspected in febrile patients who have been exposed to rodents or other mammals in known endemic areas (Fig. 6-7). The differential diagnosis of plague includes Reye's syndrome, tularemia, bacterial pneumonia, and an acute surgical abdomen. A febrile course in a prostrate patient with a bubo should suggest either plague or tularemia: a bacteriologic diagnosis in such a patient can be made readily with a smear and culture of a bubo aspirate.
Treatment: Primary therapy: streptomycin; alternatively use gentamicin, tetracyclines, or chloramphenicol.[122]

Modified from reference 619.

***Infections Caused by* Y. pseudotuberculosis.** *Y. pseudotuberculosis* is also endemic in a wide variety of animals, including fowl, and is responsible for mesenteric lymphadenitis, particularly in children in whom a clinical disease simulating appendicitis is manifested.[631] A septicemic form of *Y. pseudotuberculosis* infection occurs rarely and has been described mainly in patients with an underlying disorder, such as hepatic cirrhosis, hemochromatosis, or diabetes, with mortality rates as high as 75% despite antibiotic treatment.[395] Rare cases of urinary tract infection and chronic prostatitis due to *Y. pseudotuberculosis* have also been reported.[155,456] The vehicles and sources of *Y. pseudotuberculosis* infection are unknown. By virtue of its similarity to *Y. enterocolitica*, it has been presumed to be a possible food-borne pathogen, but the evidence for this is limited to a few suggestive outbreaks and two larger outbreaks, one in Canada linked to pasteurized milk[469] and one in Finland due to contaminated iceberg lettuce traced to a farm that used untreated water for spray irrigation of the fields.[471] The major biochemical tests that differentiate *Y. pseudotuberculosis* from *Y. enterocolitica* are ornithine decarboxylase, sucrose, and sorbitol. *Y. pseudotuberculosis* is negative for all three, whereas *Y. enterocolitica* is positive (Table 6-24).

***Infections Caused by* Y. enterocolitica.** *Y. enterocolitica* is widely distributed in aquatic and animal reservoirs, with swine serving as the major reservoir for human pathogenic strains.[59] It is the most common species of *Yersinia* recovered from clinical specimens. The portal of entry in humans is the oral digestive route, with

infection occurring in the terminal ileum that anatomically is adjacent to the appendix. The organism adheres to and penetrates the ileum, causing terminal ileitis, lymphadenitis, and acute enterocolitis, with secondary manifestations of erythema nodosum, polyarthritis,[643] and, less commonly, septicemia,[153,233] and endocarditis.[251] Septicemia with *Y. enterocolitica* is almost exclusively associated with patients in iron overload or those being treated with the iron-chelating agent deferoxamine.[59] Ironloaded patients with β-thalassemia are at greatly increased risk for severe yersiniosis even when their body iron burden (as indicated by the serum ferritin level) is only moderately

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elevated and they are not receiving iron-chelating therapy.[8] There are six biovars (1A, 1B, 2, 3, 4, and 5) and over 50 serogroups of *Y. enterocolitica;* however, only five, designated O:1,2a,3; O:3; O:5,27; O:8; and O:9, are generally considered pathogenic for humans.[48] Biogroup 1A lacks the virulence determinants of invasive isolates and is considered to be nonpathogenic.[59] The remaining biogroups are of human origin and can be divided into biogroup 1B, found mainly in the United States, and biogroups 2 to 5, found in Europe and elsewhere. Neubauer et al.[463] have proposed division of *Y. enterocolitica* into two subspecies: *Y. enterocolitica* subsp. *enterocolitica* for biogroup 1B and *Y. enterocolitica* subsp. *palaearctica* for European bioserovars.

Association of* Y. enterocolitica *With Transfusion Reactions. *Y. enterocolitica* was recovered from a unit of donor blood submitted to the University of Illinois Hospital Microbiology Laboratory for culture following a transfusion reaction. Shaking chills and a shock-like syndrome developed in the recipient of a contaminated unit of blood after about 50 mL had been transfused. Similar cases have also been reported elsewhere, illustrating this organism's ability to grow at cold temperatures.[50,106,114,314,611,620,635] Between November 1985 and November 1996, the CDC reported 21 cases of sepsis associated with receipt of transfused *Y. enterocolitica*- contaminated red cells.[114] Investigation of these cases has led to the conclusion that blood contamination resulted from asymptomatic *Y. enterocolitica* bacteremia in the blood donors at the time of donation. Arduino and colleagues[21] have demonstrated that *Y. enterocolitica*, when inoculated into units of packed erythrocytes and stored at 4°C, can proliferate and produce endotoxin after a lag phase of 2 to 3 weeks. It is clear from these reports that *Y. enterocolitica* should be looked for whenever transfusion-associated bacteremia or endotoxemia is suspected. To learn more, the reader is referred to the review article by Wagner and coauthors.[652]

Association of* Y. enterocolitica *With Household Preparation of Chitterlings. In countries where *Y. enterocolitica* has become an important cause of diarrhea, 0:3 is

the predominant serotype, and pigs appear to be the major reservoir for infection.[626] A review of clinical isolates of *Y. enterocolitica* submitted to the Yersinia Reference Laboratory of the CDC from 1970 to 1980 and from 1986 to 1988 showed a shift in the preponderant serotype of *Y. enterocolitica* in the United States from 0:8 to 0:3. This shift coincides with an outbreak of gastroenteritis caused by *Y. enterocolitica* serotype 0:3 in Atlanta from November 1988 to January 1989. The outbreak involved 15 patients (all African American), of which 14 were infants (median age, 3 months). A febrile, diarrheal illness developed in all of them, which was strongly associated with the household preparation of chitterlings (the large intestines of pigs). Although none of the infants had direct contact with the raw chitterlings, in nearly all cases, the persons caring for the infants gave a history of cleaning chitterlings.[378] *Y. enterocolitica* was cultured from unopened containers of chitterlings, from the case households as well as from containers of chitterlings purchased from local supermarkets. In 10 of the 12 exposed households, the chitterlings were prepared for a Thanksgiving, Christmas, or New Year's meal. A survey of a grocery store chain in Atlanta revealed that the sale of chitterlings is largely restricted to the period from October through January, and peaks in November. Data from other surveys support the association between chitterling preparation during the Thanksgiving to Christmas holiday period and cases of *Y. enterocolitica* infection, and suggest that routine screening for *Y. enterocolitica* is warranted at certain hospitals, particularly in Black communities, and especially for children less than 1 year of age.[6,123,379,431] Information on the safe preparation of chitterlings is available at http://www.ph.dhr.state.ga.us/epi/news/oct02/103102.shtml.[123]

Recovery of* Y. enterocolitica *From Clinical Specimens. Most strains of *Y. enterocolitica* will grow on selective enteric agars and will appear as small, lactose-negative colonies on MacConkey and SS agars in 48 hours. In some laboratories, plates of MacConkey agar inoculated with stool specimens suspected of harboring *Yersinia* species are routinely incubated at room temperature. *Y. enterocolitica*, in particular, can best be recovered from stool specimens that are incubated at 25°C. Cold enrichment of highly contaminated specimens, such as feces, by incubating cultures at 4°C for 1 to 3 weeks in phosphate-buffered saline before subculture onto enteric media, also enhances the recovery of *Y. enterocolitica*.[501] Weissfeld and Sonnenwirth[663] reported that pretreatment of stool with 0.5% potassium hydroxide at a ratio of 1:2 for 2 minutes, followed by plating onto enteric agar, resulted in the recovery of the highest number of *Yersinia* isolates. The superiority of cefsulodin–irgasan–novobiocin (CIN) agar for recovery of *Y. enterocolitica* from stool suspensions containing 102 colony-forming units or fewer has been reported by Head and colleagues.[282] The use of coldenrichment methods and specialized culture media, such as CIN agar, for the recovery of *Yersinia* is usually not required because, in

cases of enterocolitis, the organisms are usually found in relatively high concentrations. The use of EMB agar is not recommended because *Y. enterocolitica* is sucrose-positive and will appear as a coliform on this medium. A similar problem may also be encountered with the use of TSI, which also contains sucrose.

***Identification of* Y. enterocolitica.** *Y. enterocolitica* is more biochemically reactive at room temperature than at 37°C. It has been our experience that isolates of *Y. enterocolitica* generally do not give an acceptable identification when set up on the Vitek system or the API 20E at an incubation temperature of 37°C. However, API 20E strips incubated at room temperature do provide an acceptable identification. This finding has been confirmed in recent published reports.[20,592] Neubauer and colleagues compared the API 20E, API Rapid 32 IDE, and PCR-based tests and found that the API 20E provided the highest sensitivity at both the genus and the species level.[464] A later study by the same group found that the Vitek GNI card identified *Yersinia* with 96.3% accuracy to the genus level and 57.4% accuracy to the species level.[388] Although antisera that can actually be used to serotype strains of *Y. enterocolitica* are not readily available, Farmer and colleagues have described four simple tests that can be used to screen for pathogenic serotypes. These tests include the pyrazinamidase test, salicin fermentation–esculin hydrolysis, D-xylose fermentation, and Congo red–magnesium oxalate (CR–MOX) agar used to determine Congo red dye uptake, and calcium-dependent growth at 36°C.[219]

***Antibiotic Susceptibility of* Y. enterocolitica.** *Y. enterocolitica* serogroups 0:3 and 0:9 are able to produce two chromosomally mediated β-lactamases, resulting in resistance

to ampicillin, cephalothin, and carbenicillin. Serotype 0:8 strains are susceptible to ampicillin and variable for carbenicillin and cephalosporins. In vitro testing is not reliable, and the administration of broad-spectrum cephalosporins in combination with an aminoglycoside appears effective for most extraintestinal infections, including septicemia.[249] Fluoroquinolones, alone or with aminoglycosides or extended-spectrum cephalosporins, also appear effective.[249,580] Trimethoprim–sulfamethoxazole shows in vitro efficacy, but has little effect on the clinical course (duration) of *Y. enterocolitica* gastroenteritis.[6,500]

Other* Yersinia *Species. *Y. frederiksenii* is most commonly recovered from fresh water, foods, and nonirrigated soil and has only infrequently been recovered from human specimens. This organism can be recovered from stool specimens using MacConkey or SS agar incubated at 25°C for 48 hours, and cold-enrichment techniques are rarely required. *Y. frederiksenii* is believed to be part of the commensal

flora and does not cause diarrhea. Farmer and coworkers[220] cite a few human isolates, referred to the CDC, that were obtained from wound and respiratory samples.

Y. intermedia may also be recovered by cold enrichment from human fecal specimens, but it is probably not related to intestinal disease. Bottone[58] reviewed the medical literature to 1976, noting 21 case histories of extraintestinal infections with these atypical rhamnose-positive species. Of these cases, eight were from conjunctivitis, and three were from urinary tract infections. Bottone added three more cases, representing urinary tract, conjunctival, and auxiliary abscess infections.

Farmer and coworkers[220] cite six specimens received at the CDC from which *Y. kristensenii* was recovered: four from stools, one from blood, and one from urine. Its pathogenic role has not yet been determined. *Y. aleksiciae* is phenotypically similar to *Y. kristensenii* except for being lysine decarboxylase-positive. Strains have been isolated from the feces of humans, rats, moles, reindeer, and pigs and from dairy products. Their clinical significance is unknown.[604] Four species of *Yersinia* have been described that are all biochemically similar to *Y. enterocolitica*. All are very rare human isolates. *Y. rohdei* has been isolated from dog feces, water, and human feces.[16] *Y. aldovae* (formerly *Y. enterocolitica*-like group X2) has been found in surface water, drinking water, and fish.[43] *Y. mollaretii* (formerly *Y. enterocolitica* biogroup 3A) has been isolated from human feces, drinking water, meat, and raw vegetables.[660] *Y. bercovieri* (formerly *Y. enterocolitica* biogroup 3B) has been reported from human feces, water, soil, and vegetables.[660] Five additional species of *Yersinia* (*Y. entomophaga, Y. massiliensis, Y. nurmii, Y. pekkanenii, Y. similis*) have been described that have been recovered from nonhuman reservoirs and are not associated with human infections.

Tribe *Erwinieae*. The *Erwinieae* are primarily pathogens in plants and only saprophytic in humans. The species of the genus *Erwinia* may be divided into three phylogenetic groups. Cluster I represents the true erwinias and comprises *E. amylovora, E. mallotivora, E. persicinus, E. psidii, E. rhapontici*, and *E. tracheiphila*. The species of cluster II have been transferred to the genus *Pectobacterium* as *P. carotovorum* subsp. *atrosepticum*, *P. carotovorum* subsp. *betavasculorum, P. carotovorum* subsp. *carotovorum, P. carotovorum* subsp. *odoriferum, P. carotovorum* subsp. *wasabiae, P. cacticidum, P. chrysanthemi*, and *P. cypripedii*. The members of cluster III have been classified in a new genus *Brenneria* as *B. alni, B. nigrifluens, B. paradisiaca, B. quercina, B. rubrifaciens,* and *B. salicis*.[280] *Erwinia, Pectobacterium*, and *Brenneria* species cause wilt and rotting diseases on plants, food crops, and trees or are part of the epiphytic flora. There has been one report of a urinary tract infection in an 88-year-old woman caused by *Erwinia persicinus*, which previously had been isolated only from a variety of fruits and vegetables.[485] Recently, a new phytopathogenic species isolated from diseased Erythrina trees was determined to be sufficiently different from *Erwinia, Brenneria,* and *Pectobacterium* to warrant placement in a new genus and has been designated *Samsonia erythrinae*.[621]

Miscellaneous New Genera of Enterobacteriaceae

Table 6-25 is a listing of the more recently described and less common species of *Enterobacteriaceae*. New genus-andspecies designations have evolved from DNA hybridization studies and biochemical characterizations performed on atypical strains referred to the CDC and other reference laboratories for identification and classification. Many of these new genus names have been applied to bacterial strains that, at one time, were designated atypical enteric groups at the CDC. Several enteric groups remain unnamed, but will probably achieve genus status in the future when a sufficient number of strains are gathered.

Identification Characteristics of Newer *Enterobacteriaceae*. The key identifying characteristics of several of the new genera are listed in Table 6-26. Learning the new genera need not be overly difficult if one remembers that most of these bacteria represent atypical strains closely related to well-established groups. For example, the closely related genera *Buttiauxella*[228] and *Kluyvera*[222] will phenotypically present as "citrate-positive *E. coli*"; the specific characteristics by which they can be differentiated are included in Table 6-26. Bacteria belonging to *Cedecea*[225,268] resemble *Serratia* because they are lipase-positive and are resistant to cephalothin and colistin; however, unlike *Serratia*, they do not hydrolyze gelatin or DNA.

Ewingella,[267] *Rahnella*,[312] and *Tatumella*[295] will initially be grouped with *Pantoea agglomerans* because they are lysine-, ornithine-, and arginine decarboxylasenegative. The genera *Yokenella*[363] and *Obesumbacterium*[541,542] are similar to *Hafnia alvei*; *Moellerella*[289] can be considered phenylalanine-negative, lactose-positive *Providencia;* and *Leminorella*[290] can be thought of as phenylalanineand urease-negative *Proteus* species. By using this orientation, identification is made somewhat easier.

The reaction patterns of these new genera and species have been incorporated into the numerical code files of most computer-assisted identification systems. Thus, the names of these genera and species may appear in the computer-generated reports of many commercial systems. In these instances, it may be necessary to visually check the individual reactions for accuracy and assess additional characteristics using Table 6-26 or the expanded Table 6-7. Careful correlation of the

TABLE 6-25 New Genera and Species in the Family *Enterobacteriaceae*

New Designation	Previous Designation	Comments
Arsenophonus nasoniae	New species	Not isolated from humans. Cause of the sun-killer trait in the parasitic wasp, *Nasonia vitripennis*.
Averyella dalhousiensis	Enteric group 58	Clinical isolates have been from wounds and feces.
Budvicia aquatica	"HG group"	H_2S (+). Frequently found in drinking and surface water. Has been isolated from human feces, but is not associated with human disease.
Buttiauxella agrestis	"Group F"	Biochemically closest to *Kluyvera*. Key reactions are IMViC (−, +, −, +), LAO (−, −, +), Sucrose (−). Isolated from water. No human isolates reported.
Buttiauxella brennerae	New species	Isolated from mollusks, water, soil, and humans
Buttiauxella ferragutiae	"Group F" Enteric group 63	Isolated from water and soil.
Buttiauxella gaviniae	"Group F" Enteric group 64	Isolated from mollusks.
Buttiauxella izardii	New species	Isolated from mollusks.
Buttiauxella noackiae	Enteric group 59	Like Pantoea agglomerans except arginine positive. Isolated from mollusks, human sputum and wounds, and food.
Buttiauxella warmboldiae	New species	Isolated from snails.
Cedecea davisae	"Enteric group 15-Davis subgroup"	*Cedecea* resemble *Serratia* because they are lipase (+), and resistant to colistin and cephalothin, but unlike *Serratia* are gelatin and DNase (−). *C. davisae* is most common species. Sputum is the most common source.
Cedecea lapagei	"*Cedecea* species 4"	Isolated from human respiratory tract specimens.
Cedecea neteri	"*Cedecea* sp. strain 002" "*Cedecea* sp. strain 001"	Isolated from blood cultures of patient with valvular heart disease. Isolated from sputum and heart blood at autopsy.
Cedecea sp. 3	"*Cedecea* sp. strain 012"	Toe wound.
Cedecea sp. 5		
Cedecea sp. 6		
Ewingella americana	Enteric Group 40	IMViC (−, +, +, +), LAO (−, −, −). Strains may formerly have been classified as *Enterobacter agglomerans*, but differ by being arabinose (−). Isolated from sputa, wounds, and blood cultures.
Kluyvera ascorbata	Enteric Group 8	Looks like *E. coli* except malonate, esculin, and citrate (+). Dark purple pigment on non–blood-containing media. Most common source is sputum followed by urine, stool, and blood.
Kluyvera cochleae	New species	Isolated from mollusks.
Kluyvera cryocrescens	Enteric Group 8	Similar to *K. ascorbata* except grows and ferments glucose at 5°C.
Kluyvera georgiana	Enteric Group 8 *Kluyvera* sp. group 3	Isolated from human sputum and throat.
Kluyvera intermedia	*Enterobacter intermedium*	Isolated from foot wound, blood, stool, and bile.
Leclercia adecarboxylata	Enteric Group 41 *Escherichia adecarboxylata* *Enterobacter agglomerans* HG XI	IMViC (+, +, −, −), LAO (−, −, −). Yellow pigmented. Resembles E. coli on MacConkey and EMB agars. Isolated from a variety of clinical specimens, food, water, and the environment.
Leminorella grimontii	Enteric Group 57	H_2S (+), TDA (−), LAO (−, −, −), IMViC (−, +, −, +). Isolated from human feces and urine.
Leminorella richardii	Enteric Group 57	Same as *L. grimontii* except for IMViC (−, −, −, −).
Leminorella species 3	Enteric Group 57	
Moellerella wisconsensis	Enteric Group 46	Looks like *E. coli* on enteric media. IMViC (−, +, −, +), LAO (−, −, −), lactose and sucrose (+). Originally isolated from stool cultures in Wisconsin.

Photorhabdus luminescens subsp. *luminescens* *Photohabdus luminescens* subsp. *akhurstii* *Photohabdus luminescens* subsp. *laumondii* *Photohabdus temperata* subsp. *temperata* *Photohabdus asymbiotica*	*Xenorhabdus luminescens* New subspecies New subspecies New species and subspecies New species	Bioluminescent and biochemically inactive. Optimum growth at 25°C. All human clinical isolates belong to *P. asymbiotica*. Colonies are yellow pigmented, produce an unusual hemolytic reaction, and are negative for nitrate reduction. Isolates have been from human wounds and blood.
Pragia fontium		H_2S (+), biochemically similar to *Budvicia*. Most strains from drinking water in Czechoslovakia.
Rahnella aquatilis *Rahnella* species 2 *Rahnella* species 3	Group H2	LAO (−, −, −), nonmotile at 36°C, but motile at 25°C, PAD (wk +), no yellow pigment. May have been identified as *E. agglomerans* in the past. Isolates have been from water, foods, and various human sources.
Shimwellia pseudoproteus	*Flavobacterium proteus* *Obesumbacterium proteus* biogroup 2	No human isolates reported. Common brewery contaminant. Grows slowly when incubated at 36° C, making it difficult to identify.[541,542]
Tatumella ptyseos	Group EF-9	Pinpoint colonies, slow growing, relatively inert. Motile at 25°C, but nonmotile at 35°C. Flagella are polar, lateral, or subpolar rather than peritrichous. Large zones of inhibition are formed around disks containing penicillin (10 U). PAD very slow (wk +). Isolated from human clinical specimens, particularly sputum.
Trabulsiella guamensis	Enteric Group 90	H_2S (+) and biochemically similar to *Salmonella*. IMViC (−, +, −, +), Lysine (+), Arginine (50% +), Ornithine (+). Isolates have been from soil and human feces. No evidence that it causes diarrhea.
Xenorhabdus beddingii *Xenorhabdus bovienii* *Xenorhabdus japonicus* *Xenorhabdus nematophilus* *Xenorhabdus poinarii*	*Xenorhabdus nematophilus* subsp. *beddingii* *Xenorhabdus nematophilus* subsp. *bovienii* *Achromobacter nematophilus* *Xenorhabdus nematophilus* subsp. *poinarii*	Has been isolated only from nematodes.
Yokenella regensburgei	*Koserella trabulsii* Enteric Group 45 Japanese NIH biogroup 9 atypical Hafnia *Hafnia* species 3	Biochemically similar to *Hafnia alvei*. Differs by being colistin-resistant and VP (−). Isolated from blood, wounds, throat, sputum, feces, and water.
Enteric group 60 Enteric group 68 Enteric group 137		Biochemically inactive. Recovered from urine and sputum. DNase (+) but otherwise biochemically different from *Serratia*. Clinical isolates have been from urine. Closely related to *Citrobacter farmeri* and *Citrobacter amalonaticus*. Isolated from sputum, urine, and wounds.[656]

IMViC, indole, methyl red, Voges–Proskauer, citrate; LAO, lysine, arginine, ornithine; MIO, motility, indole, ornithine; PAD, phenylalanine deaminase; HG, hybridization group; (+), >90% of strains positive; (−), >90% of strains negative; (v), variable.

biochemical activity with the growth patterns and appearance of colonies on agar media is usually enough to make accurate identifications. It cannot be overemphasized that students and microbiologists must retain a fundamental orientation to the morphology, physiology, and biochemistry of bacteria if accurate identifications are to be made.

Clinical Significance of Newer *Enterobacteriaceae.*

The seemingly endless reclassification and changes in bacterial taxonomy and the

frequent addition of new genera and species might be discouraging for microbiology students and instructors alike. However, Farmer and associates conducted a study of scores of unclassified biogroups submitted for identification at the CDC and have brought order out of disarray in a landmark report that summarizes all of the old and new genera of *Enterobacteriaceae* known as of January 1985.[220] As these investigators point out, up to 95% of all *Enterobacteriaceae* recovered in clinical laboratories are *E. coli, K. pneumoniae*, and *Proteus mirabilis;* over 99% of the isolates belong to only 23 species, leaving less

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TABLE 6-26 Differentiating Characteristics of New *Enterobacteriaceae*

Genus	Genus Characteristics	Species and Differentiating Characteristics					
			B. agrestis	*Kluyvera ascorbata*	*Kluyvera cryocrescens*		
Buttiauxella Ferragut[228] (*Group F, Gavini 1976*)	Indole –	Indole	–	+	+		
	MR/VP +/–	Lysine	–	+	V (23)		
	Citrate +	Ascorbate	–	+	–		
	Lysine –	Glucose (5°C)	+	–	+		
	Arginine –	Sucrose	–	+	+		
	Ornithine +						
	Sucrose –						
			C. davisae	*C. lapagei*	*C. neteri*	*SPEC 3*	*SPEC 5*
Cedecea Grimont[268] (Enteric group 15)	ONPG +	Ornithine	+	–	–	–	V (50)
	MRVP + V (50–80)	Sucrose	+	–	+	V (50)	+
	Citrate +	Sorbitol	–	–	+	–	+
	Esculin +	Raffinose	–	–	–	+	+
	Lipase (corn oil) +	Xylose	+	–	+	+	+
	DNAse –	Melibiose	–	–	–	+	+
	Gelatin –	Malonate	+	+	+	–	–
	Colistin R						
	Cephalothin R						
	Cephalothin R						
			E. americana	*P. agglomerans*			
Ewingella Grimont[267] (Enteric group 40)	Indole –	Arabinose	–	+			
	MR/VP +/+	Xylose	V (15)	+			
	Citrate +	Yellow pigment	–	V (75)			
	Lysine –						
	Arginine –						
	Ornithine –						
			K. ascorbata	*K. cryocrescens*			
Kluyvera Farmer[222] (Enteric group 8)	Indole +	Ascorbate	+	–			
	MR/VP +/–	Glucose (5°C)	–	+			
	Citrate +	Lysine	+	V (23)			
	Malonate +						
	Esculin +						
			L. adecarboxylata	*E. coli*	*P. agglomerans*		
Leclercia Tamura et al.[622] (*Escherichia adecarboxylata*, Enteric group 41)	Indole +	Lysine	–	+	–		
	MR/VP +/–	Adonitol	+	–	–		
	Citrate –	Malonate	+	–	V		
	Lysine –	Yellow pigment	+	–	+		
	Arginine –						
	Ornithine –						
			L. grimontii	*L. richardii*	Proteus species		
Leminorella Hickman-Brenner[290] (Enteric group 57)	H_2S +	Methyl red	+	–	+		
	Phenylalanine –	Citrate	+	–	–/V (15–65)		
	Mannose –	Dulcitol	V (83)	–	–		
	Arabinose +	Phenylalanine	–	–	+		
	Xylose +	Urease	–	–	+		
	L/A/O –/–/–	Arabinose	+	+	–		
			M. wisconsensis	*Providencia species*			
Moellerella Hickman-Brenner[289] (Enteric group 46)	Indole –	Phenylalanine	–	+			
	MR/VP +/–	Lactose	+	–			
	Citrate +	Sucrose	+	V (15–50)			
	Lysine –	ONPG	+	–			
	Arginine –	Tyrosine	–	+			
	Ornithine –						
	Phenylalanine –						
	Colistin R						

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			R. aquatilis	*P agglomerans*	
Rahnella Izard et al.[312] ("Group H2")	Indole − MR/VP +/+ Citrate + Urea − Phenylalanine + wk L/A/O −/−/− Motility − (36° C)/+ (25°C)	Motility (36°C) Phenylalanine Yellow-pigmented	− + −	+ V (20) V (75)	
			S. pseudoproteus	*Hafnia alvei*	*H. alvei 1*
Shimwellia (*Obesumbacterium*) Priest[541,542]	Indole − MR/VP V (15)/− Citrate − Lysine + Ornithine + Motility −	Mannitol Salicin Arabinose	− − −	+ V (13) +	V (55) V (55) −
			T. ptyseos	*P. agglomerans*	
Tatumella Hollis[295] (Biogroup EF-9)	Indole − MR/VP −/−/(+ Coblenz) L/A/O −/−/− Phenylalanine + wk Sucrose + Gelatin −	Mannitol Phenylalanine Motility (36°C) Penicillin	− + − S	+ V (20) + R	
			T. guamensis	*Salmonella* Subgroup 4	*Salmonella* Subgroup 5
Trabulsiella McWhorter et al.[425] (Enteric group 90)	H_2S + Indole V (40) MR/VP +/− Citrate + Lysine + Arginine V (50) Ornithine + KCN +	Dulcitol Lactose ONPG Malonate Growth KCN Mucate Ferm D-Sorbitol	− − + − + + +	− − − − + − +	+ − + − + V (85) +
			Y. regensburgei	*H. alvei*	
Yokenella Kosako et al.[362] (*Koserella trabulsii* Enteric group 45)	Indole − MR/VP +/− Citrate + Lysine + Ornithine + Cellobiose + Melibiose +	VP Citrate Melibiose Colistin R	− + + +	+ − − −	

than 1% as the incidence of recovery for the several newly designated species. Thus, the bacteria listed in Table 6-25 are rarely encountered in most clinical laboratories; however, as Dr. Farmer has also stated, there are at least three reasons why these new species of *Enterobacteriaceae* are important to clinical microbiologists: (1) Some species cause serious human infections; (2) others occur in clinical specimens, but their causative role in disease is uncertain; and (3) many are biochemically similar to well-established species and thus can cause problems in identification. The clinical significance of the newer species that have been recovered from human clinical

specimens is summarized as follows:

Averyella dalhousiensis is the proposed name for CDC enteric group 58.[330] *A. dalhousiensis* has been most frequently isolated from traumatic injuries, fractures, and wounds and rarely from feces.[220] One case of septicemia associated with infection of a central venous catheter has been described.[330] *A. dalhousiensis* is not included in the databases of commercial identification systems and is most commonly misidentified as *Enterobacter* spp., *Salmonella* spp., *Serratia* spp., *Kluyvera* spp., or *Escherichia* spp.[330] There is little known about the epidemiology, pathogenesis, or clinical significance of this organism. Phenotypic reactions are listed in Table 6-7.

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The genus *Buttiauxella* now contains seven species (see Table 6-25) that occur frequently and abundantly in the intestines of snails, slugs, and other mollusks.[449] A few strains have been isolated from unpolluted soil and drinking water, surface water, sewage, soil, and fecal samples, but have not been isolated from primary sterile clinical specimens.[449] Two clinical isolates of *B. gaviniae*-like organisms have been reported from urine and leg wound specimens in Belgium. The isolates were biochemically indistinguishable from *B. gaviniae,* but 16S rRNA gene sequencing was indecisive.[174]

Cedecea davisae has been isolated from respiratory specimens,[31] blood,[1,13,165,411,516] a scrotal abscess,[30] cutaneous ulcer,[277] and an oral ulcer.[418] *C. neteri* has been reported as a cause of bacteremia.[11,225] *C. lapagei* has been reported to cause lung tissue infection,[151] peritonitis,[170] pneumonia,[681] and bacteremia.[162]

Ewingella americana was described in 1983 and named in honor of the American bacteriologist, William Ewing.[267] The original strains were from human clinical specimens, including sputum, blood, throat, toe and thumb wounds, urine, and stool.[220,267] *E. americana* has been implicated in bacteremia,[182,524] catheter-related bacteremia,[408] an outbreak of nosocomial bacteremia,[523] and an outbreak of pseudobacteremia associated with nonsterile blood collection tubes.[422,423,424] It has also been associated with wound colonization,[40] conjunctivitis,[283] and peritonitis in a patient undergoing peritoneal dialysis.[340]

The genus *Kluyvera* presently contains four species (see Table 6-25).[449,511] The original isolates of *Kluyvera* species were from human clinical specimens and the environment. The most common human sources have been sputum, followed by urine, stool, throat, and blood.[222] Environmental sources noted have been sewage, soil, kitchen food, water, milk, and a hospital sink.[222] There are only a few reports of serious infections with *Kluyvera* species that involved the urinary tract,[636] the

gallbladder,[632] the gastrointestinal tract,[10,215] and soft tissue of the forearm following a cut from a garbage can.[402] In addition, cases of catheter-related bacteremia[676] and mediastinitis and bacteremia following openheart surgery[595] have been reported. *K. intermedia* (formerly *E. intermedius* and Group H1)[650] had not been known to occur in humans until 1987 when Prats et al.[539] reported four strains of *K. intermedia* (*E. intermedius*) that had been isolated from a foot wound, blood, stool, and bile. More recently, O'Hara et al.[486] reported the isolation of *K. intermedia* from the gallbladder of a patient with cholecystitis that also grew group D *Streptococcus*, and coagulase-negative *Staphylococcus*.

The clinical significance of *L. adecarboxylata* is uncertain, but human isolates from blood, sputum, urine, stool, and wounds have been reported.[164,167,173,178,234,397,420,435,498,560,591,622,629] There have been at least two reports of *L. adecarboxylata* bacteremia in premature infants.[453,461] At the University of Illinois Hospital, *L. adecarboxylata* was found colonizing the endotrachea of a neonate and in a cystoscopic urine specimen from a patient in renal failure. In both cases, the organism was isolated along with other pathogen species; therefore, an etiologic role could not be established. In a third patient, *L. adecarboxylata* was recovered in pure culture from the blood of a patient in chronic renal failure receiving hemodialysis. Stock and colleagues studied the natural antimicrobial susceptibility patterns and biochemical profiles of *L. decarboxylata,* and reported that strains were naturally sensitive to tetracyclines, aminoglycosides, all but two β-lactams, quinolones, folate-pathway inhibitors, chloramphenicol, nitrofurantoin, and azithromycin. They were naturally resistant to penicillin G, oxacillin, erythromycin, clarithromycin, ketolides, lincosamides, streptogramins, linezolid, glycopeptides, rifampicin, and fosfomycin.[616]

Leminorella has been isolated primarily from stool and urine.[290] One case of spontaneous peritonitis caused by *L. grimontii* has been reported.[163] Blekher and associates[51] reviewed the medical records of 14 patients from which *Leminorella* spp. were isolated and classified 43% of the cases as definite pathogens, 29% as probable pathogens, and 21% as possible pathogens. Clinical syndromes included urinary tract infections (six patients), surgical site infection (three), and primary bacteremia, peritonitis, respiratory tract infection, and soft tissue infection in one patient each. In one case of asymptomatic bacteriuria, the isolate had no clinical significance.[51] *Leminorella* spp. appear as lactose-negative colonies on primary plating media and gives an alkaline slant and a weak acid reaction with H_2S in the butt of TSI after 48 hours. Similar to *Proteus, Leminorella* species are H_2S-positive, D-mannose-negative, and tyrosine-positive, but unlike *Proteus*, they are ureaand phenylalanine-negative and L-arabinose-positive.

Moellerella wisconsensis was originally found in human stool samples, mainly from the state of Wisconsin.[289] It has also been isolated from water and animals and from clinical specimens other than stool, such as the gallbladder[476,674] and a bronchial aspirate.[654] However, the majority of isolates have been from human feces, and there is evidence that *M. wisconsensis* is associated with human diarrhea.[320] On MacConkey agar, colonies appear bright red with precipitated bile around them; thus, they are indistinguishable from *E. coli* colonies.[289] Stock and colleagues report that *M. wisconsensis* is naturally sensitive to doxycycline, minocycline, all aminoglycosides, numerous β-lactams, all fluoroquinolones, folatepathway inhibitors, chloramphenicol, and nitrofurantoin.[617]

Photorhabdus species are the only terrestrial bioluminescent bacteria (bacterial bioluminescence is seen primarily in marine species). The classification within the genus is complex, with three currently recognized species: *P. luminescens* (formerly, *Xenorhabdus luminescens*), *P. temperata*, and *P. asymbiotica*.[54,231] Several subspecies are recognized (Table 6-25). *Photorhabdus* spp. inhabit the gut of some insectpathogenic nematodes, where they form a symbiotic relationship. Nematode species of this type are able to invade the larvae of susceptible insects and release *Photorhabdus* spp. The bacteria proliferate and kill the insect larvae.[250] Insectpathogenic nematodes harboring *Photorhabdus* spp. are used as biopesticides in a number of countries, including the United States and Australia. A total of 12 human infections with *Photorhabdus* spp. have been reported from the United States and Australia, most often causing skin and soft tissue infections and rarely bacteremia.[223,250,512] Colonies are formed on blood agar plates (BAP) after 24 to 48 hours at both 35°C and at room temperature, with a tendency to "swarm."[250] The defining characteristic is the presence of faint luminescence, which can be seen with the naked eye under conditions of total darkness. Gerrard and colleagues have observed that it is

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critical to allow the observer's eyes to adjust to the darkness for 10 minutes.[250] *Photorhabdus* spp. do not currently appear in the databases for either the MicroScan Rapid or overnight panels, or the Vitek GNI+ cards, which leads to misidentifications when using these systems.[250]

The genus *Rahnella* consists of three closely related species, *R. aquatilis* and two genomospecies that cannot be phenotypically differentiated from *R. aquatilis* and therefore are given the vernacular names *Rahnella* genomospecies 2 and *Rahnella* genomospecies 3.[71] The natural habitat of *Rahnella aquatilis*, as its name implies, is

water, and most isolates in the original CDC collection were from water, except two isolates from humans (one from a burn wound and the other from the bronchial washing of a patient with HIV).[278] Of the few case reports of *R. aquatilis* in the literature, most describe infections in immunocompromised patients. In addition to the CDC isolates, others have reported the isolation of this organism from sputum (in a patient with chronic lymphocytic leukemia and emphysema),[143] the urinary tract (in a patient with a renal transplant),[14] from a surgical wound possibly due to a nosocomial source,[413] and from blood of immunocompromised[88,90,242,260,298] and immunocompetent[133] patients. *R. aquatilis* has also been isolated from vegetables (primarily carrots) purchased in supermarkets[276] and has been identified as the causative agent of a smoky/phenolic odor (due to the formation of guaiacol) in spoiled, refrigerated chocolate milk.[329]

Of the original strains of *Tatumella ptyseos* studied at the CDC, 30 were from sputum, 6 from throat cultures, 3 from blood, and 1 each from a tracheal aspirate, a feeding tube, a pharynx, a stool, and urine.[295] One case of *T. ptyseos* obtained from the blood culture of a neonate with presumed sepsis has been reported.[624] There are three striking differences between *T. ptyseos* and other members of the *Enterobacteriaceae*: (1) strains produce a large zone of inhibition around penicillin; (2) it has a tendency to die on some laboratory media, such as blood agar, within 7 days; and (3) usually only one flagellum (either polar, subpolar, or lateral) is observed per cell.[295]

Trabulsiella guamensis, formerly known as enteric group 90, is H_2S-positive and biochemically resembles *Salmonella* subgroups 4 and 5.[425] Strains have been isolated from vacuum cleaner dust, soil, and human feces; however, there is no evidence that it causes diarrhea. Its main interest to clinical microbiologist may be its possible misidentification as a strain of *Salmonella* (see Table 6-26).

Yokenella regensburgei was originally identified as NIH biogroup 9 by the NIH in Japan[363] and later found to be identical to "*Koserella trabulsii*" (enteric group 45) named by workers from the CDC.[288,362] Because the name *Y. regensburgei* has priority over "*K trabulsii*" by virtue of prior publication, the use of the later name has been discontinued. In the original reports, *Y. regensburgei* had been recovered from the intestinal tracts of insects and well water, as well as human clinical specimens, including wounds of the limbs, the upper respiratory tract, urine, feces, and knee fluid.[288,363] More recently, Abbott and Janda have reported the recovery of *Y. regensburgei* from the blood of a patient with a transient bacteremia and from a knee wound of a patient with a diagnosis of septic knee.[3] The organism most closely resembles *H. alvei,* from which it must be differentiated (see Table 6-26).

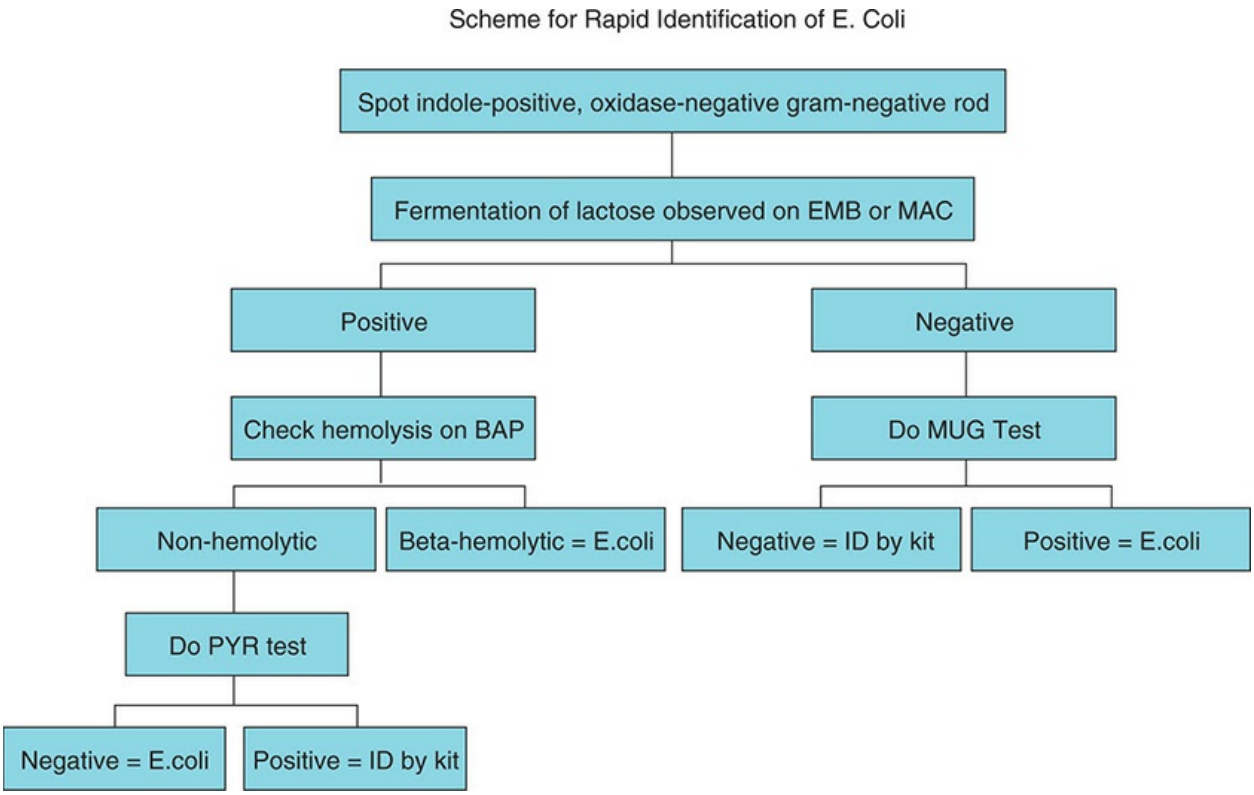
Quick Screening Methods for Rapid Identification

E. coli, the bacterial isolate most frequently recovered in clinical laboratories, is often presumptively identified if an oxidase-negative, lactose-fermenting, dry colony on Mac-Conkey agar gives a positive spot indole reaction (when tested on a colony growing on noninhibitory media, such as blood agar), particularly if the organism has been recovered in pure culture.[34] The spot indole test is also used in many laboratories for rapid speciation of swarming *Proteus* from primary isolation plates.[35] A rapid (2-minute) spot urease test has been described by Qadri and colleagues.[544] This test might be used to separate possible stool pathogens that require further biochemical testing from the nonpathogenic *Proteus–Providencia–Morganella* group. Taylor and Achanzar have reported using the catalase test (with 3% H_2O_2) as an aid in the identification of *Enterobacteriaceae*.[628] They observed vigorous catalase reactions with *Serratia, Proteus*, and *Providencia;* moderate reactions with *Salmonella, Enterobacter*, *Klebsiella*, and rare *Escherichia;* and weak reactions with *Shigella* and most *Escherichia*. They report using the rapid catalase test for screening suspicious colonies on enteric media that mimic stool pathogens. Colonies of *Serratia, Proteus, Providencia*, or *Pseudomonas* are quickly eliminated by vigorous catalase reactions, in contrast to salmonellae, which give weak-to-moderate reactions, or shigellae, which are negative and, therefore, are flagged for further workup. Chester and Moskowitz[140] have reported on the catalase activity of many of the newer members of the *Enterobacteriaceae* and further advocate the use of catalase activity as a rapid supplemental test. Mulczyk and Szewczuk[448] suggested that the test for L-pyrrolidonyl peptidase (PYR) is of great value in differentiating between *Salmonella* and *C. freundii* complex strains. Other authors have also noted the value of using the PYR test for separating *Salmonella* and *E. coli* strains (both negative from *Citrobacter* spp. (PYR+).[131,307] York and colleagues[683] have proposed an algorithm for rapid identification of *E. coli* based on spot tests for oxidase, indole, and PYR and observation of lactose fermentation on MacConkey or EMB and presence or absences of β-hemolysis on BAP (Fig. 6-8). Their algorithm permitted identification of the majority of suspected *E. coli* strains with an accuracy of greater than 99% and a 75% reduction in cost of reagents and technologist time, as well as a decrease in the time to reporting. Clearly, any of these approaches has validity in an era of cost-containment and the desire to receive test results quickly.

Commercial Screening Kits

Several commercial companies have marketed rapid detection kits for the identification of *E. coli*: E.COLI SCREEN, MUG Disk, and BactiCard *E. coli* (Remel, Lenexa,

KS); Lyfo-Kwik OMI (MicroBioLogics, St. Cloud, MN); and ColiScreen (Hardy Diagnostics, Santa Maria CA). These test systems are based on the finding that the majority of strains of *E. coli* are rapidly indole-, ONPG-, and MUG (4-methylumbelliferyl-β-D-glucuronide)-positive.[198,349] The hydrolysis of MUG releases 4-MEU, which is highly fluorescent when viewed under long-wave ultraviolet (UV) light. An interesting use of



■ **FIGURE 6-8** Identification scheme for the separation of *E. coli* from other indole-positive, oxidase-negative rods. BAP, blood agar plate; EMB, eosin methylene blue; MAC, MacConkey agar; MUG, 4-methylumbelliferyl-β-D-glucuronide; PYR, L-pyrrolidonyl-β-naphthylamide; ID, identification. (Modified from York MK, Baron EJ, Clarridge JE, et al. Multilaboratory validation of rapid spot tests for identification of *Escherichia coli*. J Clin Microbiol 2000;38:3394–3398.)

the MUG test is to screen isolates of *E. coli* for the detection of serotype O157:H7 strains, which are the Shiga-like, toxinproducing strains associated with hemorrhagic colitis. This specific serotype is both sorbitoland MUG-negative.

Also available is the RapID SS/u System (Remel), consisting of a set of 12 single-substrate chromogenic tests selected to identify rapidly the bacteria most commonly encountered in urinary tract infections (*E. coli, Proteus* species, *Klebsiella* species, *Serratia* species, *Enterobacter* species, *Pseudomonas* species, coagulase-negative staphylococci, enterococci, and others). Answers are available in 30 minutes to 2 hours, depending on the system used and the biochemical activity of the strain being tested. Another system called OMP and NGP Wee-Tabs (KEY Scientific Products, Round Rock, TX) consists of two substrate tablets that together provide eight enzymatic tests, which, combined with urease, will identify approximately 95% of the *Enterobacteriaceae*. The tablets are rehydrated in a bacterial suspension, with results available in 2 to 4 hours. Hardy Diagnostics (Santa Maria, CA) offers the EnteroScreen4, which is a single tube screening system for use in screening stools for *Salmonella* and *Shigella* species. This system detects lysine deaminase, lysine decarboxylase, H_2S, and urea in a single inoculation onetube configuration.

Chromogenic Agar Media

Chromogenic agar contains artificial substrates (chromogens) that when hydrolyzed by specific microbial enzymes produce colored compounds (Fig. 6-6). The appearance of various colony types on chromogenic agar are illustrated in Image Plates 6-5E through H. Kilian and Bülow were the first to use selective chromogenic medium for the direct identification of *E. coli* in primary culture of urine.[350] Currently, several chromogenic media allowing the direct identification of *E. coli*, *P. mirabilis*, and *Enterococcus* spp. are marketed: CPS ID2 (bioMérieux, Hazelwood, MO),[25,93,137,284,419,470,556,579,670] CHROMagar Orientation (BD Diagnostics, Sparks, MD; Hardy Diagnostics, Santa Maria, CA),[25,93,189,284,302,428,489,575,579] Rainbow UTI (Biolog, Hayward, CA),[53,93,582] and Chromogenic UTI (Oxoid, Basingstoke, United Kingdom).[25,93,137] In evaluations of multiple formulations of these media, the detection rates and identification rates are very similar.[25,93,137] In one study, the use of chromogenic agar allowed a >50% reduction in inoculation time and a >20% reduction in workup time.[189] To extend identification to additional species, colony characteristics on chromogenic agar can be combined with easy-to-use confirmatory spot tests that can be performed in seconds either on paper strips or directly on the agar medium.[489] One cautionary note, however, is that inocula taken from chromogenic agar plates may not be suitable for susceptibility testing with certain automated test systems. Reisner and Austin showed that susceptibility testing directly from CPS ID2 medium resulted in low error rates for all antimicrobial agents tested using the Vitek Legacy System (bioMérieux, Hazelwood, MO).[556] Laboratories using chromogenic media should check with their instrument manufacturer to determine the compatibility of using

inocula from chromogenic agar for antibiotic susceptibility testing on the system in use in their laboratory.

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Classic Identification Systems

Systems for the identification and naming of microorganisms are either computer-assisted or manual. Before discussing the derivation and applications of numeric coding systems, two manual bacterial identification schemes that are still in use will be reviewed: (1) the cross-hatch or checkerboard matrix, and (2) the branching or dichotomous flow charts.

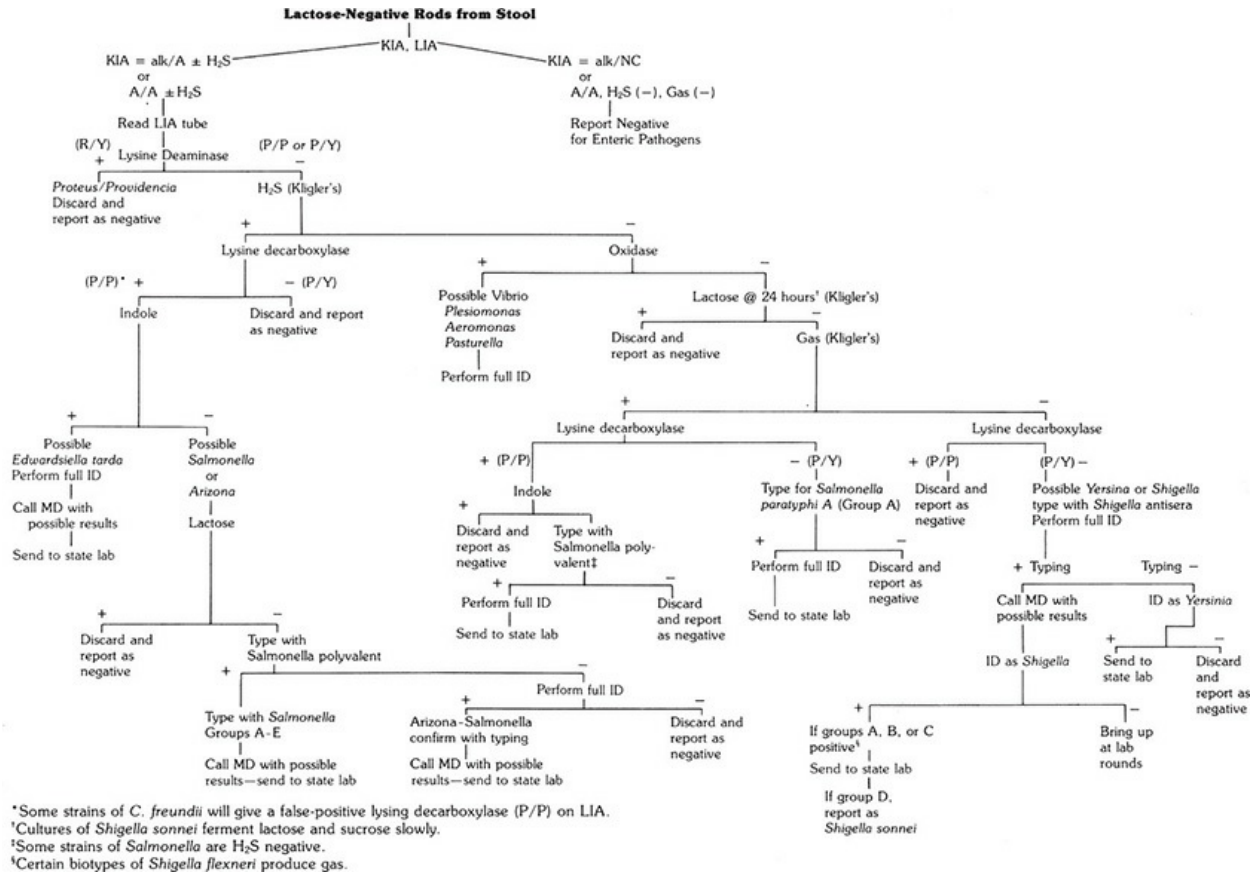
Checkerboard Matrix

Table 6-7 is a comprehensive identification table originally formulated by Farmer and colleagues for use at the CDC to identify all named species, biogroups, and unnamed enteric groups of the *Enterobacteriaceae*. This table is a classic example of the checkerboard matrix and demonstrates its advantages and disadvantages. The matrix is large, with 48 biochemical characteristics for 37 genera, 148 species, biogroups, and unnamed enteric groups. The numbers in the intersecting squares represent the percentage of strains that are positive or reactive against the various biochemical tests (listed in the vertical columns). A reaction is generally considered positive if 90% or more of the strains are reactive, negative if 10% or fewer of the strains fail to produce a result, and variable if 11% to 89% of reactions are positive. The ability to determine both the positive and negative reactions for the various characteristics being measured in this type of identification system results in a high degree of diagnostic accuracy. The major disadvantage of the checkerboard matrix is the tedium involved in matching point by point the various reactions against those derived from the test media and constructing the patterns that best match with a specific genus, species, or biogroup.

Branching Flow Diagrams

Flow diagrams were designed to reduce the tedium of reading the checkerboard matrices and to facilitate the likely bacterial identification by tracing a series of positive and negative branch points in a dichotomous algorithm (Fig. 6-8, 6-9). With the advent of automated instruments and packaged identification systems that rely on computer-assisted analyses of the various reactions of the characteristics being measured, flow diagrams are now used less frequently in clinical laboratories. One problem with flow diagrams has been the potential for inaccuracy if the reaction at a given branch point is

either aberrant (i.e., not typical for the species), misinterpreted, or the result of the reactions of a mixed culture. Many flow diagrams are constructed to repeat some species names at several junctures to accommodate reactions that may be less than 100% or in the variable category (Fig. 6-9). However, this built-in protection



■ **FIGURE 6-9** Schematic for screening of enteric pathogens from stool using KIA and modified LIA.

does not always apply for reactions that are misinterpreted, either by an automated instrument's detection system or by the human eye.

A modification of this approach is being used in many microbiology laboratories for the preliminary screening of commonly encountered bacterial species that are associated with specific infectious disease syndromes. York and colleagues[683] have presented a simple scheme for the rapid identification of *E. coli* from EMB or MacConkey agar (Fig. 6-8). To validate this scheme, five laboratories sequentially collected 1,064 fresh, clinically significant strains with the core criteria of indole-

positive, oxidase-negative, nonspreading organisms on BAP. Of the 1,064 strains tested, 1,000 were *E. coli* and 64 were non–*E. coli*. Using the scheme, three non–*E. coli* isolates were identified as *E. coli,* for an error rate of 0.3%. A total of 13 kit identifications, 657 PYR tests, and 113 MUG tests were needed to identify 1,000 *E. coli* strains with the algorithm.[683] The authors showed that the use of this rapid system saves laboratory resources, provides timely identifications, and yields rare misidentifications. The Clinical and Laboratory Standards Institute (CLSI) has produced a guideline (M35-A2, *Abbreviated Identification of Bacteria and Yeast: Approved Guideline–Second Edition*) that describes tests that can be used to identify a number of aerobic gram-negative rods and gram-positive cocci, a number of commonly isolated anaerobes, and three species of yeast.[149] Because all methods described in the guideline yield results that are highly accurate—attaining a level of reliability equal to or better than current conventional biochemical tests, multitest systems, and automated systems—the resulting identification does not require the adjective "presumptive" qualifying the resulting identification. The genus and species (or genus only, as designated by the algorithm) can be reported without any qualifying wording.[36] Laboratory workers are referred to the CLSI document for methodologic details about the tests chosen and for more information than is presented here.[149]

An algorithm using a KIA and LIA tube (supplemented with tryptophan for detection of indole) has been used for many years in one of our laboratories for the screening of enteric pathogens from stool (Fig. 6-9). Definitive biochemical and serologic procedures are performed only on isolates with reactions that are presumed to be positive, saving the cost of performing more expensive kit identifications.

In addition to saving the cost of setting up commercial test panels, these dichotomous schemes coupled with a few inexpensive biochemical tests and observations offer an additional advantage over automated systems in that they require thought, reasoning, and knowledge on the part of the student or microbiologist, who is not reduced to being a passive observer.

Computer-Aided Schemes

WIP (Web ID Program) is a free online program (developed by Adam Schreckenberger © 2014) that can be used to identify bacteria without the use of downloaded software. The WIP application works on any PC, Apple, or smartphone system with an active Internet connection. Because the program runs within an Internet browser, it requires no additional downloads. One merely needs to enter available phenotypic characteristics into the online Web form to calculate a likelihood probability and modal score. If the likelihood score is less than 95%, a list of phenotypic tests that distinguish the top two candidates will be displayed; likewise, if the modal score is poor, a list of input results that do not match well to the top

candidate prediction will be shown. The program provides statistical selection of unknown bacterial isolates through comparisons to phenotypic characteristic matrices of known strains. The program has three major functions: (1) the identification of an unknown isolate, (2) the selection of additional tests to distinguish between possible strains if identification is not achieved, and (3) the storage and retrieval of results. An assortment of WIPs, each of which targets a specific subset of gram-negative bacteria, is available on www.pschreck.com by clicking the appropriate WIP tab of choice on the site toolbar. This collection is referred to as the AID (App Identification) Cloud. Each WIP references a specific characteristic matrix that was created either from a published table in the literature or from the author's own data. References for each of the four tables are as follows:

ASHEX WIP—Gram-Negative Non-Fermenting Bacilli (c)2004–2014 A.P. Schreckenberger using test results provided by P.C. Schreckenberger.

Enterics WIP—Enterobacteriaceae courtesy of Brent Barrett using CDC *Enterobacteriaceae* Tables originally developed by J.J. Farmer, PhD, retired from CDC with revision by Caroline O'Hara, and updated with results published in the International Journal of Systemic Bacteriology, & A.P. Schreckenberger.

Vibrio WIP—Vibrio species courtesy of Brent Barrett using Tables developed by J.J. Farmer, PhD, retired from CDC (c)2005, & A.P. Schreckenberger.

Salmonella WIP—Salmonella species CDC Biochemical Tables 4-6-2009 Rev 1 (c)2009, courtesy of Brent Barrett, & WIP dev courtesy of A.P. Schreckenberger.

The table associated with the ASHEX Non-Fermenter WIP is available as a downloadable Excel spreadsheet. This file includes the characteristic matrix as well as the raw phenotypic reaction results used to compute each probability. The complete table is reproduced in Chapter 7 of this text. To access the WIP, go to www.pschreck.com and click the WIP tab of your choice in the toolbar and enter your reactions on the worksheet.

BioBASE 8.0 (BioBASE, Boston, 1994 to 2014 by Peter Alachi) is a computer-enhanced numeric identification software package for use on personal computers that allows the user to create, update, and manipulate unlimited numbers of microbial databases. The current version of BioBASE runs on any Windows-based machine, including Windows 7/Vista and 8.0. It is equipped with several database frequency files, which are used for the identification of unknown bacteria such as the *Enterobacteriaceae*. Conventional biochemical reactions on an unknown isolate are entered into the appropriate database whereby the program compares the unknown profile with that of any taxon in the database. To reach an identification verdict, BioBASE calculates identification scores, modal scores, and similarity indices of each taxon and compares them with the unknown's input data. The topscoring microorganisms are then analyzed and weighted for a decision on identification,

ranging from unacceptable to

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excellent. Miller and Alachi evaluated this program and report it to be user-friendly, rapid, and accurate, and state that it would be of value to any laboratory that uses conventional biochemicals.[438]

Numeric Coding Systems

The identification of the *Enterobacteriaceae* and many other families and groups of bacteria has been facilitated by the use of automated and packaged kit systems, by which organisms are identified with computer-assisted numeric codes. A numeric code is a system by which the several identifying characteristics of bacteria are translated into a sequence of numbers that represent one or more bacterial species. The fact that the identification of microorganisms is based on a series of positive and negative biochemical reactions makes computer programming easy because computer logic is also constructed on a sequence of positive and negative entries, using a binary numeric system. In binary logic there are only two numbers: "0" (or off) and "1" (or on). As can quickly be surmised, the identification characteristics of microorganisms can be easily translated into binary numbers by assigning a "1" to all positive reactions and a "0" to all negative reactions. This approach can be illustrated using the sequence of characteristics in the API 20E strip (bioMérieux) as a point of reference and converting the positive and negative reactions into binary numbers (Table 6-27). If the binary numbers shown in Table 6-27 are read from top to bottom and rearranged horizontally, the following 21-digit binary number is derived:

10101000011111011100

Although computers are constructed to receive 1/0 bits of data from which to calculate meaningful results, the human mind cannot efficiently manipulate binary logic; therefore, binary codes must be converted into simpler mathematic systems to become usable. Conversion of the two-digit (binary) system into an eight-digit (octal) system serves this purpose. To understand the conversion of binary into octal numbers, visualize a series of three light bulbs. By turning different lights on and off, a total of eight combinations is possible, each of which can be represented by one of eight numbers ranging from 0 to 7. If all lights are off (–), the combination—is equivalent to octal 0. If only the left bulb is turned on (+), the combination +– – is equivalent to octal 1. Octal 2 is represented by the binary pattern –+–, and octal 3 by

the pattern + +−. The octal equivalents of the eight combinations of a three-digit binary number are shown in Table 6-28.

To illustrate how binary numbers longer than three digits can be converted into their octal equivalents, use the binary number that was derived from the API 20E reactions:

10101000011111011100

Beginning from the right, because binary numbers are read from right to left, divide the binary numbers into subsets of three:

101 010 000 111 111 011 100

TABLE 6-27 Binary Conversion of Reactions of Unknown Organism on API 20-E Strip

Characteristic	Reaction	Binary Conversion
ONPG	+	1
Arginine	−	0
Lysine	+	1
Ornithine	−	0
Citrate	+	1
Hydrogen sulfide	−	0
Urease	−	0
Tryptophan deaminase	−	0
Indole	−	0
Voges–Proskauer	+	1
Gelatin	+	1
Glucose	+	1
Mannitol	+	1
Inositol	+	1
Sorbitol	+	1

Rhamnose	−	0
Sucrose	+	1
Melibiose	+	1
Amygdalin	+	1
Arabinose	−	0
Oxidase	−	0

TABLE 6-28 Octal Conversion of Binary Code

Binary	Conversion Formula	Octal
− − −	0 + 0 + 0 =	0
+− −	1 + 0 + 0 =	1
− + −	0 + 2 + 0 =	2
+ + −	1 + 2 + 0 =	3
− − +	0 + 0 + 4 =	4
+ − +	1 + 0 + 4 =	5
− + +	0 + 2 + 4 =	6
+ + +	1 + 2 + 4 =	7

Now convert each three-digit subset into its octal equivalent using the formula given in Table 6-28.

101 010 000 111 111 011 100

5 2 0 7 7 6 1

The number 5207761 is far easier to remember and simpler to enter into a computer than the binary number 10101000111111011100.

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A simpler way of remembering how to convert each binary triplet into its corresponding octal equivalent is to assign the following values (again reading from right to left): A value of 4 to a positive reaction for the first test in each triplet, a value of 2 to a positive reaction for the second test in each triplet, a value of 1 to a positive reaction for the third test in each triplet, and a value of 0 to any negative reactions (see Table 6-28).

These octal derivatives are known as **biotype numbers,** that is, a numeric representative of a series of phenotypic characteristics expressed by and unique to a particular bacterial species. It is important that everyone using biotype numbers, particularly those who are teaching students, understand that each number in the octal system is representative of three biochemical characteristics and that the number itself represents a pattern of positive and negative reactions. There is great danger that modern microbiologists consider biotype numbers as magic figures that can be read from charts or put into computers to derive automatic organism identification, thus losing sight not only of the biochemical reactions that they represent, but also of the biochemical principles on which the discipline of microbiology is based.

Reading Octal Codes in Numeric Code Registers

All manufacturers who have packaged identification kits on the market publish numeric code registers in which hundreds of biotype numbers are matched with one or more bacterial species that are unique for that number. For example, for the biotype number 5207761, derived from the API 20E set of reactions used in the previous example, the following species are listed in the API 20E Profile Index: *Serratia marcescens*: acceptable identification; *S. marcescens*: 1/243; and *S. rubidaea*: 1/2,859.

The message in the API Profile Index for biotype number 5207761 for *S. marcescens* "Acceptable Identification" indicates that *S. marcescens* can be reported. This assessment is based on a computer-derived calculation of the percentage likelihood that *S. marcescens* is the correct identification compared with all the other organisms entered into the database.

Estimated Frequency of Occurrence

The frequency figures listed with each individual species name (in the previous example, 1/243 for *S. marcescens*) indicate the number of strains selected at random that would have a biotype number similar to the strain being studied. In other words, if one were to randomly test 243 *S. marcescens*, there is a chance of 1 in 243 that you will encounter this exact biochemical pattern, whereas if you tested 2,859 randomly selected *S. rubidaea*, you would have a 1 in 2,859 chance of finding a strain of this exact biotype. This figure does not directly indicate the percentage likelihood that one of these species is the correct one, so the user cannot determine from this statistic how viable one of the choices might be.

Calculation of Likelihood

The identification of an unknown organism with a given biotype number is based on the calculation of percentage likelihood between the unknown biotype number and each taxon stored in the memory of the computer. Code registers that list percentage likelihood figures are more useful for everyday decision making in the laboratory. Any identification that has a likelihood of 90% or greater can be reported, those that are near 90% may be easily identified with one or two additional tests, and those with very low likelihood percentages can probably be discounted. The use of messages such as "excellent identification," "acceptable identification," and "very good identification" in the API system implies that there is a percentage likelihood of 90% or greater. However, when the message describes an identification as "questionable" or "doubtful" and the percentage likelihood is not given, the user is unable to make any assessments concerning the likelihood that an unknown represents a certain species. For students and microbiologists who may be trying to understand this concept for the first time, an example for calculating percentage likelihood and frequency of occurrence is given in Table 6-29.

Resolving Discrepancies

It must be pointed out that all answers derived from computer-based identification systems, whether or not they represent 90% or more confidence, must be interpreted in conjunction with other information available on the unknown organism—the colony morphology, reactions on various isolation media, cellular morphology on Gram's stain, results of presumptive biochemical reactions, antibiotic susceptibility patterns, and clinical setting.

When discrepancies occur, visualization of the tubes, reaction chambers, or microcupules, in which the reactions have taken place, may be necessary. In many instances, the visual interpretation of certain reactions may differ from that detected by instruments. When recalculated, the new biotype number may indicate an alternative bacterial identification that is much more in keeping with preliminary and supplemental observations.

Also, any given species may have several biotype numbers because one or more individual reactions may be variable. Consequently, designating bacterial species by biotype numbers may have epidemiologic value in recognizing the emergence of clusters of similar isolates in a given practice setting. For example, recovery of many *E. coli* from a given environment or series of cultures may be of limited value; however, knowing that all of the organisms have the same biotype number may be invaluable. In this way, it may be possible to trace the agents causing hospital nosocomial outbreaks or communitywide epidemics to a common source. Biotype analysis may also lead to a better understanding of the relation between virulence of bacterial variants and the presence or absence of certain biochemical characteristics.

Packaged Kit Identification Systems

The concept of combining a series of differential media or substrates in a single package, selected to aid in identifying members of a group of bacteria, is a logical development. In

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TABLE 6-29 Calculation of Frequency of Occurrence and Percentage Likelihood

The identification of an unknown profile is based on the calculation of likelihood between the unknown profile and each species of organism stored in the memory of the computer. To test your understanding of frequency of occurrence and percentage likelihood, work through the following example. For ease in explaining the calculations, this example is based on only four biochemical tests and three species.

Step 1. An unknown organism gives the following profile:

	IND	MR	VP	CIT
Unknown	+	−	+	−

Step 2. Known biochemical reactions of three species of *Enterobacteriaceae* for the four tests (shown as percentage of positive reactions).

	IND	MR	VP	CIT
Serratia marcescens	1	20	98	98
Enterobacter agglomerans	20	50	70	50
Klebsiella oxytoca	99	20	95	95

Step 3. Frequencies of occurrence of observed reactions (+ − + −) for each species.

Note: When a test result of the unknown is positive (IND and VP in this example), the probability of the positive reaction of the test listed in the database is used for the calculation. When the test result of the unknown is negative (MR and CIT), the probability of the negative reaction is 1 minus the probability of positive reactions.

	IND	MR	VP	CIT
Serratia marcescens	0.01	0.80	0.98	0.02
Enterobacter agglomerans	0.20	0.50	0.70	0.50
Klebsiella oxytoca	0.99	0.80	0.95	0.05

Step 4. Calculation of frequencies of occurrence of observed profile (+ − + −) for each species. The frequency of occurrence is calculated by multiplying together all the frequencies of occurrence of the reactions.

Serratia marcescens	$= 0.01 \times 0.80 \times 0.98 \times 0.02 =$	0.0001568
Enterobacter agglomerans	$= 0.20 \times 0.50 \times 0.70 \times 0.50 =$	0.0350000
Klebsiella oxytoca	$= 0.99 \times 0.80 \times 0.95 \times 0.05 =$	0.0376200
		0.0727768

Step 5. Identification percentages. Each frequency is divided by the sum of all the frequencies, and then multiplied by 100 to give the %ID. The sum of the percentages of identification is equal to 100.

Serratia marcescens	$\%ID = (0.001568/0.0727768) \times 100 = 0.21\%$
Enterobacter agglomerans	$\%ID = (0.0350000/0.0727768) \times 100 = 48.1\%$
Klebsiella oxytoca	$\%ID = (0.0376200/0.0727768) \times 100 = 51.7\%$

Step 6. Order of likelihood.

1. *Klebsiella oxytoca*	%ID = 51.7
2. *Enterobacter agglomerans*	%ID = 48.1
3. *Serratia marcescens*	%ID = 0.21

What is the likelihood that Klebsiella oxytoca is the correct answer among the three species in the database?

(*Answer:* From step 5, the answer is 51.7%; however, there is a 48.1% likelihood that the unknown organism is *Enterobacter agglomerans;* therefore, additional tests would have to be set up to correctly identify this unknown organism.)

How frequently will Klebsiella oxytoca give this particular reaction profile?

(*Answer:* From step 4, 3.8% of the time; in other words, not very often.)

IND, indole; MR, methyl red; VP, Voges–Proskauer; CIT, growth on citrate.

fact, the availability of packaged identification systems evolved naturally, almost as a practical necessity. The microorganisms currently known to cause infectious diseases are not only legion, but also are often fastidious and require a large battery of biochemical tests for identification. It is beyond the capability of many laboratories to maintain the diversity of conventional media required. The compact construction (requiring little storage space), easily visible chemical reactions, long shelf life, and standardized quality control provided by the manufacturers of these kits make them

very convenient for use in microbiology laboratories. They are especially useful in lowvolume laboratories, where there may not be the time or the technical expertise to make many of these identifications, and where quality control is more difficult to maintain.

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Overview of Packaged Systems

It has now become almost standard practice in many clinical laboratories to use one or more available packaged systems for the identification of certain groups of microorganisms. Extensive testing in diagnostic and research laboratories has demonstrated a 95% or greater agreement between most packaged identification systems and conventional methods in the identification of microorganisms. Thus, packaged systems have found wide acceptance in clinical laboratories for the following reasons:

1. Their accuracy has proved to be comparable with that of conventional identification systems. Evaluations of several systems have been made at the CDC by Smith and associates.[599] In all evaluations, two criteria were used for measuring the performance of a product: (1) a comparison of each test in the product with its conventional counterpart and (2) the accuracy of identification made using the product.
2. Several of the systems have a long shelf life—6 months to 1 year—so that outdating of media, a problem particularly with conventional systems, is minimized.
3. The systems require only a minimum of space for storage and incubation.
4. Some of the systems are as easy to use as conventional methods, or even easier. Inoculation is simple, reactions are generally clear-cut within 24 hours, and the availability of computer-assisted file registers makes final identification easy and accurate.

Whether to use one of the packaged identification systems and which one to select is largely a matter of personal preference. The ease of inoculation, the ability to select only the characteristics to be measured, the manipulation required in adding reagents after incubation, and the availability of interpretive charts or computer databases are the main items that potential users should consider before selecting a system. If strict attention is paid to the instructions provided by the manufacturer, essentially the same degree of accuracy and reliability of performance can be attained, with only minor differences in the sensitivity of individual tests.

Specific Identification Systems

API 20E. The API 20E identification system (bioMérieux) has become the reference method against which the accuracy of other systems is compared. The 21 characteristics that can be determined by the API 20E system make it among the largest test sets of the packaged kits. The system identifies a high percentage of bacterial species within 24 hours, without the need to determine additional physiologic characteristics (Image Plate 6-6A). This system is among the most frequently used in clinical laboratories and has a large database that includes common and atypical strains. The API Profile Index, which can be used manually or with computer assistance, provides the frequency probability of several strains that must be considered for each biotype number. Thus, the accuracy of identification of the members of the *Enterobacteriaceae* is maximized. Castillo and Bruckner found that the API 20E system correctly identified 97.7% of 339 clinical and stock isolates.[98] The system is somewhat cumbersome to inoculate—a problem, however, that is overcome quickly with practice. After inoculation, the strips must be handled carefully so that the bacterial suspensions do not spill and contaminate the surrounding environment. Practice is required to interpret occasional borderline reactions, which can affect the biotype number and the final identification. Occasionally, biotype numbers may not appear in the profile register; however, the manufacturer maintains a telephone number for consultation. The design of the system, the operating procedures, substrates included, and evaluation studies are shown in Table 6-30 (Image Plate 6-6A).

BBL Crystal Enteric/Nonfermenter ID System. The BBL Crystal E/NF identification system (Becton Dickinson Microbiology Systems, Sparks, MD) is a miniaturized identification method using modified conventional and chromogenic substrates. It is intended for the identification of clinically significant aerobic gram-negative bacteria that belong to the family *Enterobacteriaceae,* as well as some of the more frequently isolated glucose-fermenting and nonfermenting gram-negative bacilli of human origin. The E/NF kit comprises (1) BBL Crystal E/NF lids, (2) BBL Crystal bases, and (3) BBL Crystal Enteric/Stool ID inoculum fluid tubes (see Image Plate 6-6B). The BBL Crystal lid contains 30 dehydrated substrates on tips of plastic prongs. The BBL Crystal base has 30 reaction wells. Test inoculum is prepared with the BBL Crystal Enteric/Stool ID inoculum fluid and is used to fill all 30 wells in the BBL Crystal base. When the lid is aligned with the base and snapped into place, the test inoculum rehydrates the dried substrates and initiates test reactions. The tests used in the Crystal system include tests for fermentation, oxidation, degradation, and hydrolysis of various substrates, including chromogen-linked substrates.

Following inoculation, panels are incubated upside down in a non-CO_2 incubator with 40% to 60% humidity for 18 to 20 hours at 35°C to 37°C. After incubation,

panels are read upside down using the BBL Crystal light box. The wells are examined for color changes, and a 10-digit profile number is generated. The profile number and off-line test results for indole and oxidase are entered on a PC in which the BBL Crystal ID System Electronic Codebook has been installed to obtain the identification.

In an external study involving three clinical laboratories, the reproducibility of the 30 individual E/NF substrates ranged from 96.3% to 100%. The performance of the E/NF was evaluated with both fresh clinical isolates and challenge test strains. Of 299 fresh clinical isolates tested by the laboratories' current identification methods, the BBL Crystal ID System correctly reported 96.7%, including 16 instances in which two or three organisms were reported and required supplemental testing to resolve. Of 291 previously identified challenge strains confirmed by the laboratories' current identification methods, the BBL Crystal ID System correctly reported 96.9%, including eight instances in which two or three organisms were reported and required supplemental testing to resolve (BBL Crystal Package Insert, May 1994). In two independent evaluations of the Crystal system with fermentative organisms largely from the family *Enterobacteriaceae*, correct identification without supplemental testing was reported to be 91.6%[565] and 92.9%,[659] respectively. In a study using 626 stock challenge isolates conducted at the CDC, the Crystal system correctly identified 71.1% of the total isolates tested, which improved to 87.9% (88.8% for enterics; 84.3%

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TABLE 6-30 Construction, Use, and Evaluation of the API 20E Identification System

Functional Design	Operating Procedure	Substrates Included	Evaluation Studies
The system consists of a plastic strip with 20 miniaturized cupules containing dehydrated substrates and a plastic incubation chamber with a loosely fitting lid (see Image Plate 6-6*A*). Each cupule has a small hole at the top through which the bacterial suspension can be inoculated with a pipette. Bacterial action on the substrates produces color changes that are interpreted visually.	Add 5 mL of tap water to an incubation tray to provide a humid atmosphere during incubation. Place an API 20E strip into the incubation tray. Prepare a bacterial suspension of the test organism by suspending the cells from a well-isolated colony in 5 mL of sterile 0.85% saline. The turbidity of the suspension is compared with a McFarland 0.5 standard, except for same-day identifications of the *Enterobacteriaceae*, when the suspension is matched to a 1 standard. Using a Pasteur pipette, fill each cupule with the bacterial suspension through the inoculating hole. Overlay the three decarboxylase and the urease cupules with sterile mineral oil. The unit is incubated at 35°C for 5 hours (same-day identification) or for 24–48 hours before reading results.	ONPG Arginine dihydrolase Lysine decarboxylase Ornithine decarboxylase Citrate Hydrogen sulfide Urease Tryptophan deaminase (add 10% $FeCl_3$) Indole Voges–Proskauer (add KOH and α-naphthol) Gelatin Glucose Mannitol Inositol Sorbitol Rhamnose Sucrose Melibiose Amygdalin Arabinose	Aldridge and Hodges, International Clinical Laboratories, Nashville, TN: 90.5% of stock cultures and 96.6% of clinical isolates identified. Overall accuracy 92%.[a] Gooch and Hill, University of Utah, 415 cultures, same-day identification 90.2%.[b]

[a]Aldridge KE, Hodges RL. Correlation studies of Entero-Set 20, API 20E, and conventional media systems for *Enterobacteriaceae* identification. J Clin Microbiol 1981;13:120–125.
[b]Gooch WM III, Hill GA. Comparison of Micro-ID and API 20E in rapid identification of Enterobacteriaceae. J Clin Microbiol 1982;15:885–890.

for nonenterics) after additional tests suggested by the software program were included.[488]

RapID onE System. The RapID onE system (Thermo Fisher Scientific Inc., Waltham, MA USA) is a qualitative micromethod using conventional and chromogenic substrates for the identification of medically important *Enterobacteriaceae* and other selected oxidase-negative, gram-negative bacteria isolated from human clinical specimens. The system comprises (1) RapID onE panels and (2) RapID onE reagent. Each RapID onE panel has 18 reaction cavities molded into the periphery of a plastic disposable tray. Reaction cavities contain dehydrated reactants, and the tray allows the simultaneous inoculation of each cavity with a predetermined amount of inoculum (see Image Plate 6-6C). A suspension of the test organism in RapID inoculation Fluid (2 mL) is used as the test inoculum, which rehydrates and initiates test reactions. Inoculated panels are placed into the chipboard incubation trays provided in the package and incubated at 35°C to 37°C in a non-CO_2 incubator for 4 hours.

RapID onE panels contain 18 reaction cavities that provide 19 test scores. Tests labeled PRO, GGT, and PYR (cavities 15, 16, and 17) require RapID onE Reagent and are designated with a box drawn around the tests. Test 18 is bifunctional, containing two separate tests in the same cavity. This test is scored **before** the addition of reagent, providing the first test result, which is adonitol; then two drops of INOVA Spot Indole reagent are added to cavity 18 and the same cavity is scored again **after** the addition of reagent to provide the second test result, which is indole. The 19 test

results plus oxidase are scored in the appropriate boxes of the report form, and a seven-digit profile code is generated. The organism ID is obtained by finding the profile code in the RapID onE Code Compendium.

In a study by Schreckenberger and colleagues, 302 of 344 (87.8%) oxidase-negative, gram-negative bacilli tested were correctly identified to the species level, with an additional 24 (7%) organisms correctly identified to the genus or group level. Six organisms gave unacceptable or no ID, eight gave questionable IDs, and four organisms (1.1%) gave incorrect IDs.[587] Kitch and colleagues reported similar findings with an overall identification rate to species or to genus level of 95.8%, with a misidentification rate of 1.3%.[356]

Biolog GN2 Microplate. Biolog GN2 MicroPlate (Biolog, Hayward, CA) consists of a 96-well microtiter plate that tests for the ability of a microorganism to utilize (oxidize) one or more of 95 different carbon sources in the presence of a redox indicator (tetrazolium dye). One well contains no carbon and serves as a negative control or reference well. All the necessary nutrients and biochemicals are prefilled and dried into the 96 wells of the plate. Tetrazolium violet is used to colorimetrically detect the increased respiration that occurs in a cell when it is oxidizing a carbon source (see Image Plate 6-6D). Regardless of its structure, virtually any chemical substrate that is oxidized by the cell will result in formation of NADH, leading to a flow of electrons along a pathway of electron transport. Redox dyes, such as tetrazolium, tap electrons from this flow, converting the tetrazolium to a highly colored formazan. Thus, if a cell is presented with a chemical that it can oxidize, its

respiration increases and the colorless dye is irreversibly reduced to a formazan, forming a purple color. If the cell is given a chemical that it cannot oxidize, no respiratory burst occurs, and no color is formed. The test yields a pattern of purple wells that constitutes a "metabolic fingerprint" of the capacities of the inoculated organism. Bochner has published a description and overview of the system.[52] Miller and Rhoden, at the CDC, have published a preliminary evaluation on the Biolog in which they report that the system performed well with many genera, but that problems were encountered with some strains of *Klebsiella*, *Enterobacter*, and *Serratia*.[439] Holmes and colleagues[296] studied 789 strains, including 55 gram-negative taxa encountered in the clinical laboratory. They reported significantly better results when plates were read manually, rather than when they were read by the automated reader. Plates read manually gave the following performances: oxidase-positive fermenters, five taxa, 64 strains, 92% correct, 3% not identified, and 5% incorrect; biochemically active nonfermenters, eight taxa, 122 strains, 88% correct, 6% not identified, and 6%

incorrect; *Enterobacteriaceae*, 35 taxa, 511 strains, 77% correct, 8% not identified, and 15% incorrect; unreactive nonfermenters, seven taxa, 92 strains, 38% correct, 24% not identified, and 38% incorrect. These authors reported problems with identification of encapsulated strains of some *Enterobacter* and *Klebsiella* taxa, as well as the least biochemically active *Moraxella* and *Neisseria* strains.[296] The Biolog system is now available in a fully automated platform and is described in the next section on automated instruments.

Microscan System. The MicroScan System (Beckman Coulter, West Sacramento, CA) consists of plastic, standard-sized, 96-well microtiter trays in which up to 32 reagent substrates are included for the identification of the *Enterobacteriaceae* and other bacterial species (grampositive, gram-negative, and urinary tract panels are available). Some trays, called Combo trays, also include broth microdilutions of various antibiotics in certain of the microtubes for performing susceptibility tests.[176] MicroScan panels are supplied either in a frozen state or contain dehydrated substrates that make shipping more convenient and allow for room-temperature storage and a longer shelf life. Schieven and associates[583] found that both the frozen and dehydrated microdilution trays provided comparable organism identification and antimicrobial susceptibility results (only 1.3% and 4.2% discrepancy rates, respectively).

The microtubes are inoculated with a heavy suspension of the organism to be identified and incubated at 35°C for 15 to 18 hours. The panels can be interpreted visually (see Image Plate 6-6E), after which the biochemical results are converted into a sevenor eight-digit biotype number that can be translated into an identification with a code book supplied by the manufacturer. Alternatively, an automated tray reader can be used to detect bacterial growth or color changes by differences in light transmission. Differences in electronic pulses are automatically analyzed by a microcomputer that compares reaction patterns with an internal program to determine the likelihood of identifications. Rhoden and associates[559] found that the AutoScan-4, an automatic reader in the MicroScan system, correctly identified 95.4% of members of the *Enterobacteriaceae* (occasional false-negative readings for hydrogen sulfide and arginine dihydrolase reactions were the only problems resulting in misidentification). One disadvantage cited by the authors was that the instrument occasionally reports "very rare biotype," leaving the user unclear as to what rare biotype was indicated.

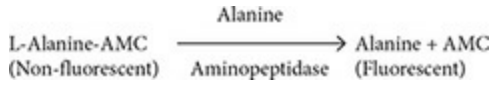
Sensititre System. The Sensititre System (Thermo Fisher Scientific Inc., Waltham, MA) may be purchased as either a manual enteric identification system or in the form of an autoidentification system. The manual plate contains media for performing 23 standard biochemical tests, plus a control, which are dried in the wells of a standard-sized 96-well microtiter tray. Each tray contains four duplicate sets of biochemical wells, permitting simultaneous identification of four organisms per tray. The system

contains conventional biochemical tests and is inoculated and read manually. Staneck and associates[606] reported that agreement between Sensititre and API 20E for 1415 isolates of *Enterobacteriaceae* was 94.6% at the species level.

Semiautomated and Automated Identification Systems

MicroScan Walkaway

The Walkaway (Beckman Coulter, West Sacramento, CA) is a fully automated instrument that incubates any combination of up to 96 conventional or Rapid MicroScan panels simultaneously, automatically adds reagents to conventional panels when required, reads and interprets panel results, and prints results, all without operator intervention (Image Plate 6-6F).[343,479,487,520,558,684] Rapid fluorescence panels in addition to the conventional MicroScan panels are available for use with the Walkaway instrument. The rapid panels use fluorescent labeled compounds and require only a 2-hour incubation for bacterial identification. Each fluorescent substrate consists of a fluorophore, either MEU or 7AMC attached to a phosphate, sugar, or amino acid compound. Two types of reactions occur: fluorogenic and fluorometric. In fluorogenic reactions, a specific enzyme, if present in the bacterial suspension, cleaves the fluorescent compound, releasing the fluorophore, which then fluoresces. For example:



Fluorometric reactions detect changes in pH such as occurs with carbohydrate fermentation. The resultant acid production causes a drop in pH and a decrease in fluorescence. In addition, eight fluorogenic rate reactions are used. These reactions measure the rate of release of the fluorophore and are used in differentiating phenotypically similar species. Results of the ID reactions are converted into 15-digit biocodes for interpretation by the computer. The Walkaway colorimetric optical system has 97 photometers illuminated

by a single tungsten-halogen source through 97 optical fibers. Light from the source passes through interference filters on a color wheel and is focused on the fiberoptics, 96 of which mirror the configuration of a 96-well panel. The 97th photometer

provides a baseline reading to which all photometer signals are ratioed. During each read cycle, the rotating color wheel provides readings at six different wavelengths through the visible spectrum. For biochemical reactions, the computer selects the wavelength reading that best discriminates the reaction occurring in each well. A review of the Walkaway technology has been published by Clayland and colleagues.[148] Several studies have shown that the Walkaway provides accurate identification of organisms belonging to the family Enterobacteriaceae.343,479,487,520,558,684

MicroScan's fluorogenic 2-hour Rapid Negative Identification Panel has been updated to significantly increase accuracy of identification and expand the number of taxa in the database. The updated panel (Rapid Gram Negative Identification Panel 3, RNID3) consists of 36 newly formulated tests and a new database consisting of 119 taxa, covering a total of 150 species. Achondo and associates reported that the RNID3 has an accuracy of 98.4% (92.5% correct to species, 1.6% correct to genus, and 4.3% correct to species with additional tests as recommended by the manufacturer) and 99.3% accuracy for clinically significant isolates.[7]

Alanine5. The accuracy and performance of the Rapid Gram-Negative Identification Type 3 (RNID3) panel were examined in a multicenter evaluation in which a total of 405 isolates comprising 54 species were tested; 96.8% of these species were identified correctly. In the same study, 465 isolates were examined for intraand interlaboratory identification reproducibility and gave an agreement of 99.8%.[39]

Vitek System

The Vitek system (Legacy) (bioMérieux) was first introduced in the early 1980s to perform automated antimicrobial susceptibility tests, with subsequent modifications to improve accuracy.[37,60,455,488] The Vitek Legacy System found wide use in clinical microbiology laboratories and has generally been accepted as a reliable approach to the rapid identification of commonly encountered gram-negative bacilli.[487,520,558] The construction and operating procedures of earlier versions of the Vitek system, initially designed to perform automated antibiotic susceptibility tests, were described in some detail in the second edition of this book (Table 15-10, pages 668–670).

The Vitek 2 system (bioMérieux) is an integrated modular system that consists of a filling-sealer unit, a reader-incubator, a computer control module, a data terminal, and a multicopy printer (see Image Plate 6-6G). In addition, the Vitek 2 system incorporates several technical improvements that automates many procedures that are performed manually with the Vitek Legacy System. The system detects bacterial growth and metabolic changes in the microwells of thin plastic cards by using a fluorescence-based technology (a colorimetric-based instrument was introduced in late 2004). The identification card for gram-negative bacilli (ID-GNB card) for the Vitek 2 is a 64-well plastic card containing 41 fluorescent biochemical tests, including 18

enzymatic tests for aminopeptidases and -oxidases. Substrates used for detection of aminopeptidases are usually coupled with 7AMC; substrates for detection of -oxidases are usually coupled with 4MEU. In addition, there are 18 fermentation tests, 2 decarboxylase tests, and 3 miscellaneous tests. There are two negative control wells, and the remaining wells are empty. Results are interpreted by the ID-GNB database after a 3-hour incubation period. In a study by Funke and colleagues, the Vitek 2 system was shown to correctly identify 84.7% of selected species representing 70 different taxa of *Enterobacteriaceae* and nonenteric bacteria within 3 hours.[241] Ling and colleagues in a much smaller study representing 31 different taxa of the most commonly recovered *Enterobacteriaceae* and nonfermenter species, obtained 95% correct identification to the species level with the Vitek 2.[390] These same researchers evaluated the Vitek 2 for rapid direct identification of gram-negative bacilli from positive blood culture bottles and reported that 97 (82.2%) of strains were correctly identified to species level and 21 (17.8%) strains were not identified. There were no misidentifications.[389] In two additional studies, O'Hara and Miller[480] reported an accuracy of 93.0% for the identification of 482 enteric stock cultures and Gavin et al.[246] reported 95.3% accuracy (*Enterobacteriaceae*, 95.9%; non-*Enterobacteriaceae*, 92.5%) with the Vitek 2.

Sensititre Gram-Negative AutoIdentification System

The Sensititre Automated Reading and Incubation System (ARIS) (Thermo Fisher Scientific Inc., Waltham, MA) is an automated system that uses fluorescence technology to detect bacterial growth and enzyme activity. The system consists of 32 newly formulated biochemical tests, including selected classic biochemical media reformulated to yield a fluorescence signal, along with newly developed fluorescence tests. Each biochemical test medium along with an appropriate fluorescence indicator is dried into the individual wells of the Sensititre plate. Each plate is designed to test three separate organisms. Because these are dried plates, they may be stored at room temperature. All autoidentification tests are read on the Sensititre AutoReader for the presence or absence of fluorescence. The results are transmitted to a computer for analysis and identification. Results may be read after 5 hours of incubation. If a satisfactory level of identification cannot be obtained at 5 hours, the plate may simply be reincubated and read after overnight incubation. Owing to the use of fluorescence technology, these plates cannot be read manually and can be read only on a correctly standardized Sensititre AutoReader. Company in-house data for 1,084 isolates of *Enterobacteriaceae* show overall agreement at 5 hours to be 92.4% and at 18 hours to be 93.4% when compared with standard methods (Sensititre Technical Product Information).

The Phoenix System

The Phoenix Automated Microbiology System (Becton Dickinson Microbiology Systems, Sparks, MD) is a newly developed, fully automated, identification and antimicrobial susceptibility test system. The system is comprised of disposable panels that combine both identification and antimicrobial susceptibility testing, and an instrument that

performs automatic reading at 20-minute intervals during incubation (Image Plate 6-6H). The gram-negative identification segment uses 45 biochemical substrates, including 16 fluorogenic, 14 fermentation, 8 carbon source, 5 chromogenic, and 2 miscellaneous substrates (urea and ornithine) to identify aerobic gram-negative bacilli in 2 to 12 hours, with the vast majority of results provided in 4 hours or less. The instrument monitors both visible spectral changes and fluorescence intensity levels, depending on the type of substrate, interprets the results, and provides an answer when the system is confident of the identification. In a study by Stefaniuk and colleagues, the Phoenix showed a high rate of agreement with conventional identification methods, ranging from 100% for gram-positive cocci to 96% for gram-negative nonfermenters and 92.5% for *Enterobacteriaceae*.[610]

The OmniLog ID System

The OmniLog ID System (Biolog, Hayward, CA) is a fully automated platform for use with Biolog's proprietary carbon source utilization test method (See description of Biolog GN2 MicroPlate in previous section). The OmniLog System simultaneously incubates, reads, and interprets the Biolog MicroPlates. It continuously processes samples but allows the user complete access at any time during a sample run. A unique feature of the OmniLog System is user-defined incubation temperature, allowing organisms to be incubated at their optimal temperature to obtain maximum growth for accurate identification. The instrument begins reading the MicroPlates 4 hours after they have been placed into the reader. The pattern is compared to the identification database, and an ID is called for if enough positive reactions have developed. If no result is obtained after 6 hours, the instrument automatically continues to incubate the Micro-Plate and begins reading again after 16 hours up to 24 hours. Biolog's databases contain over 1,400 different organisms including 501 gram-negative species, representing those important in both clinical and nonclinical microbiology. Many of these species cannot be identified with other identification systems.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrophotometry

MALDI-TOF spectra are used for the identification of microorganisms such as bacteria or fungi. A colony of the microbe in question is smeared directly on the sample target, which is a plastic or glass plate and overlaid with matrix. The matrix consists of crystallized molecules, of which the three most commonly used are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (alphacyano or alpha-matrix) and 2,5-dihydroxybenzoic acid (DHB). A solution of one of these molecules is made, often in a mixture of highly purified water and an organic solvent (normally acetonitrile (ACN) or ethanol). Trifluoroacetic acid (TFA) may also be added.

A laser is fired at the matrix crystals in the dried-droplet spot. The matrix absorbs the laser energy and the matrix is desorbed and ionized (by addition of a proton) by this event. The matrix is then thought to transfer proton to the analyte molecules (e.g., bacteria or fungi), thus charging the analyte. Ions observed after this process consist of a neutral molecule [M] and an added or removed ion. The mass spectra travels through a column which separates the ionized matrix based on charge and mass and eventually reaches a detector that generates a unique signal pattern.

The type of mass spectrometer most widely used with MALDI is the TOF (time-of-flight mass spectrometer), mainly due to its large mass range. The TOF measurement procedure is also ideally suited to the MALDI ionization process because the pulsed laser takes individual "shots" rather than working in continuous operation.

The mass spectra generated are analyzed by dedicated software and compared with stored profiles. Species diagnosis by this procedure is much faster, more accurate and cheaper than other procedures based on immunologic or biochemical tests. MALDI-TOF MS has created a revolution in microorganism identification and will likely become the standard method for bacterial identification in medical microbiologic laboratories over the next few years.[87,590,609] Overall performance of MALDI-TOF MS has been reported to be significantly better than conventional biochemical systems for identification of the *Enterobacteriaceae*.[49,157,190,465,562,572,644] One drawback is the inability to separate *E. coli* from *Shigella* using MALDI-TOF thus requiring additional confirmatory testing when *Shigella* species are being ruled out. However, in spite of this drawback laboratories will find that MALDI-TOF MS can reduce reagentand labor-costs considerably and provide identifications 1 to 2 days earlier than conventional phenotypic testing methods.[205,623]

More recently, MALDI-TOF MS has been applied directly to positive blood culture bottles,[144,186,229,370,447,543,584,613] and urine specimens[230,360] leading to reductions in turnaround time and potentially beneficial patient impacts.[648] In one study, the

application of MALDI-TOF from positive blood culture bottles resulted in a 23- to 83-hour faster time to identification for gram-positive isolates and 34- to 51-hour faster identification time for gram-negative isolates.[81] When rapid identification with MALDI-TOF MS is coupled with rapid antimicrobial susceptibility testing performed directly from the blood culture broth, results can be generated a full day earlier.[673]

In summary, commercial manufacturers continue to provide new systems and modifications of existing systems for the identification of microorganisms. To pass FDA standards, all of these systems must perform with accuracy equal to or better than reference methods. Therefore, each system can be used in clinical laboratories, but the choice depends on several variables, including volume of testing, experience of the technical staff, need for definitive identifications, and cost of operation. The *Enterobacteriaceae*, as a group, are rapidly expanding and, for the most part, are biochemically very active; therefore, they are well suited for processing by automated and semiautomated systems. Space has allowed for only a brief overview of these systems in this book. To learn more, the reader is referred to the review of automated systems written by Stager and Davis.[605] The cited references may be consulted for more detailed descriptions and evaluations of performance.

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CHAPTER 7

The Nonfermentative Gram-Negative Bacilli

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Introduction to Nonfermenting Gram-Negative Bacilli

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Yellow-Pigmented Group

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Family *Oceanospirillaceae*
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Unnamed Species
CDC Groups O-1, O-2
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Family *Oxalobacteraceae*
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CDC Group O-3

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Unnamed Species

Pseudomonas-Like Group 2

CDC Group 1c

CDC Group WO-1

OFBA-1

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Family *Rhodobacteriaceae*

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Genus *Pannonibacter* and *Achromobacter* Groups A–F

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Genus *Elizabethkingia*

Genus *Empedobacter*

Genus *Weeksella* and Genus *Bergeyella*

Genus *Myroides*

Unnamed CDC Groups IIc, IIe, IIg, IIh, IIi

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Unnamed Species
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Introduction to Nonfermenting Gram-Negative Bacilli

The nonfermentative gram-negative bacilli are a group of aerobic, non–spore-forming bacilli that do not either use carbohydrates as a source of energy or degrade them through metabolic pathways other than fermentation. Within this group are several genera and species of bacteria with special growth requirements that are not discussed in this chapter. The dividing line between what is a "nonfermenter" and what may otherwise be designated a "fastidious," "unusual," or "miscellaneous" non–glucose-fermenting, gram-negative bacillus (discussed in Chapter 9) is based more on convention than on well-defined genetic or phenotypic characteristics. The term *nonfermentative gram-negative bacilli* is used in this chapter to mean all aerobic gram-negative rods that show abundant growth within 24 hours on the surface of Kligler's iron agar (KIA) or triple sugar iron (TSI) medium, but neither grow in nor acidify the butt of these media.

The genera of nonfermenters to be discussed in this chapter include *Acetobacter, Achromobacter, Acidomonas, Acidovorax, Acinetobacter, Advenella, Agrobacterium, Alcaligenes, Alishewanella, Asaia, Azospirillum, Balneatrix, Bergeyella, Bordetella, Brevundimonas, Burkholderia, Caulobacter, Chryseobacterium, Comamonas, Cupriavidus, Delftia, Elizabethkingia, Empedobacter, Flavobacterium,*

Gluconobacter, Granulibacter, Haematobacter, Herbaspirillum, Inquilinus, Kerstersia, Laribacter, Lautropia, Massilia, Methylobacterium, Moraxella, Myroides, Naxibacter, Neisseria, Ochrobactrum, Oligella, Pandoraea, Pannonibacter, Parococcus, Pseudomonas, Psychrobacter, Ralstonia, Rhizobium, Roseomonas, Shewanella, Sphingobacterium, Sphingomonas, Stenotrophomonas, Weeksella, *Wohlfahrtiimonas*, and a few organisms that currently carry only Centers for Disease Control and Prevention (CDC) alphanumeric designations. Also included in this chapter are a few species of *Neisseria* that appear as gram-negative rods and must be differentiated from similar-appearing nonfermenting bacilli. The genera *Eikenella*, *Brucella*, and *Francisella*, although they possess the general characteristics of nonfermenters, are grouped in this book with the fastidious, gram-negative bacilli and are discussed in Chapter 9. The currently accepted organism nomenclature and a listing of previous designations are presented in Table 7-1. The synonyms for several bacterial species either previously or currently having CDC alphanumeric designations are presented in Table 7-2.

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TABLE 7-1 Nomenclature for Gram-Negative Nonfermentative Bacilli

Current Usage	Previous Designations	Comments
Achromobacter Groups B, E, and F		Unnamed *Achromobacter* species
Achromobacter denitrificans	*Alcaligenes xylosoxidans* subsp. *denitrificans* *Alcaligenes denitrificans* subsp. *denitrificans* CDC Vc	Subspecies designation no longer used
Achromobacter piechaudii	*Alcaligenes piechaudii*	Isolated primarily from human clinical specimens, but some strains from the environment. Clinical significance not known. Has been recovered from patients with bloodstream infection and chronic ear drainage.
Achromobacter xylosoxidans	*Alcaligenes xylosoxidans* subsp. *xylosoxidans* *Alcaligenes denitrificans* subsp. *xylosoxidans* CDC IIIa and IIIb	Subspecies designation no longer used
Acinetobacter baumannii	*Acinetobacter calcoaceticus* var. *anitratus* *Achromobacter anitratus* *Bacterium anitratum* *Herellea vaginicola* Morax-Axenfeld bacillus *Moraxella glucidolytica* var. *nonliquefaciens* *Pseudomonas calcoacetica*	Species name given to *Acinetobacter* genomospecies 2. Produces acid from glucose. Can be separated from *Acinetobacter calcoaceticus* (genomospecies1) by growth at 41°C and 44°C, production of β-xylosidase, and utilization of malate (*A. baumannii* positive and *A. calcoaceticus* negative). Laboratories that do not perform these tests may choose to report these organisms as *A. baumannii* complex. Most *Acinetobacter* strains isolated from human clinical specimens belong to this species.
Acinetobacter lwoffii	*Acinetobacter calcoaceticus* var. *lwoffi* *Achromobacter lwoffi* *Mima polymorpha* *Moraxella lwoffi*	Species name given to *Acinetobacter* genomospecies 8. Non–glucose-oxidizing strain found in human clinical specimens.
Alcaligenes faecalis	*Alcaligenes odorans* CDC VI	*Alcaligenes odorans* was proposed at a later date for an organism which is a strain of the earlier named *Alcaligenes faecalis*. Fruity odor.
Bergeyella zoohelcum	*Weeksella zoohelcum* CDC IIj	Rapid urea positive. Associated with dog and cat bites.
Bordetella bronchiseptica	*Alcaligenes bronchicanis* *Alcaligenes bronchiseptica* *Bordetella bronchicanis* *Brucella bronchiseptica* *Haemophilus bronchiseptica*	Rapid urea positive
Bordetella hinzii	*Bordetella avium*-like *Alcaligenes faecalis* type II TC (turkey coryza) bacterium type II *Alcaligenes* sp. strain C_2T_2	Isolated from respiratory tracts of chickens and turkeys. Human isolates reported from respiratory tract, ear discharge, and feces.
Bordetella holmesii	CDC nonoxidizer group 2 (NO-2)	Isolated from blood cultures. Oxidase negative, nonmotile; brown soluble pigment produced on heart infusion tyrosine agar. Brown diffusible pigment can also be observed on MacConkey agar.
Bordetella trematum		Isolated from wounds and ear discharge. Oxidase negative, motile.
Brevundimonas diminuta	*Pseudomonas diminuta* CDC Ia	
Brevundimonas vesicularis	*Pseudomonas vesicularis* *Corynebacterium vesiculare*	
Burkholderia cepacia	*Pseudomonas cepacia* *Pseudomonas multivorans* *Pseudomonas kingae* CDC EO-1	Yellow pigment. Recovered from numerous water sources and wet surfaces. Respiratory pathogen in CF patients. Phenotypically similar to at least 17 other species collectively referred to as the *Burkholderia cepacia* complex (Bcc).
Burkholderia gladioli	*Pseudomonas gladioli* *Pseudomonas marginata*	Primarily plant pathogen. Has been reported from sputum of patients with CF.

Burkholderia pseudomallei	*Pseudomonas pseudomallei*	Cause of melioidosis in humans.
Chryseobacterium indologenes	*Flavobacterium indologenes* CDC IIb	Phenotypically similar to *Chryseobacterium gleum*. Indole positive.
Comamonas terrigena	Various species of *Vibrio* E. Falsen group 10 *Aquasprillum aquaticum*	
Comamonas testosteroni	*Pseudomonas testosteroni* *Pseudomonas desmolytica* *Pseudomonas dacunhae* *Pseudomonas cruciviae*	
Cupriavidus pauculus	*Wautersia paucula* *Ralstonia paucula* CDC group IVc-2	Rapid urease positive
Delftia acidovorans	*Comamonas acidovorans* *Pseudomonas acidovorans* *Pseudomonas desmolytica* *Pseudomonas indoloxidans* *Achromobacter cystinovorum*	Orange indole reaction with Kovac's reagent due to production of anthranilic acid from tryptone.
Elizabethkingia meningoseptica	*Chryseobacterium meningosepticum* *Flavobacterium meningosepticum* CDC IIa	Highly pathogenic for premature infants. Indole positive
Empedobacter brevis	*Flavobacterium breve*	
Flavobacterium species		Human species previously included in the genus *Flavobacterium* have been reclassified as members of new or other genera. Remaining species widely distributed in soil and fresh water habitats. Not found in human clinical specimens. All are indole negative.
Methylobacterium mesophilicum	*Pseudomonas mesophilica* *Pseudomonas methanica* *Vibrio extorquens* *Mycoplana rubra* *Protaminobacter* sp. *Chromobacterium* sp. *Beijerinckia* sp.	Slow-growing, pink-pigmented rods. Do not stain well, appear amorphous with many nonstaining vacuoles.
Moraxella atlantae	CDC M-3	
Moraxella lacunata	*Moraxella liquefaciens*	
Moraxella nonliquefaciens	*Bacillus duplex nonliquefaciens*	
Moraxella osloensis	*Mima polymorpha* var. *oxidans*	
Myroides odoratus *Myroides odoratimimus*	*Flavobacterium odoratum* CDC M-4F	Large colonies with a tendency to spread. Indole negative, fruity odor.
Neisseria elongata subsp. *nitroreducens*	*Moraxella* sp. M-6 CDC M-6	Catalase negative. Clinical isolates associated with endocarditis.
Neisseria weaveri	*Moraxella* sp. M-5 CDC M-5	Clinical isolates associated with dog bites
Neisseria zoodegmatis	CDC EF-4b	Human infections associated with dog and cat bites
Ochrobactrum anthropi	CDC Vd-1, Vd-2 *Achromobacter* spp. biotypes 1 and 2. *Achromobacter* groups A, C, D	Only isolates have been from human clinical specimens.
Oligella ureolytica	CDC IVe	Rapid urea positive and phenylalanine deaminase positive
Oligella urethralis	CDC M-4	Clinical isolates have been from ear and urinary tract infections.
Pandoraea	New genus comprising 5 species	Isolated from human clinical samples (mostly CF patients), environment

Paracoccus yeei	CDC EO-2	Forms O-shaped cells on Gram stain
Pseudomonas aeruginosa	*Pseudomonas pyocyanea* *Bacterium aeruginosa*	Belongs to Fluorescent group, grows at 42°C. Most common clinical isolate. Grapelike odor.
Pseudomonas fluorescens		Belongs to Fluorescent group, gelatin positive. Does not grow at 42°C.
Pseudomonas luteola	CDC Ve-1 *Chryseomonas luteola* *Chryseomonas polytricha*	Yellow pigmented, oxidase negative, esculin positive.
Pseudomonas mendocina	CDC Vb-2	
Pseudomonas oryzihabitans	CDC Ve-2 *Flavimonas oryzihabitans* *Pseudomonas lacunogenes*	May cause septicemia and prosthetic valve endocarditis. Yellow pigment, oxidase negative, esculin negative.
Pseudomonas putida		Belongs to Fluorescent group, gelatin negative. Does not grow at 42°C.
Pseudomonas stutzeri	CDC Vb-1	Wrinkled colonies. Ubiquitous in soil and water.
Psychrobacter phenylpyruvicus	*Psychrobacter phenylpyruvica* *Moraxella phenylpyruvica* CDC group M-2	Phenylalanine deaminase positive, grows at 4°C, tolerates NaCl concentrations up to 9%
Ralstonia pickettii	*Burkholderia pickettii* *Pseudomonas pickettii* CDC Va-1, Va-2	Slow growing, pinpoint colonies after 24 hours on blood agar (BAP). Rarely associated with infection. Urea positive, some strains catalase negative.
Ralstonia mannitolilytica	*Pseudomonas thomasii* (Va-3)	Mannitol positive
Rhizobium radiobacter	*Agrobacterium radiobacter* *Agrobacterium tumefaciens* *Agrobacterium biovar 1* CDC Vd-3	
Roseomonas sp.	CDC "pink coccoid group"	Pink, often mucoid colonies, weakly staining gram-negative, plump, coccoid rods
Shewanella putrefaciens *Shewanella algae*	*Pseudomonas putrefaciens* *Alteromonas putrefaciens* *Achromobacter putrefaciens* CDC Ib-1, Ib-2	H_2S-positive. *S. algae* is the predominant human clinical isolate and requires NaCl for growth.
Sphingobacterium multivorum	*Flavobacterium multivorum* CDC IIk-2	Yellow pigment, oxidase positive, esculin positive, mannitol negative, rarely associated with serious infection
Sphingobacterium spiritivorum	*Flavobacterium spiritivorum* *Flavobacterium yabuuchiae* *Sphingobacterium versatilis* CDC IIk-3	Yellow pigment, oxidase positive, esculin positive, mannitol positive. Most common sources for isolation have been blood and urine.
Sphingomonas paucimobilis	*Pseudomonas paucimobilis* CDC IIk-1	Yellow pigment, oxidase positive, esculin positive, slow growing. Found in a variety of clinical specimens.
Stenotrophomonas maltophilia	*Xanthomonas maltophilia* *Pseudomonas maltophilia* CDC group I	Oxidase negative, lysine and DNase positive, can be recovered from almost any clinical site. May cause opportunistic infections.
Weeksella virosa	*Flavobacterium genitale* CDC IIf	Mucoid and sticky. Difficult to remove from agar. Clinical isolates have been associated with urinary and vaginal infections.

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TABLE 7-2 CDC Lettered and Numbered Bacterial Groups: Synonyms

CDC Designations	Current Usage
I	*Stenotrophomonas maltophilia*

Ia	*Brevundimonas diminuta*
Ib-1	*Shewanella putrefaciens*
Ib-2	*Shewanella algae*
IIa	*Elizabethkingia meningoseptica*
IIb	*Chryseobacterium indologenes*
IIc	CDC group IIc
IId	*Cardiobacterium hominis*
IIe	CDC Group IIe
IIf	*Weeksella virosa*
IIg	CDC group IIg
IIh	CDC group IIh
IIi	CDC group IIi
IIj	*Bergeyella zoohelcum*
IIk-1	*Sphingomonas paucimobilis*
IIk-2	*Sphingobacterium multivorum*
IIk-3	*Sphingobacterium spiritivorum*
IIIa, IIIb	*Achromobacter xylosoxidans*
IVa	*Bordetella bronchiseptica*
IVb	*Bordetella parapertussis*
IVc	Unnamed
IVc-2	*Cupriavidus pauculus*
IVd	*Pseudomonas*-like group 2
IVe	*Oligella ureolytica*
Va-1	*Ralstonia pickettii*
Va-2	*Ralstonia pickettii*

Va-3	*Ralstonia mannitolilytica*
Vb-1	*Pseudomonas stutzeri*
Vb-2	*Pseudomonas mendocina*
Vb-3	*Pseudomonas stutzeri*- like
Vc	*Achromobacter denitrificans*
Vd-1	*Ochrobactrum anthropi*
Vd-2	*Ochrobactrum anthropi*
Vd-3	*Rhizobium radiobacter*
Ve-1	*Pseudomonas luteola*
Ve-2	*Pseudomonas oryzihabitans*
VI	*Alcaligenes faecalis*
HB-1	*Eikenella corrodens*
HB-2	*Aggregatibacter aphrophilus*
HB-3,4	*Aggregatibacter actinomycetemcomitans*
HB-5	*Pasteurella bettyae*
M-1	*Kingella kingae*
M-2	*Psychrobacter phenylpyruvicus*
M-3	*Moraxella atlantae*
M-4	*Oligella urethralis*
M-4f	*Myroides odoratus/odoratimimus*
M-5	*Neisseria weaveri*
M-6	*Neisseria elongata* subsp. *nitroreducens*
TM-1	*Kingella denitrificans*
DF	Dysgonic fermenter
DF-1	*Capnocytophaga ochracea*

	Capnocytophaga gingivalis
	Capnocytophaga sputigena
DF-2	*Capnocytophaga canimorsus*
DF-2-like	*Capnocytophaga cynodegmi*
DF-3	*Dysgonomonas capnocytophagoides*
EO	Eugonic oxidizer
EO-1	*Burkholderia cepacia*
EO-2	*Paracoccus yeei*
EO-3	Unnamed
EO-4	Unnamed
EO-5	Unnamed
EF	Eugonic fermenter
EF-1	*Pseudomonas*-like group 2
EF-3	*Vibrio vulnificus*
EF-4a	*Neisseria animaloris*
EF-4b	*Neisseria zoodegmatis*
EF-5	*Photobacterium damsela*
EF-6	*Vibrio fluvialis*
EF-9	*Tatumella ptyseos*
EF-13	*Vibrio hollisae*
EF-19	*Comamonas terrigena*
EF-26	*Bordetella*-like species
NO	Nonoxidizer
NO-1	CDC Group NO-1
NO-2	*Bordetella holmesii*

WO	Weak oxidizer
WO-1	Unnamed
WO-2	*Pandoraea* spp.

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As more information accumulates, reclassification of bacteria between genera and species and the creation of new designations must be accepted as part of scientific progress. DNA homology studies often play a larger role in the ultimate classification of bacteria than does a scheme based on phenotypic characteristics alone. For example, within the genus *Pseudomonas,* several biovars and pathovars are now recognized, arranged according to ribosomal RNA (rRNA) and DNA homologies.[814] Full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying phenotypically aberrant microorganisms.[207] Because of the increasing microbial diversity and emergence of common pathogens having rare or unique phenotypic characteristics and the identification of new pathogens with incompletely defined phenotypes, more laboratories are relying on a combination of phenotypic and genotypic methods to report an accurate identification of many bacteria.[843] More recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been adapted for use in microbiology laboratories, where it serves as a paradigm-shifting, rapid, and robust method for accurate microbial identification.[200,828,1028] However, the initial capital required to purchase a mass spectrometry unit will delay the implementation of this technology, and therefore phenotypic profiles including Gram's stain results, colony morphologies, growth requirements, and enzymatic and/or metabolic activities using phenotypic-based tests such as those described in this chapter will likely continue to be used during the next decade. Microbiologists must also keep abreast of changes in bacterial nomenclature so that current names can be used in everyday practice and research data gathered from various investigations using previous designations will not be misinterpreted.

PART I: METABOLISM OF THE NONFERMENTERS

Bacteria that derive their energy from organic compounds are known as **chemoorganotrophs.** Most of the bacteria encountered in clinical medicine derive energy from the utilization of carbohydrates by one of several metabolic pathways.

Detection and measurement of various metabolic products are necessary to identify bacterial species that may be the cause of infectious disease. Some bacteria, such as members of the genus *Moraxella*, do not metabolize carbohydrates but rather derive energy from the degradation of other organic compounds, such as amino acids, alcohols, and organic acids. Some free-living bacteria, such as the nitrogen-fixing groups or those capable of oxidizing sulfur or iron, can derive energy from simple inorganic chemicals. These so-called **chemolithotrophs** are seldom implicated as causes of disease in humans.

Space in this textbook permits only a brief summary of the metabolic pathways used by the nonfermenters, enough to gain a working understanding of terms such as *aerobic, anaerobic, fermentation,* and *oxidation.* These metabolic processes not only define the taxonomic niche of bacteria, but also determine the tests and procedures used in the laboratory identification of microorganisms. Textbooks by Doelle[288] and Thimann[1070] should be consulted for a more detailed discussion of bacterial metabolism and physiology, and the text by MacFaddin[674] provides a review of the biochemistry of the various tests and reactions used in making identifications.

Fermentative and Oxidative Metabolism

The bacterial degradation of carbohydrates proceeds by several metabolic pathways in which hydrogen ions (electrons) are successively transferred to compounds of higher redox potential, with the ultimate release of energy in the form of adenosine triphosphate (ATP). All six-, five-, and four-carbon carbohydrates are initially degraded to pyruvic acid, an initial intermediate. Glucose is the main carbohydrate source of carbon for bacteria, and degradation proceeds by three major pathways: the Embden–Meyerhof–Parnas, the Entner–Doudoroff, and the Warburg–Dickins (hexose monophosphate) pathways. As shown in Figure 7-1, glucose is converted to pyruvic acid in each of these three pathways by a different set of degradation steps. Bacteria use one or more of these pathways for glucose metabolism depending on their enzymatic composition and the presence or lack of oxygen.

The Embden–Meyerhof–Parnas Pathway

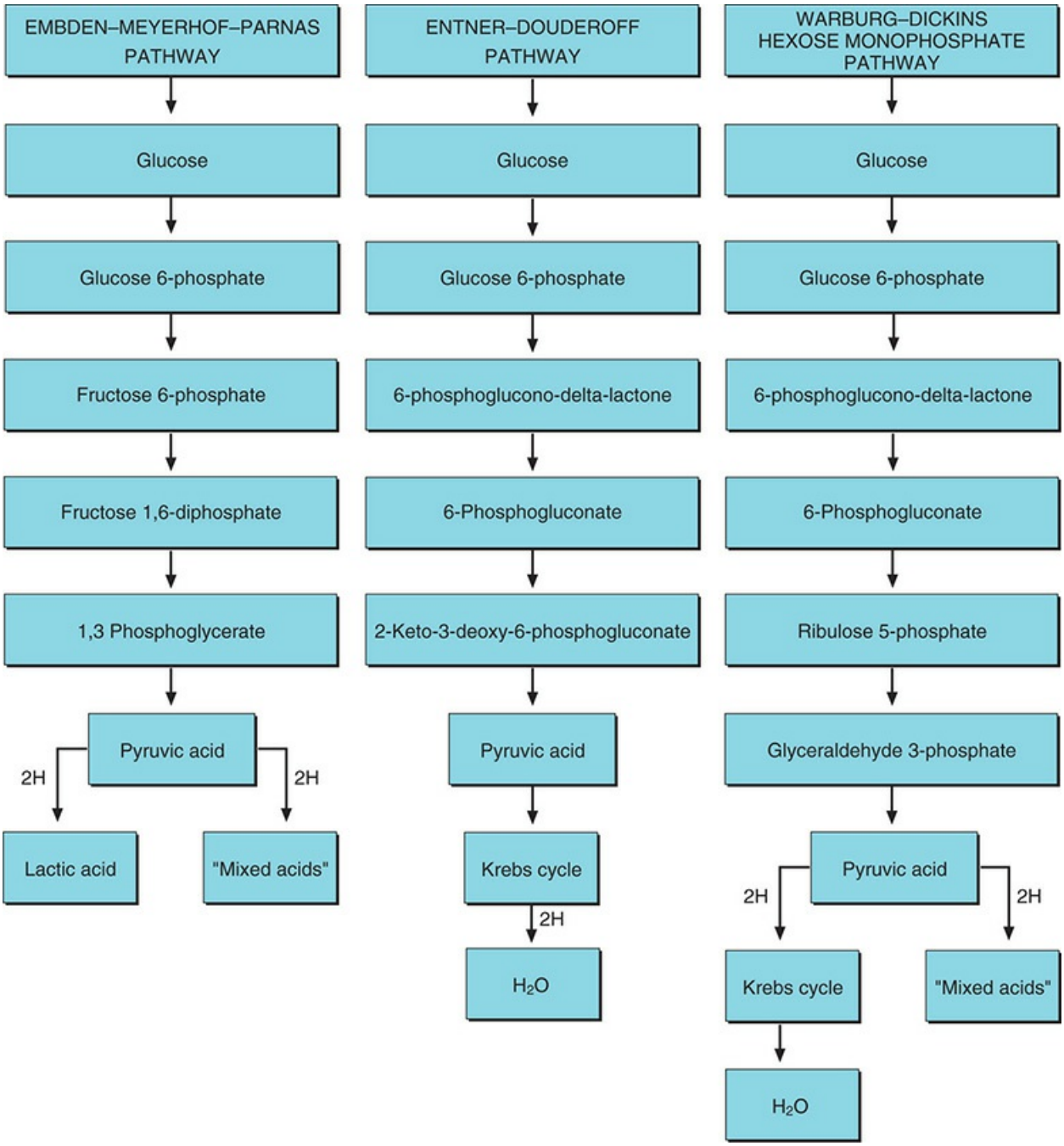
Because glucose is degraded without oxygen, the Embden–Meyerhof–Parnas (EMP) pathway has also been called the **glycolytic** or **anaerobic** pathway, used primarily by anaerobic bacteria and, to some degree, by facultatively anaerobic bacteria as well. The intermediate steps in the EMP pathway include the initial phosphorylation of glucose, conversion to fructose phosphate, and cleavage to form two molecules of glyceraldehyde phosphate, which, through a series of intermediate steps (not shown in

Fig. 7-1), forms pyruvic acid. The EMP pathway is discussed more thoroughly in Chapter 6.

Historically, the EMP pathway has also been termed the *fermentative pathway*. Fermentation and anaerobic metabolism have been considered synonymous ever since Pasteur demonstrated that acids and alcohols are the major end products of carbohydrate degradation when oxygen is excluded from the system. According to a current concept, fermentative metabolism is said to exist in a glycolytic system when organic compounds serve as the final hydrogen (electron) acceptor. Thus, as shown in the EMP pathway outlined in the left column of Figure 7-1, pyruvic acid acts as an intermediate hydrogen acceptor, but is then oxidized by giving up its hydrogen ions to sodium lactate to form lactic acid or to other organic salts to form one of several so-called mixed acids. These acids are the end products of glucose metabolism by the EMP pathway, accounting for the drop in pH in fermentation tests used for identifying bacteria. Bacteria that possess the appropriate enzyme systems can further degrade these mixed acids into alcohols, CO_2, or other organic compounds.

Although these biochemical principles seem somewhat removed from the daily work in the laboratory, microbiologists must have a basic understanding of bacterial metabolism when designing or interpreting test procedures that compare fermentation with oxidation. Fermentation must be determined in test systems that exclude oxygen. The

glycolytic products formed by fermentation have relatively strong acidity, which is easily detected by pH indicators, and large amounts of gas may be produced. This is not true for the Entner–Doudoroff pathway.



■ **FIGURE 7-1** Metabolic pathways for the bacterial degradation of glucose.

The Entner–Doudoroff Pathway

The Entner–Doudoroff (ED) pathway is also termed the *aerobic pathway* because oxygen is required for glycolysis to occur. Note in the center column of Figure 7-1 that glucose is not converted into two triose carbon molecules as in the EMP pathway; rather, it is oxidized to 6-phosphogluconate and 2-keto-3-deoxy-6-phosphogluconate

before forming pyruvic acid. Some bacteria use shunt pathways through which glucose is oxidized directly into glucuronic and ketoglucuronic acid without an initial phosphorylation step. In either event, the intermediate pyruvic acid is formed. "Oxidation" refers more to the manner in which pyruvic acid transfers its hydrogen ions than to the pathway by which it is formed. Lacking the dehydrogenase enzymes necessary to oxidize pyruvic

acid to lactic acid or other "mixed acids," oxidative bacteria transfer the available hydrogen ions from pyruvic acid into the Krebs cycle, where the ions ultimately link with elemental oxygen to form water. Thus, the **oxidative metabolism of carbohydrates** is presently defined as the energy-yielding reactions that require molecular oxygen (or other nonorganic elements) as the terminal hydrogen (electron) acceptor.

This difference in metabolism necessitates alternative, practical approaches to the identification of oxidative and fermentative bacteria. The acids that are formed in the ED pathway (glucuronic acid and its derivatives) and those produced in the Krebs cycle (citric acid and its derivatives) are extremely weak compared with the mixed acids resulting from fermentation. Because the end product of oxidative metabolism is water, gas is not formed from carbohydrates by oxidative organisms. Therefore, test systems with more sensitive detectors of acid production must be used when studying oxidative bacteria, which are discussed in detail later in this chapter. Test systems designed to detect acid production from fermentative bacteria often cannot be applied to oxidative organisms that produce insufficient acids to convert the pH indicator.

The Warburg–Dickens Hexose Monophosphate Pathway

Facultatively anaerobic bacteria have the capacity to grow on the surface of an agar plate in the presence of oxygen or in an anaerobic environment. Just because a microorganism can grow in an aerobic environment does not necessarily mean that oxygen is metabolically used. That is, not all aerobes are oxidative. The term *aerotolerant* is more appropriate for nonoxidative bacteria that are capable of growing in the presence of oxygen but grow better in an anaerobic environment.

Many of the facultative anaerobes can use either the EMP or the ED pathway, depending on the environmental conditions in which they are growing. The hexose monophosphate pathway (HMP), as shown in the right-hand column of Figure 7-1, is actually a hybrid of the EMP and ED pathways. Note that the initial steps in the degradation of glucose in the HMP pathway parallel those of the ED pathway; however, later in the HMP scheme, glyceraldehyde 3-phosphate is formed as the precursor of pyruvic acid, similar to the EMP pathway. These organisms appear

fermentative in test systems, even though the EMP pathway is not strictly used.

Note in Figure 7-1 that ribulose 5-phosphate is the precursor to the formation of glyceraldehyde 3-phosphate in the HMP pathway. Ribulose is a pentose and, for this reason, the HMP pathway has also been referred to as the pentose cycle. It provides the major avenue by which pentoses are metabolized by a number of bacterial species.

Initial Clues That an Unknown Isolate Is a Nonfermenter

The microbiologist may suspect that an unknown gram-negative bacillus is a member of the nonfermenter group by observing one or more of the following characteristics:

1. Lack of evidence for glucose fermentation (Image Plate 7-1A)
2. Positive cytochrome oxidase reaction (Image Plate 7-1B)
3. Failure to grow on MacConkey agar (Image Plate 7-1C)

Additional characteristics used to make preliminary identification of the nonfermenters are presented in Image Plate 7-1.

Lack of Evidence for Glucose Fermentation

Acids produced by nonfermenters are considerably weaker than the mixed acids derived from fermentative bacteria; thus, the pH in fermentation test media in which a nonfermenter is growing may not drop sufficiently to convert the pH indicator. The initial clue that an unknown organism is a nonfermenter is usually the lack of acid production in either KIA or TSI medium, manifested in each instance as an alkaline (red) slant and an alkaline deep (Image Plate 7-1A). Initially, it is important that an unknown organism be classified by its mode of glucose utilization to select the correct set of biochemical characteristics to make a definitive identification. Microbiologists who use packaged commercial identification kits and bypass inoculation of the unknown organism to KIA or TSI tubes may not know whether to select a fermentative or oxidative system. Therefore, before setting up differential systems, it is recommended that the oxidative-fermentative (OF) characteristic of all unknown isolates of gram-negative bacilli be assessed by inoculating a KIA or TSI slant.

Positive Cytochrome Oxidase Reaction

Any colony of a gram-negative bacillus growing on blood agar or other primary isolation media that is cytochrome oxidase positive can be suspected of belonging to the nonfermentative group (Image Plate 7-1B). However, not all oxidase-positive,

gram-negative bacilli are nonfermenters. Therefore, the mode of glucose utilization must still be measured (again demonstrating the importance of setting up a KIA or TSI tube). Cultures of oxidase-positive fermenters, such as *Pasteurella* species, *Aeromonas* species, *Plesiomonas* species, *Vibrio* species, and others, may be mistaken for nonfermenters, making identification more difficult. The procedure for performing the cytochrome oxidase test is given in Chart 1-5. To test the oxidase activity of nonfermenters, the CDC recommends using a 0.5% aqueous solution of tetramethyl- *p*-phenylenediamine hydrochloride. This solution is good for 1 week if stored in a dark bottle in the refrigerator at 4°C to 10°C. A few drops of reagent can be used to flood the surface of agar medium on which bacterial colonies are growing. The development of a blue color within a few seconds indicates a positive test. Negative reactions can be confirmed using the more sensitive Kovac's method, in which a loopful of organisms is mixed with a few drops of reagent on a piece of filter paper (Image Plate 7-1B). The development of a dark blue color within 10 seconds indicates a positive test result.

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Failure to Grow on MacConkey Agar

Although all members of the *Enterobacteriaceae* grow on MacConkey agar, some nonfermenters do not grow on MacConkey agar. Therefore, a gram-negative bacillus that grows on blood agar but grows poorly or not at all on MacConkey agar should be suspected of belonging to the nonfermentative group. This guideline, however, is far from absolute, because many of the fastidious gram-negative bacilli also do not grow on MacConkey agar. The ability of bacteria to grow on MacConkey agar is determined by inspecting with reflected light the surface of plates that have been inoculated and then incubated for 24 to 48 hours. Organisms that grow well produce colonies that are 3 mm or more in diameter and easy to see. Poorly growing strains produce either widely scattered, tiny pinpoint colonies or absolutely no growth (Image Plate 7-1C).

Tests Used in the Identification of Nonfermenters

Utilization of Glucose

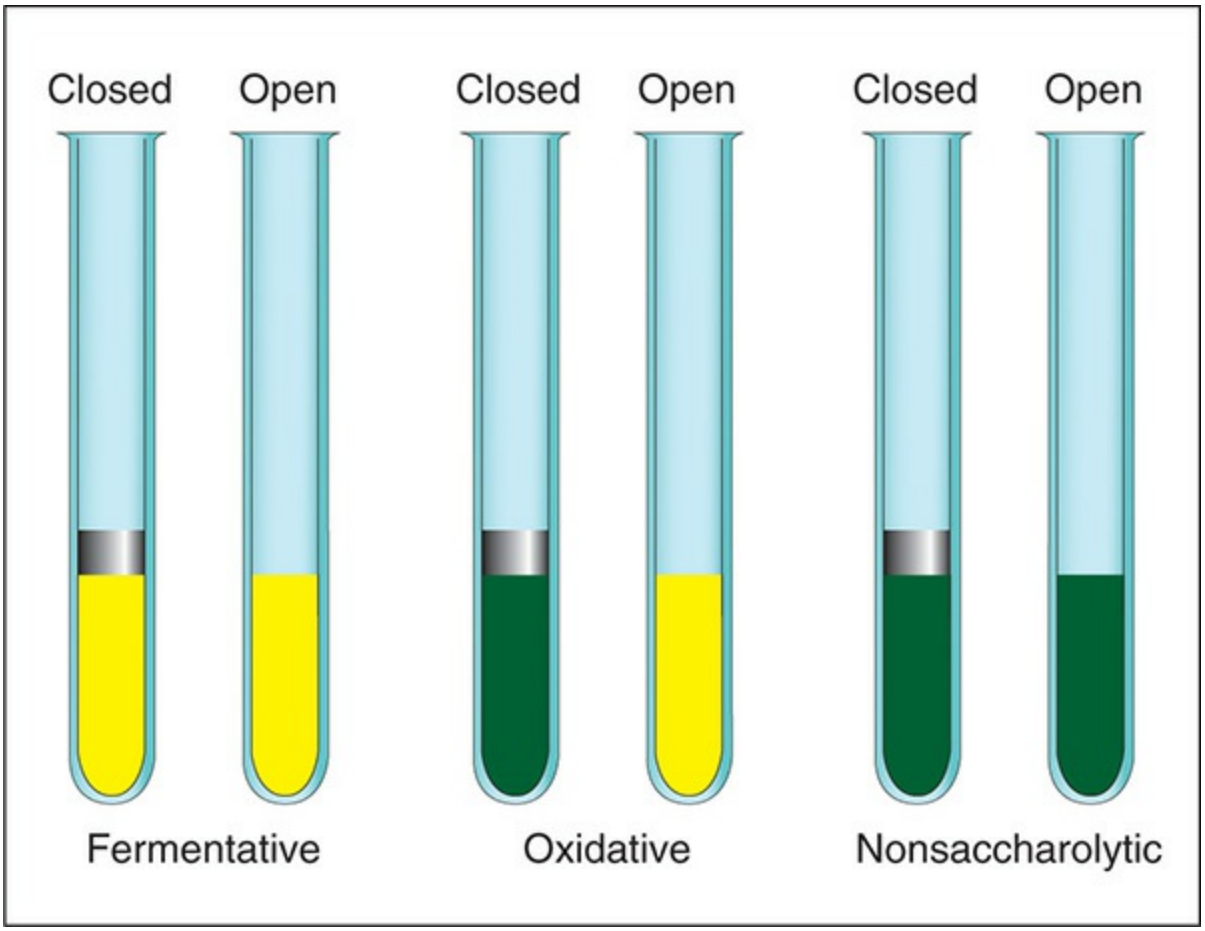
Most conventional culture media designed to detect acid production from fermentative bacteria, such as the *Enterobacteriaceae*, are not suitable for the study of nonfermentative bacilli. They do not support the growth of many strains, and the acids produced are often too weak to convert the pH indicator. Hugh and Leifson[494] were

the first to design an OF medium that accommodated the metabolic properties of the nonfermentative bacilli, as outlined in Chart 7-1.

Note that the Hugh–Leifson OF medium contains 0.2% peptone and 1.0% carbohydrate, so that the ratio of peptone to carbohydrate is 1:5, in contrast with the 2:1 ratio found in media used for carbohydrate fermentation. The decrease in peptone minimizes the formation of oxidative products from amino acids, which tend to raise the pH of the medium and may neutralize the weak acids produced by the nonfermentative bacilli. Conversely, the increase in carbohydrate concentration enhances acid production by the microorganism. The semisolid consistency of the agar, the use of bromthymol blue as the pH indicator, and the inclusion of a small quantity of diphosphate buffer are all designed to enhance the detection of acid.

Two tubes of each carbohydrate medium are required for the test. The medium in one tube is exposed to air; the other is overlaid with sterile mineral oil or melted paraffin (Fig. 7-2). Oxidative microorganisms produce acid only in the open tube exposed to atmospheric oxygen; fermenting organisms produce acid in both tubes; and nonsaccharolytic bacteria are inert in this medium, which remains at an alkaline pH after incubation. Image Plate 7-1D shows the OF reaction of an oxidative nonfermenter, with only the open tube showing the yellow color of acid production.

The OF test has limitations. Slow-growing nonfermentative bacilli may not produce color changes for several days, and species that produce amides from amino acids may cause weak acid reactions that reverse with time, thereby confusing the final interpretation. It is important that the Hugh–Leifson formula be strictly followed when performing the OF test (Chart 7-1).



■ **FIGURE 7-2** The oxidative-fermentative (OF) test. Fermentative organisms produce acid in both the closed and open tubes *(yellow)*; oxidative organisms produce acid only in the open tube. Asaccharolytic organisms that do not use carbohydrates produce no change in either tube.

Motility

A semisolid agar medium for detecting motility of fermentative organisms may not be suitable for nonfermenting species that grow only on the surface of the agar. If a semisolid agar medium is used for nonfermentative bacilli, stab-inoculate only the upper 4 mm of the medium and make an initial reading within 4 to 6 hours. Many motile strains of nonfermentative bacilli show only an early, faint haziness near the surface of the agar, which tends to disappear with prolonged incubation. Readings should again be made at 24 and 48 hours to detect the motility of slowly growing strains. Incubation at 25°C enhances the motility of some strains. We have found that Remel Motility B Medium with tetrazolium (Thermo Scientific, Lenexa, KS) works particularly well for demonstrating motility with the nonfermenting bacilli (Image Plate 7-1E).

The hanging-drop preparation may be more accurate in detecting motility of many species of nonfermentative bacilli. In this technique, a loopful of a 6- to 24-hour, actively growing broth culture that has been incubated at 25°C is placed in the center of a No. 1 coverslip that is inverted and suspended over the concavity of a depression slide. A more practical approach (preferred by the author) is to remove a small amount of growth from the surface of an 18- to 24-hour blood agar plate (BAP) and apply to the surface of a dry glass slide. A drop of saline is added to the inoculum on the slide, and a coverslip is placed over the drop. In either method, the organisms are viewed under a 40× objective with reduced light for the presence of motility. True motility must be differentiated from Brownian movement or the flow of fluid beneath the coverslip. Motile bacteria show

directional movement and change in position relative to each other; when Brownian movement is the cause of the motion, they maintain the same relative positions. The use of flagellar stains (Chart 7-2), discussed later in this chapter, is also helpful in differentiating certain motile species (Fig. 7-3).[202,623]

Pigment Production

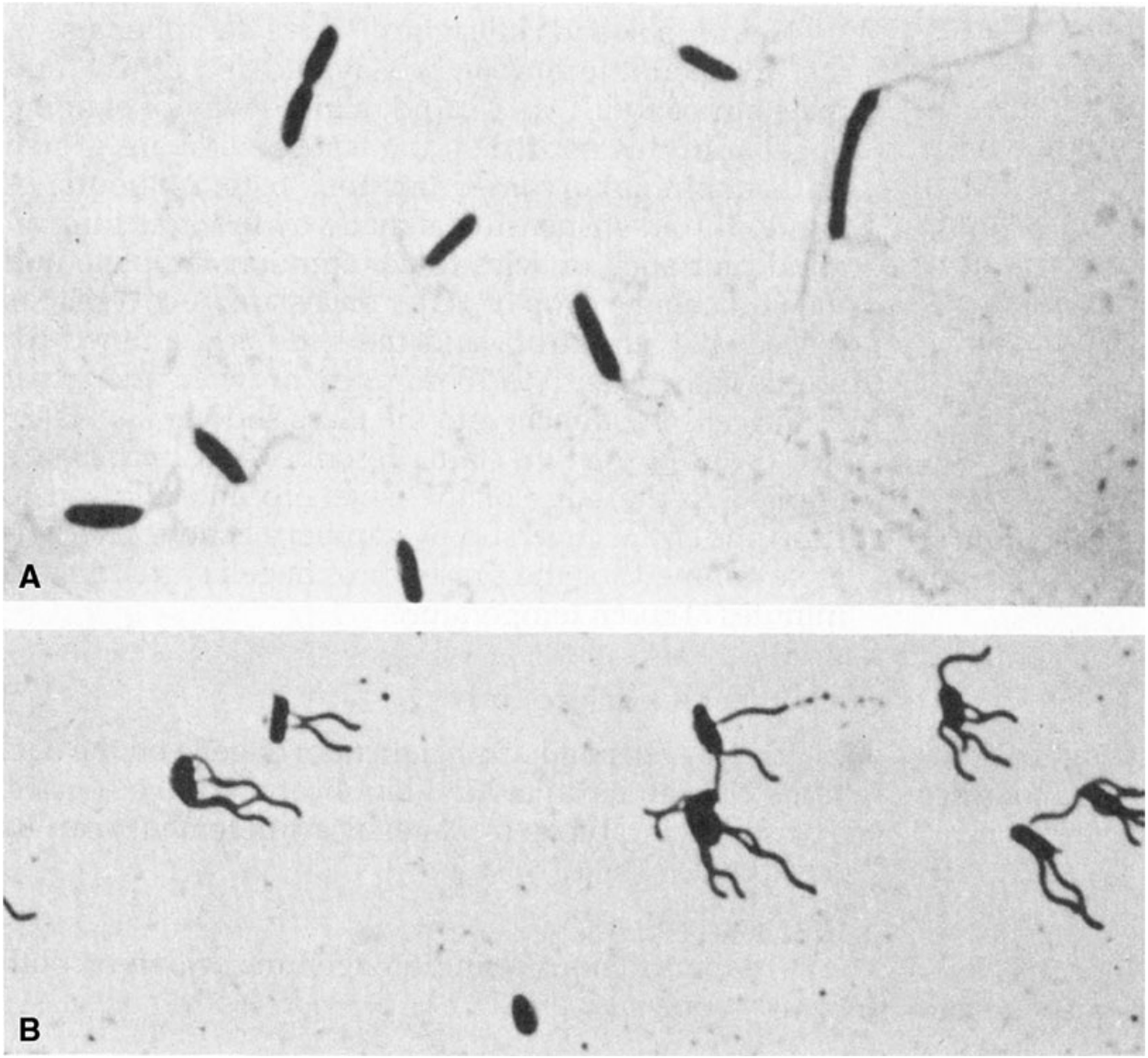
A number of pigments are produced by nonfermenters, some of which are helpful in making a species identification (Image Plate 7-1F). Water-insoluble pigments include carotenoids (yellow-orange), violacein (violet or purple), and phenazines (red, maroon, yellow) that impart distinctive colors to the colonies. Water-soluble and diffusible pigments include fluorescein (pyoverdin), pyocyanin, pyorubin, melanin, and miscellaneous other pigmented by-products that discolor the culture medium. "Tech" and "Flo" media[569] were developed to enhance formation of the water-soluble pigments pyocyanin and pyoverdin (Image Plate 7-1G). These media have special peptones and an increased concentration of magnesium and sulfate ions to enhance pigment production. King and coworkers[569] found that the kind of peptone used in the basal medium markedly affected pigment production. Bacto peptone (Difco Laboratories, Detroit, MI) proved to be superior for the production of pyocyanin but had an inhibitory effect on the elaboration of fluorescein, whereas proteose peptone 3 (Difco) enhanced the production of fluorescein and inhibited the formation of pyocyanin. An increase in phosphate concentration causes enhanced production of fluorescein but decreases pyocyanin production. Pigment production can also be enhanced by growing organisms in gelatin, potato, or milk-containing media and by incubating them at 25°C to 30°C. Pyoverdin may be demonstrated on Flo agar by observing fluorescence under ultraviolet light (using a Wood's lamp) or by the appearance of a yellow pigment in the media in visible light (Image Plate 7-1G and H).

Hydrolysis of Urea

Urea hydrolysis is presented in detail in Chart 6-6. Because many of the urea-splitting nonfermenters require enriched media for growth, Christensen's urea agar slants are used. Positive results may be achieved more rapidly by using a heavy inoculum. Bacterial species, such as *Bordetella bronchiseptica*, that avidly split urea may produce a red color change within 4 hours; weak reactors may require up to 48 hours before a positive reaction can be visualized. The appearance of a faint, delayed, pink tinge in the upper slant portion of the medium probably indicates nonspecific amino acid degradation and should be read as a negative test result (Image Plate 7-2A).

Nitrate Reduction

The basic principles and procedures for performing the nitrate reduction test are presented in Chart 6-2. The reduction of nitrate to nitrite is only the first step in a biochemical process used by some microorganisms to release oxygen—a final hydrogen acceptor at the end point of oxidative metabolism. The nitrate reduction test for nonfermenters is performed in a similar manner to that for other organisms, the end point being the appearance of a red color upon addition of sulfanilic acid and α-naphthylamine to an overnight culture in nitrate-containing media. If a red color does not develop, either



■ **FIGURE 7-3** Bacteria stained with flagellar stains. **A.** Positive flagellar stain of bacilli with polar flagella (original magnification,×900). **B.** Positive flagellar stain of bacilli with peritrichous flagella (original magnification, ×900).

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nitrate has not been reduced or reduction has proceeded beyond the nitrite stage to the formation of other compounds or to nitrogen gas (denitrification). The appearance of a red color on the addition of a small quantity of zinc dust indicates the residual presence of nitrates, denoting a negative test result; the absence of color indicates that the nitrate has been reduced to compounds other than nitrites (usually nitrogen gas), indicating that the original test was positive.

Denitrification of Nitrates and Nitrites

Certain nonfermenters have the capability of reducing either nitrate or nitrite (or both) to gaseous nitrogen (Chart 7-3). Nitrate–nitrite broth, with an inverted Durham tube or an agar slant, may be used. Because the media contain no carbohydrates, any gas that forms is derived from the nitrate or the nitrite, indicating a positive denitrification test. The broth test is easier to interpret because the collection of gas within the inverted Durham tube is readily visualized. In agar slants, the collection of gas bubbles, usually in the depths of the butt, indicates a positive test result. Most denitrifying media contain both nitrates and nitrites. In rare instances (e.g., in the identification of *Alcaligenes faecalis*, which denitrifies nitrites but not nitrates), separate denitrification tests may be in order. Combination fluorescence–denitrification or fluorescence–lactose–denitrification media are available; however, the reactions may vary from those produced in the media recommended by the CDC (Image Plate 7-2B).

Indole Production

The basic principles and procedure for determination of indole production are presented in Chart 1-4. Minor modifications may be required when detecting indole production by certain weak-reacting nonfermenters. An enriched tryptophan-containing medium, usually heart infusion broth, may be required. Because only small quantities of indole are formed by some nonfermenters, extraction of the culture media by layering a small quantity of xylene or chloroform on the surface may be helpful. Care should be taken to add only a small quantity of extractant because even minimal dilution may lower the concentration of indole below the sensitivity of detection by either Ehrlich's or Kovac's reagent. The appearance of a fuchsia color at the interface of the surface of the medium (or the extractant) with the reagent indicates indole formation and constitutes a positive test result. One organism, *Delftia (Comamonas) acidovorans*, produces a distinctive "pumpkin orange" indole reaction owing to the formation of anthranilic acid, rather than indole, from tryptophan (Image Plate 7-2C).[705]

Decarboxylation

The Møller method for detecting decarboxylation of an amino acid (described in Chapter 6) is based on a change in pH. The development of an alkaline purple color in the test medium, following inoculation with the test organism and incubation at 35°C for 24 to 48 hours, constitutes a positive test result (Chart 6-7). Many nonfermenters display only weak decarboxylase activity and may produce insufficient amines to convert the pH indicator system. This potential shortcoming in the Møller method can be overcome by using only small quantities of substrates (1 to 2 mL) and a heavy inoculum of pregrown organisms in which a high concentration of enzymes has

already accumulated. The sensitivity of detection is also increased by overlaying the culture medium with 4 mm of petrolatum. It is essential that uninoculated, amino acid-free substrate controls be used to compare the color reactions. The initial conversion of the medium to a yellow color as acids accumulate from the small amount of glucose in the medium is not seen with nonfermenters; rather, the end-point reactions are read comparing the strong alkaline purple color reactions with the lighter blue-green hue of the controls (Image Plate 7-2D). Tubes should be incubated at 35°C for up to 5 days before interpreting the reaction as negative. Other systems that use Ninhydrin reagent as an indicator may be more sensitive in detecting decarboxylase activity because the compound reacts directly with the amines to form a purple color.

Esculin Hydrolysis

Esculin hydrolysis is used primarily as a differential characteristic to distinguish between the two *Brevundimonas* species and some of the yellow-pigmented pseudomonads. An esculin medium without bile is recommended for testing of the nonfermenters, because some nonfermenter species are inhibited by bile. Esculin agar slants are inoculated with the unknown isolate and incubated at 35°C for 24 to 48 hours. Esculin in the medium fluoresces when observed with a Wood's lamp. When esculin is hydrolyzed, the medium turns reddish black and fluorescence is lost, indicating a positive test result (Image Plate 7-2E and Chart 7-4).

Flagella Stains

Although usually not required, flagellar stains are occasionally useful in identifying certain motile nonfermentative bacilli, particularly when biochemical reactions are weak or equivocal.

Leifson Method. Reliable results may be obtained using Leifson's staining technique, described in Chart 7-2, if the considerations described in Box 7-1 are given strict attention.[202,623]

Ryu Method. The use of the Ryu flagella stain, which is easy to perform and gives good results, is also recommended.[582,936] The procedure for this method is given in Chart 7-2.

Wet-Mount Technique. Heimbrook and colleagues[432] have described use of the wet-mount technique of Mayfield and Innis[716] and the stain of Ryu[936] as a rapid, simple way of staining flagella. In this approach, the test bacteria are grown on a noninhibitory medium for 16 to 24 hours. A faintly turbid suspension is made by first touching an applicator stick or wire to a colony on the plate and then touching a drop

of water on a slide. A coverslip is placed over the drop, and the slide is examined for motile cells. After 5 to 10 minutes, or when about half of the cells are attached to the glass slide or coverslip, two drops of the Ryu stain (Chart 7-2) are applied to the edge of the coverslip and allowed to

flow under the coverslip by capillary action. The cells are examined for the presence of flagella after 5 to 15 minutes at room temperature.

BOX 7-1

Considerations When Preparing a Leifson Stain

1. The slides must be scrupulously clean. Slides should be soaked in acid dichromate or acid alcohol (3% concentrated hydrochloric acid in 95% ethyl alcohol) for 3–4 days. Final cleaning can be done immediately before use by heating the slides in the blue flame of a Bunsen burner.
2. Bacteria must be grown in a carbohydrate-free medium. A low pH may inhibit formation of flagella, and any acid formation in the medium may be detrimental. The pH of the staining solution should be maintained at 5.0 or higher.
3. Bacteria should be stained during the active log phase of growth, usually within 24 or 48 hours. Room-temperature incubation for 24–48 hours may be required to promote full development of flagella in some species.
4. Care should be taken not to transfer agar to the slide because it may interfere with the staining reaction. Washing the bacteria to be stained two or three times in water (lightly centrifuging between washes) before adding to the slides may help remove surface-staining inhibitors.

Flagellar Morphology. The number and arrangement of flagella on the bacterial cell can be an aid in identification of the species. The following types of flagellar arrangements can be observed:

- Polar
  - Monotrichous—single flagellum at one or both poles
  - Multitrichous—two or more flagella at one or both poles
- Subpolar—flagella near pole with base of flagella at right angle to long axis
- Lateral—flagella projecting from the middle of bacterial cell
- Peritrichous—flagella haphazardly arranged all around bacterial cell

Representative bacteria stained with flagellar stains are shown in Figure 7-3.

PART II: TAXONOMY, BIOCHEMICAL CHARACTERISTICS, AND CLINICAL SIGNIFICANCE OF MEDICALLY IMPORTANT GENERA OF NONFERMENTERS

In the space available here, it is possible to provide only a brief summary of the medically important nonfermenters. Several references can be consulted for an in-depth discussion of the identifying features and clinical syndromes caused by this group of organisms.[439,649,958,1118,1172]

Unlike the *Enterobacteriaceae* the nonfermenting gram-negative bacilli do not fit conveniently into a single family of well-characterized genera, and the correct taxonomic placement of many nonfermentative, gram-negative bacilli (NFBs) remains unresolved. Consequently, the study of nonfermenters is often confusing for the beginning microbiologist. The major genera of nonfermenting, gram-negative bacilli have been classified into at least 22 families including: *Acetobacteraceae (Acetobacter, Acidomonas, Asaia, Gluconobacter, Granulibacter, Roseomonas), Alcaligenaceae (Achromobacter, Advenella, Alcaligenes, Bordetella, Kerstersia, Oligella), Alteromonadaceae (Alishewanella), Brucellaceae (Ochrobactrum), Burkholderiaceae (Burkholderia, Cupriavidus, Lautropia, Pandoraea, Ralstonia), Caulobacteraceae (Brevundimonas, Caulobacter), Comamonadaceae (Comamonas, Acidovorax, Delftia), Flavobacteriaceae (Bergeyella, Chryseobacterium, Elizabethkingia, Empedobacter, Flavobacterium, Myroides, Weeksella), Halomonadaceae (Halomonas), Methylobacteriaceae (Methylobacterium), Moraxellaceae (Acinetobacter, Moraxella, Psychrobacter), Neisseriaceae (Laribacter, Neisseria), Oceanospirillaceae (Balneatrix), Oxalobacteraceae (Herbaspirillum, Massilia, Naxibacter), Pseudomonadaceae (Pseudomonas), Rhizobiaceae (Agrobacterium, Rhizobium), Rhodobacteriaceae (Haematobacter, Pannonibacter, Paracoccus), Rhodospirillaceae (Azospirillum, Inquilinus), Shewanellaceae (Shewanella), Sphingobacteriaceae (Sphingobacterium), Sphingomonadaceae (Sphingomonas), and Xanthomonadaceae (Stenotrophomonas, Wohlfahrtiimonas)* (see http://www.bacterio.net/-classifgenerafamilies.html). In addition, there are a number of clinically important nonfermenters that are not yet assigned to a family and whose taxonomic position is still uncertain.

One approach to studying the nonfermenters is to group them on the basis of the presence or absence of motility and on the type of flagella present in strains that are motile. With this approach, the medically important nonfermenters can be grouped as

in Box 7-2. The biochemical characteristics of the NFBs of clinical relevance are given in Table 7-3.

Organisms That Are Motile With Polar Flagella

Pseudomonads

The genus *Pseudomonas* and some closely related genera, many of which were formerly placed in the genus *Pseudomonas*, make up a group often referred to as the pseudomonads.

BOX 7-2

Medically Important Nonfermenters

Motile with Polar Flagella

Family *Pseudomonadaceae*
(rRNA group I)
Genus *Pseudomonas*

Family *Burkholderiaceae*
(rRNA group II)
Genus *Burkholderia*
Genus *Cupriavidus*
Genus *Lautropia*
Genus *Pandoraea*
Genus *Ralstonia*

Family *Comamonadaceae*
(rRNA group III)
Genus *Comamonas*
Genus *Acidovorax*
Genus *Delftia*

Family *Caulobacteraceae*
(rRNA Group IV)
Genus *Brevundimonas*
Genus *Caulobacter*

Family *Xanthomonadaceae*
(rRNA Group V)
Genus *Stenotrophomonas*
Genus *Wohlfahrtiimonas*

Family *Acetobacteraceae*
Genus *Acetobacter*
Genus *Acidomonas*
Genus *Asaia*
Genus *Gluconobacter*
Genus *Granulibacter*
Genus *Roseomonas*

Family *Alteromonadaceae*
Genus *Alishewanella*

Family *Halomonadaceae*
Genus *Halomonas*

Family *Methylobacteriaceae*
Genus *Methylobacterium*

Family *Neisseriaceae*
Genus *Laribacter*
Genus *Neisseria*

Family *Oceanospirillaceae*
Genus *Balneatrix*

Family *Oxalobacteraceae*
Genus *Herbaspirillum*
Genus *Massilia*
Genus *Naxibacter*

Family *Rhodospirillaceae*
Genus *Azospirillum*
Genus *Inquilinus*

Family *Sphingomonadaceae*
Genus *Sphingomonas*

Family *Shewanellaceae*
Genus *Shewanella*

Organisms Whose Taxonomic Position Is Uncertain

CDC Groups 1c, O-1, O-2, O-3, Vb-3,
WO-1, *Pseudomonas*-like Group 2, OFBA-1

Motile with Peritrichous Flagella

Family *Alcaligenaceae*

Genus *Achromobacter*
Genus *Advenella*
Genus *Alcaligenes*
Genus *Bordetella* (*B. ansorpii, B. avium, B. bronchiseptica, B. hinzii, B. petrii,* and *B. trematum*)
Genus *Kerstersia*
Genus *Oligella* (*O. ureolytica*)

Family *Burkholderiaceae*

Genus *Cupriavidus* (*C. pauculus*)

Family *Rhodobacteraceae*

Genus *Pannonibacter*

Family *Rhizobiaceae*

Genus *Rhizobium*

Family *Brucellaceae*

Genus *Ochrobactrum*

Nonmotile, Oxidase-Positive

Family *Flavobacteriaceae*

Genus *Bergeyella*
Genus *Chryseobacterium*
Genus *Elizabethkingia*
Genus *Empedobacter*
Genus *Flavobacterium*
Genus *Myroides*
Genus *Weeksella*

Family *Sphingobacteriaceae*

Genus *Sphingobacterium*

Family *Moraxellaceae*

Genus *Moraxella*
Genus *Psychrobacter*

Family *Neisseriaceae*

Genus *Neisseria* (*N. zoodegmatis, N. elongata,* and *N. weaveri*)

Family *Alcaligenaceae*
Genus *Oligella* (*O. urethralis*)

Family *Rhodobacteraceae*
Genus *Haematobacter*
Genus *Paracoccus* (EO-2)

Organisms Whose Taxonomic Position Is Uncertain
CDC groups EO-3, EO-4, CDC groups IIc, IIe, IIg, IIh, IIi, Gilardi rod group 1

Nonmotile, Oxidase-Negative

Family *Moraxellaceae*
Genus *Acinetobacter*

Family *Alcaligenaceae*
Genus *Bordetella* (*B. holmesii* and *B. parapertussis*)

Organisms Whose Taxonomic Position Is Uncertain
CDC Group NO-1
CDC Group EO-5

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TABLE 7-3 Biochemical Reactions of the Named Species and Unnamed Groups of Glucose Nonfermenting Bacilli[a]

	No. isol	Motility[b]	Oxidase	Catalase	Yellow Pigment	Pink Pigment	β-Hemolysis	Growth on Mac	DNase	Starch
Achromobacter denitrificans	11	100	100	100	0	0	10	100	0	0
Achromobacter piechaudii	13	85	100	100	0	0	17	100	0	0
Achromobacter xylosoxidans	62	87	100	100	0	0	2	100	0	0
Acidovorax temperans	1	100	100	100	0	0	0	0	0	0
Acidovorax delafieldii	1	0	100	0	0	0	0	0	0	0
Acinetobacter baumannii complex	23	0	0	100	0	0	0	100	0	0
Acinetobacter haemolyticus	3	0	0	100	0	0	100	100	0	0
Acinetobacter johnsonii	1	0	0	100	0	0	0	100	0	0
Acinetobacter lwoffii	22	0	0	100	0	0	0	82	0	0
Acinetobacter ursingii	1	0	0	100	0	0	0	100	0	0
Acinetobacter species saccharolytic	2	50	0	100	0	0	100	100	0	0
Alcaligenes faecalis	9	100	100	100	0	0	0	100	0	0
Bergeyella zoohelcum	3	0	67	67	0	0	0	0	0	0
Bordetella avium	1	100	100	100	0	0	0	100	0	0
Bordetella bronchiseptica	15	80	100	100	0	0	0	100	0	0
Bordetella hinzii	2	100	100	50	0	0	0	100	0	0
Bordetella holmesii	6	0	17	50	0	0	0	0	0	0
Bordetella parapertusis	5	0	0	100	0	0	0	0	0	0
Bordetella trematum	4	100	75	100	0	0	0	100	0	0
Brevundimonas diminuta	13	100	100	92	0	0	0	92	0	0
Brevundimonas vesicularis	14	100	93	57	36	0	9	50	7	64
Burkholderia cenocepacia (III)	6	100	100	100	0	0	0	50	0	17
Burkholderia cepacia (I)	27	100	74	85	63	0	41	96	0	0
Burkholderia gladioli	6	83	33	83	0	0	67	67	0	17
Burkholderia multivorans (II)	19	95	79	95	0	0	5	89	0	0
Burkholderia pseudomallei	1	100	100	100	0	0	0	100	0	100
Burkholderia stabilis (IV)	2	100	100	100	0	0	0	100	0	0
Burkholderia thailandensis	1	100	100	100	0	0	0	100	0	0
CDC Group EO-2	4	0	100	100	100	0	0	100	0	0
CDC Group EO-3	1	0	100	100	100	0	0	100	0	0
CDC Group Ic	4	75	100	100	0	0	0	100	0	100
CDC Group IIc	6	0	100	100	33	0	33	0	0	100
CDC Group IIe	6	0	100	100	33	0	50	0	0	83
CDC Group IIg	5	0	100	100	0	0	20	40	0	0
CDC Group IIi	1	0	100	100	0	0	0	0	0	80
CDC Group NO-1	1	0	0	100	100	0	0	0	0	0
Chryseobacterium gleum	3	0	100	100	100	0	0	0	67	67
Chryseobacterium hominis	9	11	100	89	11	0	29	22	22	78
Chryseobacterium indologenes	35	0	94	97	74	0	13	9	51	46
Comamonas terrigena	5	100	100	0	0	0	0	80	0	0
Comamonas testosteroni	5	100	100	100	0	0	80	100	0	0

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Lecithinase	Lipase	PYR	LAP	ESC Spot Test	Sensitivity to Penicillin (10 U)	Sensitivity to Vancomycin (30 ug)	Sensitivity to Colistin (10 mcg)	Sensitivity to Polymyxin B (300 U)	H_2S	Pyocyanin	Pyoverdin	NO_3 reduced
0	0	63	100	0	9	0	100	100	0	0	0	100
0	0	60	100	0	0	0	92	100	0	0	0	100
0	0	80	100	0	0	0	87	98	0	0	0	90
0	0	100	100	0	100	0	100	100	0	0	0	100
0	100	100	100	0	0	0	100	100	0	0	0	100
0	30	11	100	0	4	4	100	100	0	0	0	4
0	100	0	100	0	33	0	100	100	0	0	0	0
0	100	0	100	0	0	0	100	100	0	0	0	0
0	0	25	100	0	14	9	100	100	0	0	0	0
0	100	0	100	0	0	100	100	100	0	0	0	0
0	100	50	50	50	0	50	100	100	0	0	0	0
0	0	0	100	0	11	0	100	100	0	0	0	22
0	0	50	50	50	100	100	0	0	0	0	0	0
0	0	100	100	0	0	0	100	100	0	0	0	100
0	0	0	100	0	0	0	93	89	0	0	0	93
0	0	50	50	50	100	0	100	100	0	0	0	0
0	0	0	83	0	0	0	100	100	0	0	0	0
0	0	0	100	0	0	0	100	100	0	0	0	0
0	0	25	100	0	0	0	100	100	0	0	0	75
0	0	0	67	0	8	23	46	0	0	0	0	0
0	0	33	83	50	29	93	14	64	0	0	0	21
0	83	17	100	17	0	0	0	0	0	0	0	0
4	100	5	95	58	0	0	0	0	0	0	0	7
0	100	0	100	0	67	0	0	0	0	0	0	50
0	84	5	100	0	0	0	0	5	0	0	0	95
0	0	0	100	0	0	0	0	0	0	0	0	100
0	50	0	100	0	0	0	0	0	0	0	0	50
0	100	0	100	0	0	0	0	0	0	0	0	100
0	0	0	100	0	100	100	100	100	0	0	0	100
0	0	50	50	50	0	0	100	50	0	0	0	0
0	0	0	100	0	0	0	100	100	0	0	0	100
0	0	17	100	0	100	100	0	33	0	0	0	100
0	0	0	100	0	83	100	0	50	0	0	0	0
0	0	60	100	0	100	0	100	100	0	0	0	0
0	0	0	100	100	0	100	0	0	0	0	0	0
0	0	50	50	50	100	100	100	100	0	0	0	100
0	100	0	100	33	0	33	0	0	0	0	0	33
0	0	14	100	43	78	100	0	0	0	0	0	11
0	96	6	100	38	11	89	0	0	0	0	0	34
0	0	0	100	0	0	0	60	100	0	0	0	100
0	0	60	100	0	0	0	100	100	0	0	0	100

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	Gas from NO_3	NO_2 reduced	Gas from NO_2	OF Fructose	OF Dextrose	OF Lactose	OF Maltose	OF Mannitol	OF Xylose	OF Sucrose
Achromobacter denitrificans	100	91	78	0	0	0	0	0	0	0
Achromobacter piechaudii	0	0	0	0	0	0	0	0	0	0
Achromobacter xylosoxidans	77	87	84	10	100	0	3	2	100	4
Acidovorax temperans	0	100	100	100	100	0	0	0	0	0
Acidovorax delafieldii	0	0	0	100	100	0	0	100	100	0
Acinetobacter baumannii complex	0	0	0	0	100	100	39	9	100	0
Acinetobacter haemolyticus	0	0	0	0	0	0	0	0	0	0
Acinetobacter johnsonii	0	0	0	0	0	0	0	0	0	0
Acinetobacter lwoffii	0	0	0	0	0	0	0	0	0	0
Acinetobacter ursingii	0	0	0	0	0	0	0	0	0	0
Acinetobacter species saccharolytic	0	0	0	0	100	100	0	0	100	0
Alcaligenes faecalis	0	100	80	0	0	0	0	0	0	0
Bergeyella zoohelcum	0	0	0	0	0	0	0	0	0	0
Bordetella avium	0	0	0	0	0	0	0	0	0	0
Bordetella bronchiseptica	11	0	0	0	0	0	0	0	0	0
Bordetella hinzii	0	0	0	0	0	0	0	0	0	0
Bordetella holmesii	0	0	0	0	0	0	0	0	0	0
Bordetella parapertusis	0	0	0	0	0	0	0	0	0	0
Bordetella trematum	25	25	33	0	0	0	0	0	0	0
Brevundimonas diminuta	0	0	0	8	23	0	0	0	8	0
Brevundimonas vesicularis	11	14	0	0	50	7	29	0	14	0
Burkholderia cenocepacia (III)	0	0	0	100	100	83	83	83	100	100
Burkholderia cepacia (I)	0	0	0	78	100	96	93	67	89	100
Burkholderia gladioli	0	17	0	100	100	17	0	100	100	0
Burkholderia multivorans (II)	0	0	0	100	100	100	100	79	100	0
Burkholderia pseudomallei	100	100	100	100	100	100	100	100	100	0
Burkholderia stabilis (IV)	0	0	0	100	100	100	100	100	100	0
Burkholderia thailandensis	100	100	100	100	100	100	100	100	100	100
CDC Group EO-2	0	0	0	100	100	75	0	25	75	0
CDC Group EO-3	50	0	50	100	100	0	0	0	100	50
CDC Group Ic	0	0	0	100	100	0	100	0	50	25
CDC Group II c	17	83	17	67	83	17	83	0	17	83
CDC Group II e	0	0	0	0	67	0	67	0	0	0
CDC Group II g	0	100	0	0	0	0	0	0	0	0
CDC Group II i	0	100	20	60	100	0	60	0	0	0
CDC Group NO-1	0	0	0	0	0	0	0	0	0	50
Chryseobacterium gleum	60	33	0	67	33	0	33	33	0	0
Chryseobacterium hominis	14	0	0	11	78	0	67	0	0	14
Chryseobacterium indologenes	0	37	0	60	60	0	66	0	14	0
Comamonas terrigena	0	0	0	0	0	0	0	0	0	0
Comamonas testosteroni	0	20	0	0	0	0	0	0	0	0

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Arginine	Lysine	Ornithine	Acetamide	Esculin	Gelatin	Indole	Malonate	PAD	Urea 2 hours	Urea 48 hours	6.5% NaCl	10% Lactose	ONPG	Growth 42°C
0	0	0	64	0	0	9	91	0	0	0	73	0	0	55
0	0	0	46	0	0	0	88	0	0	8	92	0	0	38
0	0	0	73	2	0	0	26	0	0	5	74	0	0	87
0	0	0	0	0	0	0	0	0	0	100	0	0	0	100
100	0	0	0	0	0	0	100	0	0	100	0	0	0	0
0	0	0	17	0	0	0	48	13	0	22	17	100	0	91
0	0	0	0	0	33	0	33	67	0	33	0	0	0	33
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	53	24	0	9	27	0	8	27
0	0	0	0	0	0	0	100	100	0	0	0	0	0	0
0	0	0	50	0	50	0	0	100	0	0	0	100	0	0
0	0	0	100	0	0	0	100	0	0	11	100	0	0	78
0	0	0	0	0	67	67	0	0	100	100	0	0	0	67
0	0	0	0	0	0	0	100	0	0	0	100	0	0	100
0	0	0	0	0	0	0	47	7	93	100	73	0	0	87
0	0	0	0	0	0	0	50	0	0	0	100	0	0	100
0	0	0	0	0	0	0	17	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	80	100	0	0	0	0
0	0	0	25	0	0	0	100	0	0	0	50	0	0	50
8	0	0	0	0	46	0	57	0	0	31	38	0	0	62
7	0	0	0	86	14	0	7	7	0	7	29	0	54	7
0	50	67	67	50	67	0	67	0	0	67	0	83	33	67
0	96	30	37	89	93	4	93	26	0	96	11	100	96	44
0	0	0	0	17	100	0	25	0	0	67	17	0	25	0
0	32	0	0	0	0	0	84	0	0	68	5	100	74	100
100	0	0	0	100	100	0	100	0	0	0	0	100	0	100
0	100	100	100	0	50	0	100	0	0	100	0	100	0	0
100	0	0	0	100	100	0	100	0	0	0	0	100	0	100
0	0	0	0	0	0	0	25	0	75	100	25	100	0	50
0	0	0	0	0	0	0	0	0	0	100	100	100	0	0
100	0	0	0	0	0	0	0	0	0	50	100	0	0	100
0	0	0	0	100	67	100	0	50	0	0	0	0	17	0
0	0	0	0	0	0	100	0	83	0	0	0	0	0	17
0	0	0	0	0	0	100	0	100	0	0	0	0	0	100
0	0	0	0	100	0	100	0	100	20	20	20	20	100	20
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	100	100	100	33	0	0	33	0	0	67	33
0	0	0	0	100	44	100	0	44	11	22	33	0	0	33
0	0	0	0	83	100	100	9	18	0	23	6	0	27	40
0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
0	0	0	0	0	0	0	20	40	0	0	0	0	0	40

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	No. isol	Motility[b]	Oxidase	Catalase	Yellow Pigment	Pink Pigment	β-Hemolysis	Growth on Mac	DNase	Starch
Cupriavidus campinensis	1	100	100	100	0	0	0	100	0	0
Cupriavidus gilardii	1	100	100	100	0	0	0	100	0	0
Cupriavidus metallidurans	1	100	100	100	0	0	0	100	0	0
Cupriavidus pauculus	11	100	100	100	0	0	0	100	0	0
Delftia acidovorans	18	94	100	100	6	0	0	100	0	0
Elizabethkingia meningoseptica	26	4	100	100	19	0	40	0	96	31
Empedobacter brevis	1	0	100	0	0	0	50	100	100	100
Inquilinus limosus	1	0	100	100	0	0	0	0	0	0
Kerstersia gyiorum	4	75	0	100	0	0	0	100	0	0
Laribacter hongkongensis	2	100	100	100	0	0	0	0	0	0
Methylobacterium species	13	8	100	100	0	100	0	0	0	46
Moraxella atlantae	1	0	100	100	0	0	50	100	0	0
Moraxella lacunata	4	0	100	100	0	0	0	0	0	0
Moraxella lincolnii	1	0	100	100	0	0	0	0	0	0
Moraxella nonliquefaciens	7	0	100	100	0	0	0	29	0	0
Moraxella osloensis	4	0	100	100	0	0	0	0	0	0
Myroides odoratimimus	3	0	100	100	100	0	67	33	100	0
Myroides odoratus	11	0	100	100	91	0	13	45	82	9
Neisseria elongata ss. *elongata*	3	0	100	0	0	0	0	0	0	0
Neisseria elongata ss. *glycolitica*	2	0	100	100	0	0	0	0	0	0
Neisseria elongata ss. *nitroreducens*	7	0	100	0	0	0	0	0	0	0
Neisseria weaveri	4	0	100	100	50	0	0	0	100	0
Ochrobactrum anthropi	10	100	100	100	0	0	0	100	0	0
Ochrobactrum intermedium	10	100	100	90	0	0	17	100	0	0
Oligella ureolytica	3	0	100	67	0	0	0	67	0	0
Oligella urethralis	22	0	100	100	0	0	0	14	0	0
Pandoraea species	4	100	25	100	0	0	0	100	0	0
Pandoraea apista	1	100	0	100	0	0	0	100	0	0
Pseudomonas aeruginosa	52	67	100	100	0	2	73	100	10	25
Pseudomonas alcaligenes	9	100	100	100	0	0	0	89	0	0
Pseudomonas fluorescens	22	91	95	100	5	9	72	100	5	50
Pseudomonas fulva/parafulva	1	100	0	100	100	0	100	100	0	100
Pseudomonas luteola	13	100	0	92	85	0	0	92	15	54
Pseudomonas mendocina	12	100	100	75	25	8	0	100	0	17
Pseudomonas oryzihabitans	17	100	0	100	88	0	8	100	0	65
Pseudomonas pseudoalcaligenes	13	69	100	85	0	0	0	100	0	8
Pseudomonas putida	29	100	97	97	17	0	13	100	0	24
Pseudomonas stutzeri	22	95	100	95	64	0	0	100	0	86
Pseudomonas stutzeri (Vb-3)	6	100	100	100	33	17	0	100	0	100
Pseudomonas sp CDC Group 1	1	100	100	100	0	0	0	100	0	0
Psychrobacter immobilis (asaccharolytic)	5	0	100	100	0	0	20	80	0	0

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Lecithinase	Lipase	PYR	LAP	ESC Spot Test	Sensitivity to Penicillin (10 U)	Sensitivity to Vancomycin (30 ug)	Sensitivity to Colistin (10 mcg)	Sensitivity to Polymyxin B (300 U)	H_2S	Pyocyanin	Pyoverdin	NO_3 reduced
0	0	100	100	0	0	0	100	100	0	0	0	100
0	0	0	100	0	0	0	100	100	0	0	0	0
0	0	100	100	0	0	0	0	0	0	0	0	100
0	0	75	100	0	9	0	100	100	0	0	0	0
0	0	43	100	0	0	0	11	14	0	0	0	94
0	0	56	100	84	0	96	0	0	0	0	0	4
0	0	50	50	50	100	100	0	0	0	0	0	0
0	0	100	100	100	0	0	0	0	0	0	0	0
0	0	0	100	0	0	0	100	100	0	0	0	0
0	0	0	100	0	0	0	100	100	0	0	0	100
0	0	0	33	0	31	0	0	67	0	0	0	38
50	50	50	50	50	100	0	100	50	0	0	0	0
0	0	0	100	0	50	100	100	100	0	0	0	50
0	0	0	100	0	0	0	100	100	0	0	0	0
0	0	0	100	0	71	86	100	100	0	0	0	100
0	0	0	100	0	50	75	100	100	0	0	0	0
0	0	100	33	0	0	100	0	0	0	0	0	0
0	13	17	83	0	18	91	0	0	0	0	0	0
0	0	50	50	50	100	67	100	100	0	0	0	0
0	0	0	100	0	100	100	100	100	0	0	0	0
0	0	0	14	0	86	86	100	100	0	0	0	100
0	0	0	100	0	100	100	100	100	0	0	0	0
0	0	100	100	0	0	0	90	100	0	0	0	60
0	0	100	75	0	0	0	0	17	0	0	0	80
0	0	50	100	0	0	0	67	50	0	0	0	67
0	0	0	100	0	55	5	100	100	0	0	0	0
0	0	0	100	0	0	0	0	0	0	0	0	0
0	0	0	100	0	0	0	0	0	0	0	0	0
11	31	35	97	0	0	0	100	100	0	23	40	75
0	0	100	100	11	22	0	89	100	0	0	0	67
70	37	23	100	0	0	0	100	100	0	0	68	9
0	0	0	100	0	0	0	100	100	0	0	0	0
0	0	0	100	100	8	0	100	100	0	0	0	46
0	33	0	100	0	0	0	100	100	0	0	0	100
0	0	36	100	0	0	0	100	100	0	0	0	0
0	0	0	100	0	8	0	100	100	0	0	0	100
0	0	0	100	0	0	0	100	100	0	0	79	17
0	82	7	93	0	5	0	95	100	0	0	0	100
0	100	50	50	50	0	0	100	100	0	0	0	100
0	100	0	100	0	0	0	100	100	0	0	0	100
0	40	0	80	0	80	80	100	100	0	0	0	60

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	Gas from NO_3	NO_2 reduced	Gas from NO_2	OF Fructose	OF Dextrose	OF Lactose	OF Maltose	OF Mannitol	OF Xylose	OF Sucrose
Cupriavidus campinensis	0	0	0	0	0	0	0	0	0	0
Cupriavidus gilardii	0	0	0	0	0	0	0	0	0	0
Cupriavidus metallidurans	100	100	100	0	0	0	0	0	0	0
Cupriavidus pauculus	0	0	0	0	0	0	0	0	0	0
Delftia acidovorans	0	0	0	100	6	0	6	100	0	0
Elizabethkingia meningoseptica	0	54	0	85	81	27	92	85	12	5
Empedobacter brevis	0	0	0	0	0	0	0	0	0	50
Inquilinus limosus	0	0	0	100	100	0	0	100	0	0
Kerstersia gyiorum	0	0	0	0	0	0	0	0	0	0
Laribacter hongkongensis	0	0	0	0	0	0	0	0	0	0
Methylobacterium species	0	0	0	69	62	0	0	0	69	0
Moraxella atlantae	50	0	50	0	0	0	0	0	0	50
Moraxella lacunata	0	0	0	0	0	0	0	0	0	0
Moraxella lincolnii	0	100	0	0	0	0	0	0	0	0
Moraxella nonliquefaciens	0	0	0	0	0	0	0	0	0	0
Moraxella osloensis	0	0	0	0	0	0	0	0	0	0
Myroides odoratimimus	0	100	64	64	0	0	0	0	0	0
Myroides odoratus	0	73	63	18	9	9	0	0	0	0
Neisseria elongata ss. *elongata*	0	0	0	0	0	0	0	0	0	0
Neisseria elongata ss. *glycolitica*	0	0	0	0	0	0	0	0	0	0
Neisseria elongata ss. *nitroreducens*	0	100	0	0	0	0	0	0	0	0
Neisseria weaveri	0	0	0	0	0	0	0	0	0	0
Ochrobactrum anthropi	50	80	75	70	90	0	50	20	80	0
Ochrobactrum intermedium	50	90	67	50	100	0	20	10	100	50
Oligella ureolytica	0	67	0	0	0	0	0	0	0	0
Oligella urethralis	0	95	54	0	0	0	0	0	0	0
Pandoraea species	0	0	0	0	0	0	0	0	50	0
Pandoraea apista	0	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	45	72	62	83	96	13	8	50	81	12
Pseudomonas alcaligenes	0	0	0	0	0	0	0	0	0	0
Pseudomonas fluorescens	0	0	0	100	100	9	9	82	100	23
Pseudomonas fulva/parafulva	0	0	0	100	100	0	0	0	100	0
Pseudomonas luteola	0	8	0	100	100	23	100	100	100	0
Pseudomonas mendocina	100	92	100	92	100	0	0	25	92	0
Pseudomonas oryzihabitans	0	0	0	100	100	18	82	100	100	38
Pseudomonas pseudoalcaligenes	0	0	0	100	31	0	15	8	0	0
Pseudomonas putida	4	7	8	97	100	7	7	28	93	5
Pseudomonas stutzeri	94	86	88	95	100	0	86	82	91	12
Pseudomonas stutzeri (Vb-3)	50	67	50	100	100	0	100	67	50	0
Pseudomonas sp CDC Group 1	100	100	100	0	0	0	0	0	0	0
Psychrobacter immobilis (asaccharolytic)	0	0	0	0	0	0	0	0	0	0

Arginine	Lysine	Ornithine	Acetamide	Esculin	Gelatin	Indole	Malonate	PAD	Urea 2 hours	Urea 48 hours	6.5% NaCl	10% Lactose	ONPG	Growth 42°C
0	0	0	0	0	0	0	100	0	0	100	0	0	0	0
0	0	0	0	0	0	0	100	0	0	0	0	0	0	100
0	0	0	0	0	0	0	100	0	0	100	0	0	0	0
0	0	0	0	0	0	0	73	0	100	100	55	0	0	73
0	0	0	100	0	0	0	79	0	0	6	11	0	0	17
0	0	0	0	100	96	92	8	0	0	35	15	4	100	12
0	0	0	0	0	0	100	0	0	0	0	0	0	0	0
0	0	0	0	100	0	0	100	0	0	0	0	0	100	100
0	0	0	0	0	0	0	50	0	0	0	100	0	0	100
100	0	0	0	0	0	0	100	0	100	100	0	0	0	100
0	0	0	15	0	0	0	100	0	0	69	15	0	0	8
0	0	0	0	0	0	0	0	0	0	0	100	0	0	100
0	0	0	0	0	50	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	100	0	100	0	100	100	100	0	0	64
0	0	0	0	0	100	0	36	45	27	82	55	0	0	18
0	0	0	0	0	0	0	0	33	0	0	0	0	0	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	25	0	0	0	0	0	25	100
40	0	0	0	50	0	0	30	90	60	100	80	0	0	40
0	0	0	0	60	0	0	0	100	70	100	60	0	0	70
0	0	0	0	0	0	0	67	67	100	100	33	0	0	0
0	0	0	0	0	0	0	40	100	0	0	68	0	6	86
0	0	0	50	0	0	0	100	0	0	25	0	0	0	0
0	0	0	0	0	0	0	100	0	0	100	0	0	0	100
98	0	0	50	4	50	0	49	0	0	69	62	11	9	92
0	0	0	0	0	0	0	33	22	0	56	44	0	0	56
95	0	0	14	0	100	0	68	0	0	82	59	14	0	0
100	0	0	0	0	0	0	0	0	0	100	100	0	0	100
85	0	0	0	92	23	0	33	0	8	77	77	50	83	54
100	0	9	0	0	0	0	92	0	0	83	100	0	0	100
0	6	6	6	12	12	0	50	71	0	94	47	38	0	24
38	0	0	23	0	0	0	29	8	0	8	92	0	0	100
100	0	0	10	0	0	0	54	0	0	86	69	23	0	0
0	5	0	14	0	0	0	100	41	0	59	100	5	0	95
100	0	0	0	0	0	0	100	0	0	83	100	0	25	83
100	0	0	0	0	0	0	0	0	0	0	100	0	0	100
0	0	0	0	0	0	0	0	80	0	0	80	20	0	20

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	No. isol	Motility[b]	Oxidase	Catalase	Yellow Pigment	Pink Pigment	β-Hemolysis	Growth on Mac	DNase	Starch
Psychrobacter immobilis (saccharolytic)	4	0	100	100	0	0	25	100	0	0
Psychrobacter phenylpyruvicus	5	0	100	100	0	0	0	80	0	0
Ralstonia mannitolilytica (Va-3)	7	100	100	14	0	0	0	71	0	14
Ralstonia pickettii (Va-1)	14	79	100	0	14	0	0	57	0	36
Ralstonia pickettii (Va-2)	4	100	100	0	0	0	0	0	0	0
Rhizobium (Agrobacterium) radiobacter	13	85	92	100	0	0	33	73	0	0
Roseomonas species	11	18	55	100	0	100	0	9	0	64
Shewanella algae	6	100	83	100	0	17	25	100	100	0
Shewanella putrefaciens	5	80	100	60	20	20	0	100	100	0
Sphingobacterium multivorum	6	50	100	100	33	0	20	100	17	83
Sphingobacterium spiritivorum	9	0	100	89	11	0	0	0	100	0
Sphingobacterium thalopophilum	2	0	100	100	0	0	50	100	100	100
Sphingomonas paucimobilis	20	50	55	85	100	0	42	5	25	75
Stenotrophomonas maltophilia	46	87	22	93	30	0	14	96	80	0
Weeksella virosa	22	0	100	95	50	0	50	0	50	0

	Gas from NO_3	NO_2 reduced	Gas from NO_2	OF Fructose	OF Dextrose	OF Lactose	OF Maltose	OF Mannitol	OF Xylose	OF Sucrose
Psychrobacter immobilis (saccharolytic)	0	0	0	0	100	0	0	0	100	0
Psychrobacter phenylpyruvicus	0	20	0	0	0	0	0	0	0	0
Ralstonia mannitolilytica (Va-3)	0	0	0	100	100	100	100	100	100	29
Ralstonia pickettii (Va-1)	67	0	0	86	100	100	100	0	100	0
Ralstonia pickettii (Va-2)	100	25	25	100	100	25	25	0	100	0
Rhizobium (Agrobacterium) radiobacter	17	69	0	100	100	62	100	92	100	100
Roseomonas species	0	0	0	100	100	0	0	36	18	0
Shewanella algae	0	83	0	0	17	0	17	0	17	0
Shewanella putrefaciens	0	60	0	20	60	0	40	0	0	0
Sphingobacterium multivorum	60	50	60	100	100	50	100	17	100	40
Sphingobacterium spiritivorum	0	0	0	89	100	100	100	100	100	100
Sphingobacterium thalopophilum	50	0	50	100	100	100	100	0	100	50
Sphingomonas paucimobilis	0	0	0	85	95	85	90	0	90	89
Stenotrophomonas maltophilia	0	4	0	87	85	46	91	0	30	46
Weeksella virosa	0	5	0	0	0	0	0	0	0	0

[a]Data from clinical isolates identified using the methods described in reference 958.
[b]Numbers are percentages of strains giving positive reaction.
PYR, pyrrolidonyl arylamidase test; LAP, leucine amino peptidase test; ESC spot test, esculin hydrolysis; H_2S, hydrogen sulfide; PAD, phenylalanine deaminase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

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Lecithinase	Lipase	PYR	LAP	ESC Spot Test	Sensitivity to Penicillin (10 U)	Sensitivity to Vancomycin (30 ug)	Sensitivity to Colistin (10 mcg)	Sensitivity to Polymyxin B (300 U)	H_2S	Pyocyanin	Pyoverdin	NO_3 reduced
0	75	0	100	0	100	100	100	100	0	0	0	50
0	33	0	100	0	60	60	60	100	0	0	0	40
0	43	29	100	0	0	0	0	0	0	0	0	14
0	70	56	100	10	21	0	0	0	0	0	0	86
0	50	25	100	0	0	0	0	0	0	0	0	100
0	0	100	83	33	8	23	62	100	0	0	0	92
0	0	20	40	0	0	0	9	14	0	0	0	18
0	75	67	100	0	0	0	83	100	100	0	0	100
0	0	0	100	0	0	0	100	100	80	0	0	100
0	60	80	100	40	0	17	67	80	0	0	0	50
0	0	0	100	100	11	56	0	0	0	0	0	0
50	50	50	50	50	0	100	0	50	0	0	0	100
0	0	0	100	100	45	100	40	55	0	0	0	5
0	78	0	94	97	0	8	76	87	0	0	0	45
0	0	0	100	0	91	100	81	100	0	0	0	5

Arginine	Lysine	Ornithine	Acetamide	Esculin	Gelatin	Indole	Malonate	PAD	Urea 2 hours	Urea 48 hours	6.5% NaCl	10% Lactose	ONPG	Growth 42°C
0	0	0	0	0	0	0	0	75	0	0	100	75	0	0
0	0	0	0	0	0	0	0	100	40	100	40	0	0	20
0	0	0	0	0	14	0	71	14	0	100	14	86	0	100
0	0	0	14	0	43	0	71	7	0	100	21	79	0	7
0	0	0	0	0	25	0	75	50	0	100	0	0	0	50
0	0	0	0	100	0	0	8	85	31	100	46	23	100	46
0	0	0	0	0	0	0	64	10	0	91	18	0	0	64
0	0	100	0	0	100	0	50	0	0	100	100	0	0	100
0	0	60	0	0	60	0	0	20	0	0	20	0	0	0
0	0	0	17	50	0	0	60	50	17	100	67	40	40	33
0	0	0	0	100	0	0	0	100	78	89	0	67	100	22
0	0	0	0	100	0	0	0	100	100	100	0	100	50	100
0	0	0	0	100	5	0	0	15	0	5	15	35	100	20
0	98	0	0	93	89	0	57	41	0	7	35	2	44	48
0	0	0	0	0	100	100	18	86	0	0	0	0	0	95

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Members of this group share the characteristics of being straight or slightly curved gram-negative bacilli that are strict aerobes; most strains are motile by means of one or

more polar flagella; they utilize glucose and other carbohydrates oxidatively; and are usually cytochrome oxidase positive. The key differentiating features of the pseudomonad group are given in Table 7-4.

Palleroni[814] separated the pseudomonads into five rRNA homology groups based on rRNA–DNA homology studies. Gilardi, however, separated the pseudomonads into seven major groups based on phenotypic characteristics: fluorescent, stutzeri, alcaligenes, pseudomallei, acidovorans, facilis–delafieldii, and diminuta.[368] An updated scheme that combines some of the features of both the genotypic and phenotypic classification is outlined in Box 7-3.

Family Pseudomonadaceae—rRNA Group I

Genus *Pseudomonas*. Although most of the pseudomonads were originally classified in the genus *Pseudomonas*, it is now known that each of the five rRNA groups represents taxonomically distinct genetic groups, and as a result different genus names have been assigned to each of the rRNA groups. Only the members of rRNA group I retain the genus designation of *Pseudomonas*.

Fluorescent Group. The species within this group are all characterized by the production of a water-soluble pyoverdin pigment that fluoresces white to blue-green under long-wavelength (400-nm) ultraviolet light. Production of fluorescent pigments is particularly enhanced in media with a high phosphate concentration.[569] Although all three members of this group produce pyoverdin, only one species, *P. aeruginosa*, produces the distinctive blue, water-soluble pigment pyocyanin (Image Plate 7-1G). The key biochemical features that separate the members of the fluorescent group are given in Table 7-5.

Pseudomonas aeruginosa. *P. aeruginosa* produces a characteristic appearance when grown on BAP. It appears as large gray colonies with a spreading periphery and exhibits β-hemolysis. Colonies often have an alligator skin appearance and exhibit a metallic sheen (Image Plate 7-2F). Rapid identification of *P. aeruginosa* in culture can be made whenever the following characteristics are observed: typical colony morphology (Image Plate 7-2F), production of diffusible pigments (Image Plate 7-2G), the presence of a fruity odor, and oxidase positivity (Box 7-4).[208] We have occasionally observed strains that produce a pungent, "rotten-potato" odor. There has been at least one report of a nosocomial outbreak caused by strains of malodorous *P. aeruginosa*.[595]

Pseudomonas aeruginosa is the pseudomonad most frequently recovered from clinical specimens. *P. aeruginosa* infection is especially prevalent among patients with burn wounds, cystic fibrosis (CF), acute leukemia, organ transplants, and intravenous drug addiction.[106] Infections commonly occur at any site where moisture tends to accumulate—tracheostomies, indwelling catheters, burns, the external ear (i.e.,

"swimmer's ear"), and weeping cutaneous wounds. The exudation of bluish pus, with a grapelike odor from the production of pyocyanin, is characteristic of an infection caused by this organism. *P. aeruginosa* also causes urinary tract and lower respiratory tract infections; the latter can be severe and even life-threatening in immunocompromised hosts. The organism can also cause devastating infections of the eye. *Pseudomonas* keratitis, infection of corneal ulcers, and endophthalmitis must be approached as a medical emergency that can be fulminant and threaten permanent loss of vision. Individual case reports of endocarditis, meningitis, brain abscess and infections of bones and joints from hematogenous spread appear with regular frequency in the literature.[106] Most patients with endocarditis caused by *P. aeruginosa* require valve replacement because the infection is difficult to eradicate.[900] *P. aeruginosa* dermatitis and otitis externa outbreaks associated with swimming-pool and hot-tub use are well described.[71] Sporadic *P. aeruginosa* infections following ear piercing have also been reported.[548]

P. aeruginosa produces several substances that are thought to enhance the colonization and infection of host tissue.[106] These substances, together with a variety of virulence factors, including lipopolysaccharide (LPS), exotoxin A, leukocidin, extracellular slime, proteases, phospholipase, and several other enzymes (Box 7-5), make *P. aeruginosa* the most clinically significant bacteria among the NFBs. An unusual mucoid morphotype of *P. aeruginosa* is frequently recovered from respiratory secretions of patients with CF who are chronically infected with *P. aeruginosa* (Image Plate 7-2H). The mucoid morphotype is due to the production of large amounts of a polysaccharide (called alginate) that surrounds the cell. The production of alginate is ultimately responsible for the poor prognosis and high mortality rates among patients with CF. Comprehensive reviews of this subject are available.[373,715,757,1032]

The accuracy of antimicrobial susceptibility testing of *P. aeruginosa* has been evaluated in several studies.[509,528,938] Sader et al.[938] evaluated the MicroScan WalkAway, Vitek, and Vitek 2 and found that all systems exhibited an unacceptable level of very major (false-susceptible) errors for piperacillin-tazobactam, unacceptable minor error rates for cefepime (Vitek 2 and Vitek) and for aztreonam (all three systems). Jekarl and colleagues evaluated Vitek 2 and BD Phoenix for susceptibility testing of *P. aeruginosa* and reported that both systems showed unacceptable overall categoric agreement for imipenem and cefepime and for ceftazidime with Vitek 2.[509] The accuracy of β-lactam susceptibility testing of *P. aeruginosa* was evaluated with four automated systems (BD Phoenix, MicroScan WalkAway, Vitek, and Vitek 2) by Juretschko et al.[528] They found unacceptable levels of error (minor, major, and very major) with aztreonam, cefepime and ceftazidime, piperacillin–taxobactam, and imipenem with all four automated systems and recommended that laboratories seek alternative methods when testing *P. aeruginosa*. Agar diffusion methods (disk

diffusion and Etest) were the most accurate when compared to reference minimal inhibitory concentration (MIC) testing of *P. aeruginosa*.[528]

Treatment of *P. aeruginosa* infections can be difficult. A relatively narrow spectrum of antimicrobials is effective against *P. aeruginosa*, including the carboxypenicillins (carbenicillin, ticarcillin), the ureidopenicillins (mezlocillin, piperacillin), the antipseudomonal cephalosporins (ceftazidime), monobactams (aztreonam), carbapenems (imipenem, meropenem), quinolones (ciprofloxacin, levofloxacin) and aminoglycosides (gentamicin, tobramycin,

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TABLE 7-4 Key Characteristics of Pseudomonads

	Oxidase	Motility	Pyoverdin	Yellow	Glucose	Maltose	Lactose	Mannitol	Arginine	Lysine	NO_3-NO_2	NO_3-N_2	Urea	ONPG	DNase	Acetamide	Esculin	H_2S in KIA	Polymyxin
Genus *Pseudomonas*																			
Fluorescent group																			
P. aeruginosa	+	+	+	–	+	V	–	V	+	–	+	V	V	–	–	+	–	–	S
P. fluorescens	+	+	+	–	+	V	–	+	+	–	V	–	V	–	–	–	–	–	S
P. putida	+	+	+	–	+	V	–	V	+	–	–	–	V	–	–	–	–	–	S
Stutzeri group																			
P. stutzeri	+	+	–	–	+	+	–	V	–	–	+	+	V	–	–	–	–	–	S
P. mendocina	+	+	–	–	+	–	–	–	+	–	+	+	V	–	–	–	–	–	S
CDC group Vb-3	+	+	–	–	+	+	–	+	+	–	+	+	V	–	–	–	–	–	S
Alcaligenes group																			
P. alcaligenes	+	+	–	–	–	–	–	–	–	–	V	–	V	–	–	–	NA	–	S
P. pseudoalcaligenes	+	+	–	–	–	–	–	–	V	–	+	–	–	–	–	–	–	–	S
Pseudomonas sp. group 1	+	+	–	–	–	–	–	–	V	–	+	+	–	–	–	–	NA	–	S
Yellow-pigmented group																			
P. luteola	–	+	–	+	+	+	–	+	V	–	V	–	V	+	–	–	+	–	S
P. oryzihabitans	–	+	–	+	+	+	–	+	–	–	–	–	V	–	–	–	–	–	S
Genus *Burkholderia*																			
B. pseudomallei	+	+	–	–	+	+	+	+	+	–	+	+	V	–	–	–	V	–	R
B. cepacia complex	w	+	–	V	+	+	+	+	–	V	V	–	V	V	–	V	V	–	R
B. gladioli	–	+	–	–	+	–	–	+	–	–	V	–	+	+	–	–	–	–	R
Genus *Ralstonia*																			
R. pickettii Va-1	+	+	–	–	+	+	+	–	–	–	V	V	+	–	–	–	–	–	R
R. pickettii Va-2	+	+	–	–	+	–	–	–	–	–	+	+	+	–	–	–	–	–	R
R. mannitolilytica	+	+	–	–	+	+	+	+	–	–	V	V	+	–	–	–	–	–	R
Genus *Delftia*																			
D. acidovorans	+	+	–	–	–	–	+	–	–	–	+	–	–	–	–	+	–	–	S
Genus *Brevundimonas*																			
B. diminuta	+	+	–	–	w	–	–	–	–	–	–	–	–	–	V	–	–	–	V
B. vesicularis	+	+	–	+	w	V	–	–	–	–	–	–	–	V	–	–	+	–	S
Genus *Stenotrophomonas*																			
S. maltophilia	–	+	–	–	+	++	+	–	–	+	V	–	–	+	+	–	+	–	S
Genus *Shewanella*																			
S. algae	+	+	–	–	+	–	–	–	–	–	+	–	–	–	+	–	–	+	S
S. putrefaciens	+	+	–	–	+	+	V	+	–	–	+	–	–	–	+	–	V	+	S
Genus *Sphingomonas*																			
S. paucimobilis	+	+	–	+	+	+	+	–	–	–	–	–	–	+	–	–	+	–	S

+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; ++, strong positive reaction; w, weak positive; S, susceptible; R, resistant. Key reactions are shaded.
Data from reference 367.

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BOX 7-3

Phenotypic Classification of the Pseudomonads (Polar Flagellated NFBs)

rRNA Group I (Tables 7-5–7-7)

Fluorescent Group (Table 7-5)

Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas putida

Stutzeri Group (Table 7-6)

Pseudomonas stutzeri
Pseudomonas mendocina
CDC Group Vb-3

Alkaligenes Group (Table 7-7)

Pseudomonas alcaligenes
Pseudomonas pseudoalcaligenes
Pseudomonas species group 1

Other Clinically Relevant *Pseudomonas*

Pseudomonas andersonii
Pseudomonas fulva
Pseudomonas otitidis

rRNA Group II (Tables 7-8–7-10)

Pseudomallei Group

(Colistin-Resistant Group)

Burkholderia mallei
Burkholderia pseudomallei
Burkholderia cepacia complex
Burkholderia gladioli
Cupriavidus species
Inquillinis limosus
Lautropia mirabilis
Pandoraea species
Ralstonia species

rRNA Group III (Table 7-7)

Weak Oxidizer Group

Acidovorax delafieldii
Acidovorax facilis
Acidovorax temperans

Comamonas terrigena (*C. terrigena* DNA 1)
Comamonas aquatica (*C. terrigena* DNA 2)
Comamonas kerstersii (*C. terrigena* DNA 3)
Comamonas testosteroni
Delftia acidovorans
CDC WO-1

rRNA Group IV (Table 7-7)

Diminuta Group

Brevundimonas diminuta
Brevundimonas vesicularis

rRNA Group V (Table 7-11)

Stenotrophomonas maltophilia
Wohlfahrtiimonas chitiniclastica

Acetic Acid Bacteria

Acetobacter
Acidomonas
Gluconobacter
Granulibacter bethesdensis

Yellow-Pigmented Group (Table 7-12)

Pseudomonas luteola
Pseudomonas oryzihabitans
Sphingomonas paucimobilis
Sphingomonas parapaucimobilis
Agrobacterium yellow Group
Balneatrix alpica
Massilia oculi
Massilia haematophilus
Massilia timonae
CDC O-1
CDC O-2

Curved Rod Group (Table 7-13)

Herbaspirillum species 3
Laribacter hongkongensis
CDC O-3

H_2S Positive Group (Table 7-14)

Shewanella putrefaciens
Shewanella algae

Halophilic Group (Table 7-14)

Alishewanella fetalis
Halomonas venusta
CDC halophilic nonfermenter group 1

Pink Pigmented Group (Table 7-15)

Asaia species
Azospirillum species
Methylobacterium species
Roseomonas species

Unnamed Species

Pseudomonas-like group 2
CC group WO-1
CDC group 1c
OFBA-1

amikacin). Almost all strains are resistant to other penicillins and cephalosporins, including ampicillin, cefuroxime, and cefotaxime.[348,656]

P. aeruginosa has the capacity to carry multiresistance plasmids, and this feature has led to the appearance of some *P. aeruginosa* strains that are resistant to all reliable antibiotics.[656] Klebsiella pneumoniae carbapenemase (KPC)-producing *P. aeruginosa* isolates have now been reported in several countries including the United States.[9,501,863,1139] More recently, pan-resistant *P. aeruginosa* isolates harboring both KPC and metallo-β-lactamase (MBL) carbapenemases have emerged.[230,711] The extent to which KPC and MBL carbapenemases-producing *P. aeruginosa* has spread throughout the world is difficult to gage since phenotypic detection

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TABLE 7-5 Key Characteristics of the Fluorescent Group

Test	*P. aeruginosa*	*P. fluorescens*	*P. putida*
Pyoverdin	+	+	+
Pyocyanin	+	–	–
Acetamide	V	–	–
Growth at 42°C	+	–	–
NO_3 reduction	V (74)	V (19)	–
Gelatin hydrolysis	V (46)	+	–

+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; numbers in parentheses are percentage of strains giving positive reaction.
Data from reference 367.

BOX 7-4

Minimum Requirements for Definitive Identification of *P. aeruginosa*

Identification based on all of the following:

1. Gram-negative rod
2. Oxidase-positive
3. Typical smell (fruity grapelike odor or corn tortilla)
4. Recognizable colony morphology
   a. On blood or chocolate agar, appear as large colonies with metallic sheen, mucoid, rough, or pigmented (pyocyanin) and oftenβ-hemolytic (Image Plate 7-2F)
   b. On MacConkey, appear as lactose negative with green pigmentation, or metallic sheen (Image Plate 7-2G)

Limitations:

1. Rare *Aeromonas* isolates may resemble *P. aeruginosa* (lacking the typical smell) but will be spot indole positive (*P. aeruginosa* are indole negative).

2. Some *Burkholderia cepacia* isolates from patients with CF may exhibit morphotypes that resemble *P. aeruginosa*.

From reference 208.

of carbapenemases in *P. aeruginosa* is difficult. The use of molecular techniques may be the only way to determine the extent to which carbapenemase-producing *P. aeruginosa* isolates have disseminated. If undetected by the laboratory, KPC-producing *P. aeruginosa* may become a reservoir for the transmission of this resistance mechanism.[910]

BOX 7-5

Virulence Factors of *Pseudomonas aeruginosa*

Virulence Factor	Biologic Activity
Alginate	Capsular polysaccharide that allows infecting bacteria to adhere to lung epithelial cell surfaces and form biofilms which, in turn, protect the bacteria from antibiotics and the body's immune system
Pili	Surface appendages that allow adherence of organism to GM-1 ganglioside receptors on host epithelial cell surfaces
Neuraminidase	Removes sialic acid residues from GM-1 ganglioside receptors, facilitating binding of pili
Lipopolysaccharide	Produces endotoxin, causes sepsis syndrome: fever, shock, oliguria, leukopenia or leukocytosis, disseminated intravascular coagulation, metabolic abnormalities
Exotoxin A	Tissue destruction, inhibition of protein synthesis; interrupts cell activity and macrophage response
Enterotoxin	Interrupts normal gastrointestinal activity, leading to diarrhea
Exoenzyme S	Inhibits protein synthesis
Phospholipase C	Destroys cytoplasmic membrane; destroys pulmonary surfactant; inactivates opsonins

Elastase	Cleaves immunoglobulins and complement components, disrupts neutrophil activity
Leukocidin	Inhibits neutrophil and lymphocyte function
Pyocyanins	Suppress other bacteria and disrupt respiratory ciliary activity; cause oxidative damage to tissues, particularly oxygenated tissues such as lung

Pseudomonas fluorescens* and *Pseudomonas putida. *P. fluorescens* and *P. putida* both include complexes of closely related species that are difficult to separate phenotypically and with MALDI-TOF MS. For this reason, laboratories are advised to report any organisms included in the *P. fluorescens* or *P. putida* complex as "*Pseudomonas fluorescens* complex," or "*Pseudomonas putida* complex." A list of organisms included in the *P. fluorescens* and *P. putida* complexes are shown in Box 7-6. Organisms included in the *P. fluorescens* and *P. putida* complexes occur in water and soil and may exist in water sources in the hospital environment. For practical purposes, if an isolate is encountered that produces fluorescein (pyoverdin) pigment and does not produce pyocyanin or grow at 42°C, laboratories may wish to report the isolate as "*P. fluorescens/putida* complex," with no further testing, since most laboratory isolates are not considered clinically significant. Both *P. fluorescens* and *P. putida* may exist as normal pharyngeal flora and in only rare occasions have been reported to cause infection in humans. *P. putida* has

BOX 7-6

Species Included in the *P. fluorescens* and *P. putida* Complexes[a]

Pseudomonas fluorescens Complex

P. antarctica
P. azotoformans
P. brassicacearum
P. brenneri
P. cedrina
P. congelans

P. corrugata
P. costantinii
P. extremorientalis
P. fluorescens
P. gessardii
P. libanensis
P. mandelii
P. marginalis
P. mediterranea
P. meridiana
P. migulae
P. mucidolens
P. orientalis
P. panacis
P. poae
P. protegens
P. proteolytica
P. rhodesiae
P. synxantha
P. thivervalsensis
P. tolaasii
P. trivialis
P. veronii

Pseudomonas putida Complex

P. fulva
P. monteilii
P. mosselii
P. oryzihabitans
P. plecoglossicida
P. putida

[a] Data from reference 31 and Schreckenberger P., personal communication.

been reported to cause catheter-related sepsis in patients with cancer[26,712] and septic arthritis.[675,679] Both groups have been associated with bacteremia from transfused blood.[557,763,870,962,1050,1061] *P. fluorescens* was reported to cause bloodstream infections associated with the use of syringes preloaded with heparin IV catheter flush in 36 patients in 4 states in 2004 to 2005. Twenty-eight additional patients developed delayed-onset bloodstream infections 84 to 421 days after last potential exposure to

the contaminated flush solution.[164] Of increasing importance are reports of *P. fluorescens* pseudobacteremias, the presence of positive blood cultures in the absence of true bacteremia and any related symptoms, most often attributed to contaminated catheters and catheter-related devices.[770,1003]

TABLE 7-6 Key Characteristics of the Stutzeri Group

Test	*P. stutzeri* Vb-1	*P. mendocina* Vb-2	CDC Group Vb-3
Oxidase	+	+	+
OF glucose	A	A	A
OF maltose	A	–	A
OF lactose	–	–	–
OF mannitol	V (70)	–	A
NO_3 reduction	+	+	+
NO_3 to gas	+	+	+
Arginine	–	+	+
Lysine	–	–	–
Starch hydrolysis	+	–	V (75)
Polymyxin B	S	S	S
Wrinkled colonies	+	–	–

+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; A, acid reaction; numbers in parentheses are percentage of strains giving positive reaction; S, susceptible.
Data from reference 367.

Stutzeri Group. The organisms in the stutzeri group are all soil denitrifiers and can grow anaerobically in nitrate-containing media, with production of nitrogen gas. Strains are motile by way of polar monotrichous flagella. They can grow with NH_4 as the sole source of nitrogen and acetate as the sole source of carbon for energy. Characteristics

that differentiate members of the stutzeri group are given in Table 7-6.

Pseudomonas stutzeri. *P. stutzeri* (formerly CDC group Vb-1) is ubiquitous in soil and water and has been recovered from humus, manure, straw, sewage, stagnant water, baby formula, hospital equipment, eye cosmetics, and various clinical specimens.[366,368,829] It has only rarely been associated with infections, such as otitis media,[366] conjunctivitis,[684,829] pneumonia,[146,151,806] septic arthritis,[678] endocarditis,[407,921] meningitis in an HIV-positive patient,[915] infections of synthetic vascular grafts,[357]infections of traumatic wounds,[366,371] and vertebral osteomyelitis.[897] It is susceptible to most antibiotics. Freshly isolated colonies are adherent and have a characteristic wrinkled appearance (Image Plate 7-3A), which may be lost after repeated laboratory subculture. *P. stutzeri* comprises a heterogeneous set of strains that includes at least 18 genomic groups without taxonomic status called genomovars.[199,352,927,930,973,993] However, the *P. stutzeri* genomovars are characterized by a high level of heterogeneity of phenotypic properties, which does not allow the taxa to be further categorized into different species.[927–929,993] Only one of the described *P. stutzeri* genomovars has been reclassified and validly published, as the new species, *Pseudomonas balearica*.[88] No human clinical isolates of *P. balearica* have yet been reported.

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Pseudomonas mendocina. *P. mendocina* (formerly CDC group Vb-2) and the unnamed species CDC group Vb-3 are rarely isolated from clinical specimens. *P. mendocina* colonies are smooth and have the appearance and consistency of butter. Rare but serious infections have been reported because of *P. mendocina*, including four reports of infective endocarditis,[38,517,726,1041] one report of sepsis,[792] and one patient with spondylodiscitis.[186] CDC group Vb-3 isolates resemble *P. stutzeri* except they are arginine positive. Potvliege and colleagues[866] have reported a patient with Vb-3 septicemia in a patient with multiple myeloma.

***Alcaligenes* Group.** Organisms in the alcaligenes group are characterized by being asaccharolytic or only weakly saccharolytic in OF glucose medium. Members of this group include *P. alcaligenes*, *P. pseudoalcaligenes*, and *Pseudomonas* species CDC group 1. The latter unnamed species is similar to *P. alcaligenes*, except that *Pseudomonas* species group 1 strains reduce both nitrate and nitrite to gas.[368] Two former subspecies of *P. pseudoalcaligenes, P. pseudoalcaligenes* subsp. *citrulli* and *P. pseudoalcaligenes* subsp. *konjaci* have been tranfered to the genus *Acidovorax* as *A. citrulli* and *A. konjaci,* respectively.[31] Characteristics that differentiate this group from other similar alkaline pseudomonads are given in Table 7-7. Although members

of this group are recovered from clinical specimens, their ability to act as human pathogens has only rarely been documented. There have been reports of *P. alcaligenes* causing eye infections, empyema, and one patient with fatal endocarditis.[1095]

Other Clinically Relevant *Pseudomonas* Species

Pseudomonas andersonii. *"P. andersonii"* is the name proposed for a new species of *Pseudomonas* associated with granulomatous lung lesions.[419,996] The five isolates described to date have all been recovered from surgically resected lung nodules. *Mycobacterium avium* complex was concomitantly isolated in two of the patients and fungal structures were identified histopathologically in two of the other patients.[996] All organisms grew overnight as on sheep blood agar as small, smooth, opaque, and light-brown colonies. They were positive for oxidase, catalase, and urease. All were negative for bile–esculin hydrolysis and citrate utilization and failed to assimilate most substrates tested. The isolates did not grow at 42°C and did not produce pyocyanin or pyoverdin pigments.[996] All five isolates were susceptible to most antibiotics that are active against gram-negative bacteria.[419,996] The relatively slow growth rate and absence of profiles in most identification system databases will likely lead to underrecognition of this organism.

Pseudomonas fulva. *P. fulva* is included in the *Pseudomonas putida* complex. Colonies are smooth, entire, flat to convex, and pale brownish yellow on nutrient agar. Smooth, yellow-pigmented colonies appear on sheep blood agar after overnight incubation.[886] Fluorescein (pyoverdin) and pyocyanin pigments are not produced and growth does not occur at 42°C.[1089] It is a strict aerobe, weak oxidase positive, catalase positive, nitrate negative, arginine dihydrolase positive, starch and casein hydrolysis negative.[1089] It can be identified reliably by MALDI-TOF MS using the MicroFlex LT instrument and BioTyper (version 3.1) software (Bruker Daltonics, Billerica, MA).[886] *P. fulva* has been reported to cause bloodstream infections,[655,972] and meningitis in patients with external ventricular drains.[19,886] In all instances, the isolates were incorrectly identified as *P. putida* by commercially available identification systems. Laboratories are alerted to conduct further testing, either MALDI-TOF MS or 16S rRNA sequencing on yellow-pigmented isolates that identify as *P. putida* on commercial identification systems.

Pseudomonas otitidis. *P. otitidis* is a novel *Pseudomonas* species recovered exclusively from infected human ears.[201] Cells are motile, gram-negative rods that appear as circular, concave, nonpigmented and nonhemolytic colonies on trypticase soy agar supplemented with blood.[201] Growth occurs at 7°C to 45°C. Colonies typically do not produce fluorescent pigment, and all strains are positive for oxidase and catalase, but negative for urease. Tween 80 and gelatin are hydrolyzed, but casein

and esculin are not.[201] Isolates oxidize glucose, but not mannose, mannitol, or xylose.[618] Roland and Stroman were the first to report *Pseudomonas "otitidis"* otic infections in the United States in 2002.[917] Subsequently, Motoshima et al. reported two isolates of *P. otitidis* from otic infections in Japan,[758] and Lee and colleagues[618] reported isolation of *P. otitidis* from a 53-year-old Korean man with purulent ear discharge. All strains appear to be susceptible to aminoglycosides, fluoroquinolones, polymyxin B, piperacillin, cefotaxime, ceftazidime, and aztreonam, while showing resistance or decreased susceptibility to carbapenems.[201,618,1067] All strains have been found to express MBL activity and to carry a new subclass B3 MBL gene, named $bla_{\text{POM-1}}$.[1067] As such, *P. otitidis* MBL-1 (POM-1) represents the first example of a resident MBL found in a pathogenic *Pseudomonas* species.[1067] Isolates are most often misidentified as *P. aeruginosa* on commercial identification systems; therefore, laboratories are advised that *Psedomonas* isolates that are oxidase positive and grow at 42°C but do not produce a blue-green pigment and demonstrate unusual β-lactam susceptibility patten including testing nonsusceptible to carbapenems should be identified further by 16S rRNA gene sequencing.

Family Burkholderiaceae—rRNA Group II

rRNA Group II consists of organisms that have been referred to in the past as the *Pseudomallei* group. Most species in this group have the unique property of exhibiting resistance to the polymyxin group of antibiotics (polymyxin B and colistin). The biochemical characteristics used to distinguish the members of this group are given in Tables 7-8, 7-9, and 7-10. This group comprises many species, including several plant-associated species. Only those that have been associated with human disease are included in this textbook. Two of the pathogenic species, *B. pseudomallei* and *B. mallei* are listed as category B biothreat agents by the CDC's Strategic Planning Workgroup because of their availability and potential to cause illnesses with high morbidity and mortality.[932]

Genus *Burkholderia*—*Pseudomallei* Group. At the time of this writing, there were 87 species recognized in the genus *Burkholderia*. *Burkholderia* species are human and plant pathogens as well as environmentally important bacteria. They are gram-negative, rod-shaped, motile, and generally obligately aerobic.

Burkholderia mallei. *B. mallei* is an obligate parasite of animals (primarily horses, mules, and donkeys) causing a respiratory tract infection known as glanders. The acute form

TABLE 7-7 Key Characteristics of Alkaline Pseudomonads[a]

	Comamonas				*Delftia*	*Pseudomonas*		*Brevundimonas*	
Test	***C. terrigena***	***C. aquatica***	***C. kerstersii***	***C. testosteroni***	***D. acidovorans***	***P. alcaligenes***	***P. pseudoalcaligenes***	***B. diminuta***	***B. vesicularis***
Oxidase	+	+	+	+	+	+	+	+	+
Growth on MacConkey	+ (91)	NA	NA	+	+	+	+	+	V (26)
OF glucose	Alk	Alk	Alk	Alk	Alk	Alk	Wk (19)	Wk (29)	Wk (57)
OF fructose	Alk	Alk	Alk	Alk	A	Alk	A	Alk	Alk
OF mannitol	Alk	Alk	Alk	Alk	A	Alk	Alk	Alk	Alk
NO_3 reduction	+	+	+	+	+	V (61)	+	–	–
NO_3 to gas	–	–	–	–	–	–	–	–	–
Gelatin hydrolysis	–	–	–	–	–	–	–	V (58)	V (38)
Esculin hydrolysis	–	–	–	–	–	–	–	–	+
Starch hydrolysis	–	–	–	–	–	V (16)	–	–	+
Tyrosine hydrolysis	+	–	+	+	NA	NA	NA	NA	NA
DNase	–	NA	NA	–	–	–	–	V (12)	–
Acetamide	–	NA	NA	–	+	–	–	–	–
Indole	–	–	–	–	Orange[b]	–	–	–	–
PYR[c]	+	–	–	+	+ (96)	–	–	V (12)	–
Susceptibility to 250 µg desferrioxamine[d]	S	S	S	R	R	V	V	S (92)	S
Alkaline phosphatase	–	–	–	–	–	–	–	+	+
Assimilation on API ID 32 GN of:									
3-Hydroxybenzoate	–	–	–	+	NA	NA	NA	NA	NA
4-Hydroxybenzoate	–	V	+	+					
L-Alanine	+	–	–	+					
Growth at 42°C	–	–	+	–	V (8)	V (48)	V (75)	V (19)	–
Flagellar arrangement	Polar or bipolar tufts, long wavelength (3.0 µm)					Single polar, normal wavelength (1.5 µm)		Single polar, short wavelength (0.5 µm)	

[a]Data from references 367 and 1160.
[b]Pumpkin orange color develops on addition of Kovac's reagent due to formation of anthranilic acid from tryptophan.
[c]PYR data from reference 598.
[d]Procedure described by Lindsay JA and Riley TV.[647]
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; A, acid reaction; Alk, alkaline reaction; NA, results not available; numbers in parentheses are percentage of strains giving positive reaction; R, resistant; S, susceptible; Wk, weak acid; PYR, pyrrolidonyl arylamidase.

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TABLE 7-8 Key Characteristics of *Burkholderia mallei*, *B. pseudomallei*, and *P. stutzeri*[a]

Test	*P. stutzeri*	*B. pseudomallei*	*B. mallei*
Oxidase	+	+	V
Motility	+	+	–
Growth on MacConkey	+	+	V
OF glucose	A	A	A
OF maltose	A	A	V
OF lactose	–	A	V
OF mannitol	V	A	V
NO_3 reduction	+	+	+
NO_3 to gas	+	+	–
Arginine	–	+	+
Lysine	–	–	–
Starch hydrolysis	+ (92)	–	NA
Polymyxin B	S	R	R
Wrinkled colonies	+	+	–
Pigment	Gray or slight yellow	Cream or tan	Gray

[a]Data from references 367 and 1172.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; A, acid reaction; numbers in parentheses are percentage of strains giving positive reaction; S, susceptible; R, resistant; NA, not available.

of the disease can kill a horse in a couple of weeks. In rare instances, it can be transmitted to humans, usually through an abrasion of the skin.[1146] A patient with laboratory-acquired glanders occurred in the year 2000.[163] Glanders shares with anthrax and plague the distinction of use in war. There is documentation that *B. mallei* was used as a biowarfare agent in World War I.[1173] *B. mallei* is a small gram-negative coccobacillus. On sheep blood agar, it appears as smooth, gray, translucent colonies in 2 days, without pigment or distinctive odor. It is the only nonmotile species in the genus. Other differentiating features are given in Table 7-8.

Burkholderia pseudomallei. *B. pseudomallei* causes melioidosis, a glanders-like disease in animals and humans (Box 7-7). From the rRNA and DNA–DNA results of Yabuuchi et al.,[1197] it is evident the *B. mallei* and *B. pseudomallei* belong to a single genomospecies, but it has been proposed that these two species be kept separate for epidemiologic and zoonotic reasons.

Epidemiology. *B. pseudomallei* has a specific ecologic niche, existing in soil and stagnant water in an area of latitudes 20 degrees north and south of the equator, primarily in Thailand, Vietnam, and parts of northern Australia.[237,244] Other reports confirm that it is endemic in China, Taiwan, and Laos as well, but the true incidence in most countries is unknown.[242,488] Very few patients with melioidosis have been reported from the Indian subcontinent despite similarities in environmental conditions with Southeast Asian countries, although, a patient with melioidosis brain and lung abscess after travel to Sri Lanka was reported in 1999.[831] Most melioidosis cases

reported in other regions arise from travel to areas endemic for *B. pseudomallei*. In the United States, patients with locally acquired melioidosis have been reported in the states of Oklahoma and Georgia.[718,794] The first Oklahoma strain was isolated in 1973 from a 27-year-old farmer with a deep leg wound heavily contaminated with soil as a result of a farming accident. Similar isolates were recovered from the soil near the accident site.[718] The Georgia isolate was recovered in 1977 from a patient that was involved in an automobile accident that resulted in the individual being propelled into a clay embankment. The patient sustained multiple facial fractures and lacerations and developed ulcers in the left eye leading to enucleation. Cultures from the noninfected anophthalmic orbit 8 weeks after enucleation yielded *B. pseudomallei*.[794] Because molecular studies of the Oklahoma strain suggested that it may be a separate species, Glass and colleagues reexamined the Oklahoma and Georgia isolates using standard microbiologic procedures, 16S rRNA gene sequencing, multilocus sequence typing (MLST), and DNA–DNA hybridization and determined that the isolates comprised a novel *Burkholderia* species and in 2006, proposed the name *Burkholderia oklahomensis* for these isolates.[378]

Clinical syndromes. Most infections are asymptomatic or present as a self-limited, short-term, flu-like illness and can be diagnosed only by serology.[49] It is estimated that thousands of US military personnel became infected with *B. pseudomallei* while serving in Southeast Asia in the 1960s and 1970s. Serologic surveys have revealed positive titers for this organism in 1% to 9% of US soldiers returning from Vietnam.[204,574,1024] Thus, with an estimated 3 million military

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TABLE 7-9 Key Characteristics of Select *Burkholderia Cepacia* Complex and Related Species

Test	*Burkholderia cepacia* Complex											*Pandoraea* species[a,b]	*Inquilinus limosus*[f]	*Ralstonia pickettii*		*Ralstonia mannitolilytica*
	B. cepacia[a] GV I	*B. multivorans*[a] GV II	*B. pseudomultivorans*[j]	*B. cenocepacia*[a] GV III	*B. stabilis*[a] GV IV	*B. Vietnamiensis*[a] GV V	*B. dolosa*[a,b] GV VI	*B. ambifaria*[a,c] GV VII	*B. anthina*[d] GV VIII	*B. pyrrocinia*[e] GV IX	*B. gladioli*[a]			Va-1	Va-2	Va-3
Oxidase	100[c]	100	100	100	100	100	100	100	100	+	0	67	+	100	100	100
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	−/D[g]	−/D[g]	−/D[g]
Lipase	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
OF glucose	100	100	100	95	100	100	100	100	100	+	100	11	0	100	100	100
OF maltose	39	98	100	78	93	97	100	100	100	V	0	0	NA	100	0	100
OF lactose	61	100	100	79	93	97	100	100	100	+	0	0	43	100	0	100
OF xylose	87	98	100	88	44	75	100	100	100	NA	96	0	NA	100	100	100
OF sucrose	87	0	78	88	0	94	0	95	V	+	0	0	0	0	0	0
OF adonitol	70	91	67	79	78	0	100	100	V	NA	93	0	NA	0	NA	NA
10% Lactose	100	100	NA	36	100	100	NA	NA	NA	NA	8		NA	81	0	100
ONPG	100	98	100	99	0	100	100	100	V	−	100	0	75	0	0	0
Lysine	100	53	100	99	100	100	0	100	V	+	0	0	0	0	0	0
Ornithine	30	0	0	71	100	0	0	0	0	NA	0	0	0	0	0	0
Nitrate reduced	4	94	V	31	4	47	100	67	V	+	33	11	0	87	100	20
Gelatin	74	2	0	55	93	0	0	94	0	V	70	0	38	77	40	80
Esculin	56	2	67	33	0	0	0	56	V	V	11	0	NA	0	0	0
Urease	91	100	NA	8	60	100	0	0	NA	−	75	63	25	100	100	100
Colistin, 10 µg	R	R	NA	R	R	R	R	R	R	R	R	R	R	R	R	R
Growth on MacConkey	83	96	100	84	93	83	100	100	100	+	96	100	NA	77	100	100
Growth at 42°C	43	100	100	84	0	100	100	26	V	−	4	89	100	26	60	60
Malonate utilization	100	90	NA	42	100	100	NA	NA	NA	NA	0	NA	NA	71[i]	75[i]	71[i]
Yellow pigmented	78	2	0	3	0	0	0	0	0	−	44	0	0	0	0	0

[a]Data from references 438 and 1108.
[b]Data from reference 213.
[c]Data from reference 215.
[d]Data from reference 1104.
[e]Data from reference 1034.
[f]Data from references 212 and 857.
[g]The catalase reaction for *R. pickettii* is often weak or delayed (Schreckenberger P, personal observation).
[h]Data from reference 209.
[i]Data from reference 832.
[j]Data from Table 7-3 (Schreckenberger P, personal observation).
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; numbers are percentage of strains giving positive reaction; S, susceptible; R, resistant; NA, not available; D, different results reported. Oxidation test results were recorded after 3 days of incubation; Roman numerals represent genomovars (GV) of *B. cepacia* complex; Va-1, Va-2, and Va-3 indicate CDC designations.

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TABLE 7-10 Key Characteristics of *Ralstonia* and *Cupriavidus* and Phenotypically Similar Nonfermenters[a]

Test	*Ralstonia*				*Cupriavidus*				*Alcaligenes*	*Bordetella*
	R. pickettii biovar VA-1	*R. pickettii* biovar VA-2	*R. mannitolilytica* biovar 3/thomasii	*R. insidiosa*	*C. gilardii*	*C. paucula*	*C. respiraculi*	*C. taiwanesis*	*A. faecalis*	*B. bronchiseptica*
Oxidase	D[b]	D[b]	+	+	+	+	+	+	+	+
Catalase	D[c]	D[b]	V (87)[d]	+	+	+	+	+	+	+
Lipase	+	+	+	+	–	+	–	+	–	–
OF glucose	+	+	+	+	–	–	–	–	–	–
OF maltose	+	–	+	–	–	–	–	–	–	–
OF lactose	+	–	+	–	–	–	–	–	–	–
OF xylose	+	+	+	+	–	–	–	–	–	–
OF sucrose	–	–	–	–	–	–	–	–	–	–
OF adonitol	NA	NA	NA	NA	–	–	–	–	–	–
OF mannitol	–	–	+	–	–	–	–	–	–	–
10% Lactose	V (81)[e]	–	+	NA	–	–	–	–	–	–
ONPG	–	–	–	–	–	–	–	–	–	–
Lysine	–	–	–	–	–	–	–	–	–	–
Ornithine	–	–	–	–	–	–	–	–	–	–
Nitrate reduced	+ (87)[e]	+ (100)[e]	– (20)[e]	+	V[f]	–	V	+	–	+ (100)[e]
Nitrite reduced	NA	NA	NA	NA	–	–	NA	NA	+	–
Gelatin	V (77)[e]	V (40)[e]	– (80)[e]	–	–	–	–	NA	– (4)[e]	–
Esculin	–	–	–	NA	–	–[d]	NA	+	–[d]	–[d]
Urease	+	+	+	V	–	++	–	–	–	++
Colistin 10 µg	R	R	R	R	S	S	S (97)[e]	S	S (100)[e]	S (96)[e]
Desferrioxamine[g]	S	S	R	NA	R	R	NA	NA	S	R
PYR[h]	+	+	+	+	–/+w	+	+	NA	–	–
Growth on MacConkey	+ (77)[e]	+	+	NA	NA	+ (90)[e]	+ (90)[e]	NA	+	+
Growth at 42°C	V (26)[e]	V (60)[e]	+ (60)[e]	NA	+	V (68)[e]	V (68)[e]	–	V (67)[e]	V (46)[e]
Growth in 6.5% NaCl	–	–	–	NA	–	–	–	–	+	–
Growth on acetamide	–	–	–	NA	–	–	–	NA	+	–
Malonate utilization	+	+	+	NA	V	NA	NA	NA	+	+
Yellow pigment	V (36)[d]	NA	V (33)[d]	NA	–	–	–	NA	–	–

[a]Except where noted, data from references 180, 210, 211, 221, and 1159.
[b]De Baere T et al.[262] report these reactions positive, Vandamme et al.[1103] report these reactions negative.
[c]The catalase reaction for *R. pickettii* is often weak or delayed (Schreckenberger P, personal observation.)
[d]Data from reference 1172.
[e]Data from reference 367.
[f]Data from reference 1122.
[g]Procedure described in reference 647.
[h]PYR data from reference 598.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; numbers are percentage of strains giving positive reaction; S, susceptible; R, resistant; NA, not available; +w, weak reaction; D, different results reported; PYR, pyrrolidonyl arylamidase; ++, strong positive reaction (4 hours).

BOX 7-7

Epidemiology, Diagnosis, Treatment, and Prevention and Reporting of Melioidosis (*Burkholderia pseudomallei*)

Epidemiology

- Melioidosis is an infectious disease caused by the bacterium *Burkholderia pseudomallei.*
- It is clinically and pathologically similar to glanders disease caused by *Burkholderia mallei.*
- Melioidosis is predominantly a disease of tropical climates, especially in Southeast Asia, where it is endemic, with the greatest concentration of cases reported in

Vietnam, Cambodia, Laos, Thailand, Malaysia, Myanmar (Burma), and northern Australia.
- The bacteria causing melioidosis are found in contaminated water and soil. Infection is acquired by inhalation of dust, ingestion of contaminated water, and contact with contaminated soil especially through skin abrasions, and for military troops, by contamination of war wounds. Person-to-person transmission can occur.
- Glanders is contracted by humans from infected domestic animals.
- *Burkholderia pseudomallei* and *Burkholderia mallei* are considered potential agents for biologic warfare and biologic terrorism.

Clinical Findings

- Four clinical presentations of melioidosis are described:
  - *Acute, localized infection*: This form of infection is generally localized as a nodule and results from inoculation through a break in the skin. The acute form of melioidosis can produce fever and general muscle aches, and may progress rapidly to infect the bloodstream.
  - *Pulmonary infection*: This form of the disease can produce a clinical picture of mild bronchitis to severe pneumonia. The onset of pulmonary melioidosis is typically accompanied by high fever, headache, anorexia, and general muscle soreness. Chest pain is common, but a nonproductive or productive cough with normal sputum is the hallmark of this form of melioidosis.
  - *Acute bloodstream infection*: Patients with underlying illness such as HIV, renal failure, and diabetes are affected by this type of the disease, which usually results in septic shock. The symptoms generally include respiratory distress, severe headache, fever, diarrhea, development of pus-filled lesions on the skin, muscle tenderness, and disorientation. This is typically an infection of short duration, and abscesses will be found throughout the body.
  - *Chronic suppurative infection*: Chronic melioidosis is an infection that involves the organs of the body. These typically include the joints, viscera, lymph nodes, skin, brain, liver, lung, bones, and spleen.
- The incubation period (time between exposure and appearance of clinical symptoms) is not clearly defined, but may range from 2 days to many years.

Laboratory Testing

- Organisms appearing as small gram-negative rods may be observed in direct Gram stain from respiratory specimens or abscess materials.
- *Burkholderia pseudomallei* can be recovered from the blood, urine, sputum, or skin lesions using standard laboratory media incubated at 35°C–37°C in ambient atmosphere.
- Detecting and measuring antibodies to the bacteria in the blood is another means of diagnosis.

Recommended Treatment

- For acute or chronic infections, parenteral administration of imipenem or ceftazidime for 2–4 weeks followed by oral therapy with amoxicillin-clavulanate or a combination of doxycycline and trimethoprim-sulfamethoxazole for 3–6 months is recommended.[488]
- Treatment should be initiated early in the course of the disease. Although bloodstream infection with melioidosis can be fatal, the other types of the disease are nonfatal.

Prevention and Reporting

- There is no vaccine for melioidosis.
- Prevention of the infection in endemic-disease areas can be difficult, since contact with contaminated soil is so common. Persons with diabetes and skin lesions should avoid contact with soil and standing water in these areas. Wearing boots during agricultural work can prevent infection through the feet and lower legs.
- Laboratory-acquired infections have been documented. In healthcare settings, using common precautions while handling blood and body fluids can prevent transmission.
- All patient specimens and culture isolates should be handled while wearing gloves and gowns in a biosafety cabinet. Plates should be taped shut when incubating. "Sniffing" of plates containing *B. pseudomallei* is dangerous and should not be done.
- Report possible cases of melioidosis to health authorities.

From the CDC (http://www.cdc.gov/melioidosis/index.html) and the American Society for Microbiology (http://www.asm.org/images/PSAB/Burkholderia101714.pdf).

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personnel serving in Vietnam from 1965 to 1973, as many as 250,000 may have become infected with *B. pseudomallei*.[752] An important feature of this disease is its ability to produce latent infection that can reactivate many years after primary exposure. For this reason, melioidosis has been dubbed the "Vietnamese time bomb" because the disease may still be incubating in American veterans of the Vietnam conflict.[387,752]

Infections are acquired by contact with the organism either by inhalation of dust or direct contact through breaks in the skin. Three forms of melioidosis have been described: (1) acute disease, presenting as septicemia with metastatic lesions; (2) subacute disease, presenting as a tuberculosis-like pneumonia with cellulitis and lymphangitis; and (3) chronic disease, presenting as a localized chronic cellulitis (Box 7-7). Fifty percent of patients with culture-confirmed melioidosis present with

pneumonia and another 20% of patients with other primary melioidosis presentations develop secondary pneumonia, especially those with positive blood cultures.[730] There has been one report of *B. pseudomallei* lymphadenitis and mediastinitis in a patient with chronic granulomatous disease.[295] Diabetes mellitus has been shown to be a risk factor for the development of bacteremic melioidosis.[1046] It is important to treat suspected cases with antibiotics before any additional treatment of patients, such as draining lesions, in order to avoid sepsis. The mortality rate is 95% in patients with acute disease who are not treated. *B. pseudomallei* is intrinsically resistant to many antibiotics, including the penicillins, first- and second-generation cephalosporins, macrolides, rifamycins, colistin, and the aminoglycosides.[245,513] It is generally susceptible to chloramphenicol, the tetracyclines, trimethoprim-sulfamethoxazole, ureidopenicillins, third-generation cephalosporins, carbapenems, and (unusual for a pseudomonad) amoxicillin-clavulanate.[1175,1204] The fluoroquinolones have only weak activity, and have proved very disappointing in clinical trials.[48,170] This unusual antibiotic profile (i.e., gentamicin- and colistin-resistant and amoxicillin-clavulanate–susceptible) in an oxidase-positive gram-negative rod is useful for confirming the identification of *B. pseudomallei* in the microbiology laboratory.[246] Ceftazidime has been the treatment of choice for treating severe melioidosis, but response to high-dose parenteral treatment is slow.[1175] Carbapenem antibiotics have been shown to be highly active against *B. pseudomallei* in vitro,[245,1008] and in one study, imipenem alone was shown to be an effective treatment for acute severe melioidosis, resulting in fewer treatment failures than ceftazidime alone.[999] In another study, the use of combination therapy with cefoperazone-sulbactam plus trimethoprim-sulfamethoxazole or ceftazidime plus trimethoprim-sulfamethoxazole appeared to be equally efficacious for treatment of severe melioidosis.[183]

Laboratory diagnosis. Definitive diagnosis of melioidosis, which has been called "the great mimicker," depends on the isolation and identification of *B. pseudomallei* from clinical specimens. *B. pseudomallei* grows well on standard laboratory media and often produces wrinkled colonies and thus morphologically may resemble *P. stutzeri*. It can be recovered from blood using standard blood-culture techniques.[1193] Selective agars, either Ashdown's selective agar (ASA)[46] or *Burkholderia pseudomallei* selective agar (BPSA)[482] or selective broths[1155] are recommended for isolation of *B. pseudomallei* from nonsterile body sites, particularly sputum from patients clinically suspected to have melioidosis, and for follow-up during treatment of the disease.[1194]The biochemical properties useful in identifying this organism are given in Table 7-8. Two biotypes of *B. pseudomallei* are categorized by their ability to assimilate L-arabinose.[1004] The arabinose nonassimilators (Ara–) are virulent and can be isolated from both clinical specimens and the environment, whereas the arabinose

assimilators (Ara+) are usually avirulent and with rare exceptions are found only in the environment.[627,1004] Identification of *B. pseudomallei* is usually not difficult in laboratories in areas where the organism is endemic. However, with the increase in international travel and the threat of bioterrorism, it has become more likely that laboratories in areas where *B. pseudomallei* is not endemic could encounter this organism. As many laboratories rely on commercial identification systems, Lowe and colleagues compared the accuracy of four systems, the manual APE 20NE and 20E, and the automated Vitek 1 and 2 systems (bioMérieux Inc. Hazelwood, MO) for their ability to correctly identify 103 strains of *B. pseudomallei*. The API 20NE, API 20E, and Vitek 1 systems gave the correct identification in 98%, 99%, and 99% of the isolates, respectively. The Vitek 2 failed to identify a large number of the *B. pseudomallei* strains largely because of differences in the biochemical reactions achieved compared to expected values in the database.[668] Koh and colleagues, using 47 strains of *B. pseudomallei,* tested what was then a relatively new automated identification system, the BD Phoenix System. Although *B. pseudomallei* is not in the Phoenix database, only four strains were read as no identification. The remaining strains were all incorrectly identified with high confidence as *B. cepacia* (34), *Burkholderia/Ralstonia* spp. (6), or another nonfermenter species (3).[583]

Rapid latex agglutination tests for detection of *B. pseudomallei* antigen in urine[279,1006,1007] have been developed that offer a simple, rapid and highly specific method for diagnosing melioidosis and are particularly useful in areas with limited laboratory facilities.

Laboratory workers are advised to use biologic safety hoods when working with this organism because laboratory-acquired infection with *B. pseudomallei* has been reported.[47,401,856,954] Additional information on the clinical and laboratory features of melioidosis can be found in other published reviews.[242–244,622,1175]

***Burkholderia cepacia* Complex.** *Burkholderia cepacia* is a phytopathogen that causes onion bulb rot in plants and foot rot (i.e., jungle rot) in humans.[1059] Since the early 1980s, *B. cepacia* has emerged as a cause of opportunistic human infections, particularly in patients with chronic granulomatous disease[114,597,802] and CF.[381,499,997,1071] Recent taxonomic advances have demonstrated that *B. cepacia* is actually a cluster of at least 18 closely related genomic species (or genomovars) now called the *B. cepacia* complex (Bcc) which can be differentiated on the basis of molecular and biochemical tests.[213,215,219,375,438,1100,1104,1107,1108,1110,1134] See Box 7-8 for a list of species included in the Bcc. Although all genomovars of Bcc have been cultured from the sputa of patients with CF, *B. cenocepacia* (genomovar III) and *B. multivorans* (genomovar II) account for the majority of the isolates recovered from patients in North America[302,652,826,888,1019] and Europe.[7,281,302,692] A recent study conducted at two CF centers in Argentina showed that *B. contaminans* was the

species

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BOX 7-8

Species Included in the *Burkholderia cepacia* Complex (Bcc)[a]

B. ambifaria (VII)[b]
B. anthina (VIII)
B. arboris
B. cenocepacia (III)
B. cepacia (I)
B. contaminans
B. diffusa
B. dolosa (VI)
B. lata
B. latens
B. metallica
B. multivorans (II)
B. pseudomultivorans
B. pyrrocinia (IX)
B. seminalis
B. stabilis (IV)
B. ubonensis
B. vietnamiensis (V)

[a] Data from references 832 and 1100.

[b] Roman numerals in parentheses indicate original genomovar designations.

most frequently recovered from CF patients (57.6%) followed by *B. cenocepacia* (15%).[710] Patients with CF who are colonized with *B. cepacia* have a higher mortality in the year following colonization and have a more precipitous decline in overall pulmonary function following colonization.[635] These bacteria are also those most frequently associated with epidemic spread and with "cepacia syndrome," which is manifested by severe progressive respiratory failure and bacteremia.[43,219,280,390,648,682,1190] Jones et al.[519] reported that genomovar status of Bcc may influence both the likelihood of progression from initial to chronic infection

and the overall survival of the patients. In their study, patients with either *B. multivorans* or *B. cenocepacia* developed chronic infection with deaths from "cepacia syndrome" occurring in both groups.[519] Chaparro et al.[171] found a poorer outcome for CF patients with *B. cepacia* infection following lung transplantation, and De Soyza et al.[280] reported a worse outcome following transplantation with *B. cenocepacia* than with other Bcc species. Aris et al.[43] also found that decreased survival after lung transplantation was linked to genomovar status with a worse outcome for CF patients with *B. cenocepacia* infection. Although the evidence suggests that access to lung transplantation should not be denied to all Bcc-infected CF patients, both the short- and long-term survival are significantly lower when *B. cenocepacia*-infected patients are compared to those infected with other Bcc genomovars.[800]

In addition, several epidemiologic studies have indicated that certain Bcc strains are transmissible between patients and that cross-infection probably occurs by direct person-to-person spread.[161,389,650,651,1002] Virulence markers such as the cable (cbl) pilus encoded by the cable pilin subunit gene (*cblA*) that mediates adherence to mucus glycoproteins and enhances adherence to epithelial cells,[302,382,943,944,1043] and the *B. cepacia* epidemic strain marker (BCESM)[681] designated such because of its association with *B. cepacia* strain types infecting multiple patients with CF, occur almost exclusively in *B. cenocepacia*.

B. cepacia complex has been isolated from numerous water sources and wet surfaces, including detergent solutions and intravenous fluids. Hospital outbreaks of *B. cepacia* infection are usually due to a single contaminated source such as anesthetics,[111] disinfectants,[821,1011] intravenous solutions,[290,1125] nebulizer solutions,[417,495] mouthwash,[162,708,1222] and medical devices, including respiratory therapy equipment.[667,1162] Disinfectants in which *B. cepacia* will grow include povidone–iodine, quaternary ammonium compounds, and chlorhexidine.[91,234,1011] Pseudobacteremias (false-positive blood cultures) have been reported following the use of *B. cepacia*-contaminated disinfectant solutions.[91,234,813,821] *B. cepacia* can also grow in distilled water with a nitrogen source owing to the ability of this organism to fix CO_2 from air. Clinical infections include pneumonia and pneumonitis in patients receiving contaminated anesthetics, urinary tract infection in patients receiving contaminated irrigation fluids following catheterization or cystoscopy, septicemia following heart surgery, endocarditis caused by contaminated heart valves, conjunctivitis, and septic arthritis.[813] Peritonitis following peritoneal dialysis has been associated with povidone–iodine solution contaminated with *B. cepacia*.[821] Infections involving the central nervous system include one child with congenital hydrocephalus who developed bacteremia secondary to a contaminated Holter ventriculoatrial shunt[73] and an adult who developed a brain abscesses secondary to chronic suppurative otitis

media.[445]

Selective media with bacteriostatic dyes, antibiotics, or low pH have been described for the selective isolation of *B. cepacia*. These include *Pseudomonas cepacia* medium (PCM) containing crystal violet, polymyxin B, and ticarcillin[374]; oxidation-fermentation basal medium containing polymyxin B, bacitracin, and lactose (OFPBL)[1164]; and *Burkholderia cepacia*- selective agar (BCSA) containing lactose, sucrose, polymyxin B, gentamicin, and vancomycin.[436] Comparative evaluations of these media have shown that the recovery of *B. cepacia* from patients with CF is enhanced with the use of such media.[437,1128,1135,1192] Overall, BCSA (predominantly used in the United States) and Mast *B. cepacia* medium (predominantly used in Europe) have been found to be the most suitable for growth of all *B. cepacia* complex isolates.[1135,1192]

Identification of *B. cepacia* in the clinical laboratory is problematic because *B. cepacia* is not a single phenotype but a complex of at least 18 separate genetic species (Box 7-8).[722,1100] Commercial identification systems have performed poorly in identifying these organisms. Kiska et al.[575] compared four commercial systems, including the RapID NF Plus (Remel), API Rapid NFT (renamed API 20NE (bioMérieux), Vitek GNI (bioMérieux), and Uni-N/F Tek (Remel). Correct identification for *B. cepacia* was 86% for Uni-N/F Tek, 81% for RapID NF Plus, 50% for Vitek GNI and 43% for API 20NE. Van Pelt and colleagues reported that 90% of *B. cepacia* strains were identified correctly with the API 20NE and Vitek GNI, with 68% identified correctly with the MicroScan WalkAway overnight urine combo type 1 panels.[1128] Shelly et al.[977] evaluated the performance of nine

different commercial systems used in 108 clinical microbiology laboratories from 91 US cities that referred isolates to a CF reference laboratory for confirmatory testing using a polyphasic approach that included genus and species-specific rRNA-based polymerase chain reaction (PCR) assays. Positive predictive values ranged from 71% to 98%, negative predictive values ranged from 50% to 82%, and all systems gave misidentifications for *B. cepacia* complex. The species most frequently misidentified as *B. cepacia* was *Burkholderia gladioli*. Brisse et al.[132] compared the BD Phoenix and Vitek 2 automated instruments for identification of isolates of the *B. cepacia* complex. Rates of correct identification were 50% for BD Phoenix and 53% for Vitek 2 when all *B. cepacia* complex isolates were considered, but differed markedly for *B. cenocepacia* (genomovar III) (71% for Phoenix and 38% for Vitek 2) and *B. multivorans* (genomovar II) (58% for Phoenix and 89% for Vitek 2). Correct identification to the species level has been reported to be as low as 33.3% for *B.*

cepacia using the Vitek MS v2.0 system.[691] The results of these studies support the recommendation that when using commercial systems for identification of *B. cepacia* complex isolates, results should be confirmed by performing supplemental biochemical tests (Table 7-9) or by molecular methods.

MALDI-TOF MS of intact cells has also emerged as a powerful and rapid method for the identification of NFBs including members of the Bcc. However, even though genus-level identifications are reported to be in the 90th percentile, identification to species level for the Bcc is often less than 80%.[100,270,278,328,477,604,691,701,719,1126] Laboratories are advised that when using MALDI-TOF for identification, any organisms listed in Box 7-8 should be reported as *B. cepacia* complex.

Unlike other common pseudomonads, *B. cepacia* is resistant to aminoglycoside antibiotics but is usually susceptible to trimethoprim-sulfamethoxazole, which has become the drug of choice in treating *B. cepacia* infections. Daniel et al.[253] performed in vitro susceptibility studies on 36 *B. cepacia* blood isolates and found that most strains were susceptible to minocycline (94.4%), ceftazidime (86.1%), ciprofloxacin (83.3%), and trimethoprim–sulfamethoxazole (83.3%). In two- and three-drug synergy studies performed by Bonacorsi and colleagues,[109] the addition of ciprofloxacin significantly enhanced the killing activities of piperacillin, imipenem, and meropenem, while the three-drug combination of β-lactam–ciprofloxacin–tobramycin gave the most consistently synergistic effect. Aaron and colleagues[1] also performed in vitro testing using multiple antibiotic combinations for *B. cepacia* isolates. They reported that using double-antibiotic combinations improved bactericidal activity for the following drug combinations: meropenem–minocycline, meropenem–amikacin, and meropenem–ceftazidime. Triple-antibiotic combinations that contained tobramycin, meropenem, and an additional antibiotic were most effective and were bactericidal against 81% and 93% of isolates.[1] In another study, Nzula and colleagues showed that antibiotic MICs vary widely for the different strains of the *B. cepacia* complex.[795] Although all strains were resistant to polymyxin B, MICs for chloramphenicol ranged from 4 to 128 mg/L and those for trimethoprim from 0.25 to 64 mg/L, with the majority of strains exhibiting resistance. Similarly, the MIC ranges for tobramycin, ciprofloxacin, and ceftazidime varied widely, suggesting the need for laboratories to perform susceptibility testing on individual isolates. Vermis et al.[1135] noted that the MICs of clinical isolates originating from patients with CF were higher than those isolated from patients who did not have CF and that in general, *B. cepacia* complex isolates were more susceptible to rifampicin, trimethoprim–sulfamethoxazole, and imipenem and were most resistant to polymyxin B.

For further information on the biology, taxonomy, mechanisms of virulence, and epidemiology of *B. cepacia*, several excellent reviews can be consulted.[219,373,390,520,648,680,1100,1135]

Burkholderia gladioli. *B. gladioli* (formerly named *P. marginata*) is primarily a plant pathogen causing "flower rot" in gladiolus and other plants. It is one of the few pseudomonads that is negative for cytochrome oxidase, and it produces nonfluorescent yellow colonies after 48 to 72 hours of incubation. It has been reported to cause human pulmonary disease and sometimes bacteremia and soft tissue infections in patients with CF,[66,195,395,521,538,558,753] chronic granulomatous disease,[444,924] diabetes,[983] and other immunologic deficiencies.[395] *B. gladioli* has also been reported to cause keratitis, endophthalmitis, and corneal ulcers,[629,909] as well as osteomyelitis in association with chronic granulomatous disease.[126]

B. gladioli usually grows as yellow colonies because of production of a diffusible, nonfluorescent yellow pigment. Biochemical tests that enable differentiation from *B. cepacia* complex include negative reactions for lysine, maltose, and lactose. Most strains are oxidase negative or weakly oxidase positive and catalase positive. Further differentiating characteristics are given in Table 7-9. Laboratory workers should be aware that phenotypic methods for differentiation of *B. gladioli* from the organisms of the *B. cepacia* complex are unreliable and may lead to misidentification.[205] Commercial identification systems often fail to discriminate *B. gladioli* from related species.[977,1128] Therefore, molecular-based methods should be used when confirmation of an isolate as *B. gladioli* is deemed necessary.[76,205,1174] The antibiotic susceptibility pattern may also serve as a clue that an organism might be *B. gladioli,* if it tends to be susceptible to aminoglycosides, imipenem, ciprofloxacin, and trimethoprim–sulfamethoxazole and resistant to aztreonam and cephalosporins.[924]

Genus *Ralstonia* and Genus *Cupriavidus.* Palleroni[814] and later Li et al.[638] demonstrated two DNA homology groups within the *Pseudomonas* RNA homology group II. In 1992, Yabuuchi proposed the generic name *Burkholderia* for seven species but noted that *B. pickettii* and *B. solanacearum* were similar to each other and different from the other five *Burkholderia* species named at the time.[1197] In 1995, Yabuuchi et al.[1198] proposed the transfer of *B. pickettii, B. solanacearum,* and *Alcaligenes eutrophus* to the new genus *Ralstonia* with *R. pickettii* serving as the type species. Subsequently, several new species or new combinations were added to the genus *Ralstonia*. Comparative 16S rRNA sequence analysis now indicates that two distinct sublineages exist within the genus *Ralstonia*. The first, which is called the *Ralstonia eutropha* lineage, is composed of *R. basilensis, R. campinensis, R. eutropha, R. gilardii, R. metallidurans, R. oxalatica, R. paucula, R. repiraculi,* and *R. taiwanensis*. The second is the *Ralstonia pickettii* lineage, which is composed of *R. insidiosa, R. mannitolilytica, R. pickettii, R. solanacearum,* and *R. syzygii*. This separation is supported

BOX 7-9

Characteristics for Differentiation of *Ralstonia* and *Cupriavidus*

Characteristic	*Ralstonia*	*Cupriavidus*
Flagellation	Polar, 1–4	Peritrichous
Colistin (10-µg disks)	Resistant	Susceptible
Acid from carbohydrates	Positive	Negative

by phenotypic differences. Members of the *R. eutropha* lineage have peritrichous flagella, do not produce acids from glucose and are colistin-susceptible, in contrast members of the *R. pickettii* lineage have one or more polar flagella, produce acid from several carbohydrates and are colistin resistant (Box 7-9).[262,1122] Vaneechoutte and colleagues proposed that the species of the *R. eutropa* lineage be reclassified into the new genus *Wautersia*.[1122] Subsequently, DNA–DNA hybridization experiments and an evaluation of phenotypic characteristics, DNA base ratios, and 16S rRNA gene sequences demonstrated that *Wautersia eutropha*, the type species of the genus *Wautersia*, is a later synonym of *Cupriavidus necator*, the type species of the genus *Cupriavidus*. Therefore, all members of the genus *Wautersia* have been reclassified into the genus *Cupriavidus*.[1099]

Ralstonia pickettii. *Ralstonia pickettii* was created for a group of clinical isolates[876] and also turned out to include strains of CDC groups Va-1 and Va-2, which were regarded as two different biovars of *R. pickettii*.[851] Shortly before this, King et al.[567]reported that *R.* ("*Pseudomonas*") *pickettii* contained several biovars, including the strains originally isolated from St. Thomas Hospital, London,[845,846] which had been designated "*Pseudomonas thomasii*," although the name was never validly published. In 1994, Pickett[848] noted that *R. pickettii* should be recognized as having three biovars designated biovar Va-1, biovar Va-2, and biovar 3/*thomasii*. De Baere et al.[262] subsequently confirmed by DNA–DNA hybridization that *R. pickettii* biovar 3/*thomasii* was a separate species and proposed the name *Ralstonia mannitolytica,* later corrected to *Ralstonia mannitolilytica*.[653]

R. pickettii is rarely associated with human infections, but it has been reported to cause nosocomial infections, including bacteremia and urinary tract infections. Nosocomial outbreaks have occurred with contaminated respiratory care solutions,[596] heparin solutions,[706] "sterile" saline solutions,[160,184] distilled water[549,703] and a contaminated water irrigation system.[1209] Pseudo-outbreaks have also been reported because of contaminated blood-culture bottle caps[116] and water splashed from a sink.[431] There have been several reports of primary infection caused by *R. pickettii* including vertebral osteomyelitis and diskitis in a patient receiving long-term hemodialysis,[1169] community-acquired pneumonia,[816,880] bacteremia in a cord-blood-transplant recipient,[1187] bacteremia in cancer patients,[734,835] sepsis in a hemodialysis patient,[1035] neonatal sepsis in a premature infant[1142] and meningitis in a presumably healthy rancher[430] and a child with hydrocephalus.[110]

R. pickettii is slow-growing and produces only pinpoint colonies on BAPs after 24 hours. All strains are urease positive and some strains may be catalase negative. Motility is weak or delayed and may not be detectable. Biovar Va-1 can be separated from Va-2 by the oxidation of lactose and maltose. For additional biochemical characteristics see Table 7-10.

Ralstonia mannitolilytica. *R. mannitolilytica* is the name given to organisms formerly classified as *R. pickettii* biovar 3/*thomasii* (see discussion above).[262] It has been recovered from the sputum of patients with CF[216,220,329] and one patient each with recurrent meningitis and hemoperitoneum,[1117] peritoneal fluid in patients undergoing peritoneal dialysis,[297,1221] bloodstream infection in a premature infant associated with a humidifying respiratory therapy device[103] and catheter-related bacteremia in five patients on a hematology-oncology ward.[409] A national outbreak of *R. mannitolilytica* colonization or infection in pediatric patients associated with use of a contaminated oxygen delivery device (Vapotherm 2000i, Vapotherm Inc., Stevensville, MD) was reported in the period of December 2004 through August 2005. Pulse-field gel electrophoresis showed related strains of *R. mannitolilytica* were recovered from 38 patients at 18 hospitals in 12 states in the United States.[514] *R. mannitolilytica* can be distinguished from other *Ralstonia* species by its acidification of D-arabitol and mannitol, its lack of nitrate reduction and alkalinization of tartrate. Other characteristics are given in Table 7-10.

Ralstonia insidiosa. *R. insidiosa* strains have been isolated from the environment as well as human clinical samples, including respiratory secretions of patients with CF.[211,1122] Strains of *R. solanacearum* and *R. syzygii* have not been isolated from human clinical specimens. Characteristics for identification of *Ralstonia* species of human origin are given in Table 7-10.

Antibiotic susceptibility of *R. pickettii* and *R. insidiosa* has been reported by Ryan

and Adley.[935] The majority of isolates tested showed susceptibility to most antibiotics. The most effective were found to be quinolones and sulfamethoxazole/trimethoprim.[935]

The genus *Cupriavidus* is composed of former *Ralstonia* species that are asaccharolytic, colistin-susceptible, and motile, with peritrichous flagella, except for *C. gilardii*, which has a single polar flagellum (Box 7-9). At the time of this writing, the genus included 14 species: *C. alkaliphilus*, *C. basilensis, C. campinensis, C. gilardii, C. laharis, C. metallidurans, C. necator* (type species formerly *Ralstonia eutropha*), *C. numazuensis, C. oxalaticus, C. pampae, C. pauculus, C. pinatubonensis, C. respiraculi,* and *C. taiwanensis*.[1099] Selected species that have been associated with human infection are discussed in more detail below.

Cupriavidus gilardii. *C. gilardii* (formerly *Ralstonia gilardii*) was named in honor of G.L. Gilardi, an American microbiologist who contributed much to our knowledge of NFBs. It is phenotypically similar to *Alcaligenes faecalis,* and was referred to as *A. faecalis*–like by Gilardi. It is a gram-negative, nonfermenting, asaccharolytic bacillus that is motile by means of peritrichous flagella,[1159] although in the original description by Coenye et al.[210] they were described as being motile by means of a single polar flagellum. Catalase and oxidase are positive. Nitrate and nitrite are not reduced. It can be differentiated from *A. faecalis* by the absence of nitrite reduction and failure to grow on acetamide and in 6.5% NaCl. Human isolates have been from the respiratory tract,

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a furuncle, cerebrospinal fluid (CSF), bone marrow, thigh muscle abscess,[1062] and blood.[210,541,1159] Coenye et al.[216] reported *C. gilardii* in the respiratory secretions of 10 patients with CF, which represented 9% of the *Ralstonia/Cupriavidus* species isolated from CF patients.

Cupriavidus metallidurans. *C. metallidurans* (formerly *Ralstonia metallidurans*) is known for its ability to survive in high concentrations of toxic heavy metals: metallidurans literally translates to "metal-enduring."[386] Coenye et al.[216] reported *C. metallidurans* in the bronchial secretions of two patients with CF, but its pathogenic or colonizing role could not be determined. There have been two reports of nosocomial septicemia,[607,742] and one report of catheter-related bacteremia[286] caused by *C. metallidurans.* At one of our hospitals, *C. metallidurans* >100,000 CFU/mL was isolated from the urine of an 80-year-old renal transplant patient who was admitted due to respiratory distress caused by fluid overload. The identification was obtained using the Bruker MALDI-TOF MS with a value score of 2.537 and was confirmed with 16S rRNA sequencing. The isolate was susceptible to ampicillin, cefepime,

ceftazidime, ceftriaxone, ciprofloxacin, ertapenem, imipenem, meropenem, and piperacillin/tazobactam, trimethoprim/sulfamethoxazole and resistant to amikacin, cefazolin, gentamicin, nitrofurantoin, and tobramycin using the Clinical and Laboratory Standards Institute (CLSI) non- *Enterobacteriaceae* interpretive breakpoints (Schreckenberger P, personal communication).

Cupriavidus pauculus. *C. pauculus* (formerly *Ralstonia paucula* and CDC group IV c-2) is an oxidase-positive, nonsaccharolytic gram-negative rod that is motile by means of peritrichous flagella and is strongly urease positive. It therefore phenotypically resembles *Alcaligenes*, *Bordetella,* and *Oligella ureolytica*, from which it must be differentiated (Table 7-10). Most reports of human infection have been immunocompromised patients with bacteremia.[30,39,41,235,240,713,738,789,881,945,1069,1213] There have also been two reports of peritonitis following continuous ambulatory peritoneal dialysis, one with accompanying septicemia[1213] and one with a mixed infection with IVc-2 and *Alcaligenes faecalis*[422]; and one patient with tenosynovitis of the hand following a cat bite.[764] In the latter case, it is not clear whether the source of the infectious agent was the cat bite or the tap water used to rinse the lesion. *C. pauculus* has also been reported to cause bacteremia in a child on extracorporeal membrane oxygenation (ECMO),[1092] concomitant meningitis and septicemia in a neonate,[303] and community-acquired pneumonia in a newborn.[55]

Cupriavidus respiraculi. *C. respiraculi* (formerly *Ralstonia respiraculi*) has been recovered from the respiratory tract of patients with CF, although the isolates did not grow on BCSA.[221] Characteristics that differentiate *C. respiraculi* from other *Cupriavidus* species are given in Table 7-10.

Cupriavidus taiwanensis. *C. taiwanensis* (formerly *Ralstonia taiwanensis*) has also been isolated from the sputum of a patient with CF.[180] It is a nonsaccharolytic gram-negative rod that is oxidase, catalase, nitrate, and esculin positive. Additional biochemical characteristics are given in Table 7-10.

Genus *Lautropia*

Lautropia mirabilis. *L. mirabilis* is a motile facultatively anaerobic gram-negative coccus that ferments glucose, fructose, sucrose, and mannitol; reduces nitrate and nitrite; and produces positive reactions for oxidase, urease, and, sometimes weakly, catalase.[361] Phylogenetic characterization based on 16S rRNA gene sequence analysis places this species in the β-subgroup of the *Proteobacteria*, separate from all other described genera, but most closely related to *Burkholderia*.[361] Interestingly, the cellular fatty acid composition of *L. mirabilis* is most similar to the cellular fatty acid of *Acidovorax delafieldii*, *Comamonas terrigena,* and CDC weak oxidizer group 1 (WO-1).[247] Coccoid morphology and the ability to ferment glucose separates *L.*

mirabilis from *C. terrigena*, *A. delafieldii*, WO-1 and members of the *Burkholderia* group. *L. mirabilis* displays extremely polymorphic cell morphology. At least three colony morphologies are seen: (1) flat, dry, circular colonies in young cultures, (2) larger, wrinkled, crisp, and crater forms on prolonged incubation and (3) smooth, glistening, raised, round, mucoid colonies. The colony diameter varies between pinpoint and more than 5 mm, and colonies are usually adherent to the agar.[361] *L. mirabilis* has been recovered from oral and upper respiratory sites,[361] the sputum of a patient with CF,[86] and the oral cavities of children infected with HIV.[931] Its pathogenic potential is unknown.

Genus *Pandoraea*. In 2000, Coenye et al.[209] described a new genus, *Pandoraea* (referring to Pandora's box in Greek mythology) of gram-negative nonfermenters isolated primarily from sputa of patients with CF and from soil. Originally, this genus contained five named species (*P. apista*, *P. norimbergensis*, *P. pnomenusa*, *P. pulmonicola,* and *P. sputorum*) and one unnamed genomic species.[209] Daneshvar et al.[250] described three additional unnamed genomospecies, previously classified as CDC weak oxidizer group 2 (WO-2). More recently four additional named species (*P. thiooxydans, P. oxalativorans, P. faecigallinarum, P. vervacti*) from environmental or animal sources have been added bringing the total of named species to nine.[27,939] *Pandoraea* spp. have been isolated mainly from the respiratory secretions of patients with CF, but have also been found in other clinical samples, including blood and lung tissue.[53,231,524,746,1038] Epidemic spread of *P. apista* from an index patient to five other patients with CF participating in winter camps and/or who had been hospitalized has been reported.[524] At present, the prevalence, patient-to-patient transmissibility, and clinical impact of *Pandoraea* organisms in patients with CF are unknown because the usual manual and automated phenotypic identification methods used in most clinical laboratories are not satisfactory in identifying *Pandoraea* spp.[214,746] *Pandoraea* species will usually grow well on *B. cepacia*-selective medium and therefore, can be misidentified as members of the *B. cepacia* complex. *Pandoraea* species grow well on BAP after overnight incubation at 35°C. Isolated colonies appear circular, convex, semiopaque, entire, and smooth and are 0.5 to 1 mm in diameter. Hemolysis is variable. All species are motile, with polar flagella, and are positive for growth on MacConkey agar, production of catalase, and alkalinization of citrate. All species are negative for reduction of nitrite; denitrification; indole production; gelatin, esculin, and tween hydrolysis; lysine and ornithine decarboxylase; and arginine dihydrolase, β-galactosidase, and DNase activity. No acid is

produced from OF base mannitol, lactose, sucrose, maltose, or fructose. Strains produce weak acid or are negative in OF glucose.[209,250] For features that differentiate *Pandoraea* from phenotypically similar organisms, see Table 7-9.

Family Rhodospirillaceae

Genus *Inquilinus*. The genus *Inquilinus* belongs to the family *Rhodospirillaceae* and includes two species, *I. ginsengisoli* and *I. limosus*. Only *I. limosus* has been associated with infection in humans. *I. limosus* is a rod-shaped gram-negative bacterium that measures 1.5 to 2 mm in width by 3.5 mm in length. It grows at 35°C and 42°C, but poorly at 25°C. It forms very slimy, nonpigmented colonies on nonselective media. Growth on MacConkey agar is very slight after 3 days. Some strains are motile with one or two polar flagella, but motility is difficult to demonstrate because of the mucoid nature of the colonies. It is polymyxin B resistant and lipase positive, making it appear phenotypically similar to the *B. cepacia* complex (Table 7-9).[212,857] All strains are positive for oxidase, catalase, β-glucosidase, phosphatase, proline aminopeptidase, pyrrolidonyl aminopeptidase, esculin hydrolysis, and acetoin production and negative for lysine, arginine, ornithine, denitrification, gelatinase, indole production, and citrate utilization.[189,212] Acid is formed in OF fructose, dextrose, and mannitol (Table 7-3). H_2S is positive by the lead acetate paper strip test but not in TSI agar (Christopher Doern, personal communication). *I. limosus* has been recovered from respiratory secretions of patients with CF in the USA,[212,429,857] Germany,[956,1165] France,[98,189,270] Italy,[197] Spain,[948] and the UK.[227] There is one report of *Inquilinus* sp. being recovered from blood samples of a patient without CF who had prosthetic valve endocarditis.[570] Definitive identification is difficult because it is not contained in the databases of commercial identification kits and its mucoid appearance may lead to confusion with mucoid *P. aeruginosa* strains.[857,1165] Isolates can be recovered on colistin containing *B. cepacia*- selective media, but are inhibited on BCSA, which contains gentamicin.[189] The mucoid nature of *I. limosus* may contribute to its colonization and resistance to many antimicrobial agents.[98,440] All isolates are reported to be resistant to penicillins and cephalosporins, kanamycin, tobramycin, colistin, doxycycline, and co-trimoxazole, but susceptibile to imipenem and ciprofloxacin.[189,1165] There has been no patient-to-patient transmission reported.

Family Comamonadaceae—*rRNA Group III*

Willems and colleagues have proposed that the organisms belonging to rRNA group III be recognized as a new bacterial family, the *Comamonadaceae*.[1176]

***Acidovorans* Group.** This group consists of the organisms formerly named

Pseudomonas acidovorans, Pseudomonas testosteroni and *Comamonas terrigena*. In 1987 Tamaoka[1055] proposed that the organisms known as *Pseudomonas acidovorans* and *P. testosteroni* be placed in the genus *Comamonas,* along with the species *C. terrigena*. Subsequently, *C. acidovorans* was placed into a new genus *Delftia* as the type species *D. acidovorans*.[1166] All are motile by way of a polar tuft of up to six flagella, with the distinctive feature of having a long wavelength (3.0 mm between the top of adjacent waves). Acid is not produced in OF glucose medium, and thus these organisms are grouped among the alkaline pseudomonads (Table 7-7). As a group, the comamonads have a wide geographical distribution and are common soil and water saprophytes. They have been isolated from animal sources, foodstuffs, hospital equipment and human clinical specimens, but they are rarely clinically significant.

Genus *Delftia*

Delftia acidovorans. The genus *Delftia* consists of four speciens: *D. acidovorans, D. lacustris, D. litopenaei,* and *D. tsuruhatensis. D. acidovorans* is the most common of this group to be isolated from clinical specimens. It can be easily distinguished from other alkaline pseudomonads because of the production of a weak acid or neutral reaction in OF fructose and OF mannitol. It is acetamide positive and reduces nitrate without gas formation. It is indole negative, but most strains produce an orange color in the medium on the addition of Kovacs' reagent (so-called orange indole) because of the production of anthranilic acid from tryptone (Image Plate 7-2C).[705] The orange indole reaction can also be demonstrated by adding a drop of Kovacs' reagent to colonies growing on the surface of BAPs. When the indole test is performed with xylene extraction and the addition of Ehrlich's reagent, these same strains produce a vivid yellow reaction in the test medium. *D. acidovorans* has been isolated from a variety of clinical specimens and is usually considered to be nonpathogenic. Isolates have been recovered from sputum, urine, the right and left ureters of a transplanted kidney, renal preservation fluid, corneal scrapings from a patient who had undergone multiple previous eye surgeries, and the blood of a patient with tuberculosis (Schreckenberger P, personal communication). There are reports in the literature of *D. acidovorans* associated with catheter-related bacteremia,[155,156,192,313,545,602,606] suppurative otitis,[894] urinary tract infection,[274] ocular infections,[131,621,625,884,1033] and peritonitis in a patient who had undergone continuous ambulatory peritoneal dialysis.[663] *D. acidovorans* endocarditis has also been described in a 42-year-old intravenous drug abuser.[480] One patient with AIDS developed pneumonia caused by this organism,[334] and an infected immunocompetent man developed a chronic empyema in the pleural cavity.[196] Camargo and colleagues reported isolating *D. acidovorans* isolates in pure cultures at counts above 1,000,000 CFU/mL from tracheal aspirates of 21 patients hospitalized in an intensive care unit,

indicating a common, but undetermined source responsible for the colonization.[145] Characteristic for identification of *D. acidovorans* are given in Table 7-7.

D. lacustris, D. litopenaei,* and *D. tsuruhatensis. These three new species of *Delftia* that have been isolated from mesotrophic lake water in Denmark,[525] a freshwater shrimp culture pond in Taiwan,[181] and sludge collected from a wastewater treatment plant in Japan.[982] Two of these species have subsequently been reported to cause human infection. *D. tsuruhatensis* has been reported to cause catheter-related bloodstream infections in patients with long-term indwelling central venous catheters.[556,869,1049] *D. lacustris* has been reported to cause septicemia in a patient with

pheochromocytoma[1012] and was cultured from bile fluid of a patient with hepatocellular carcinoma, from empyema fluid of a chest cavity following chest trauma and from blood cultures of two patients with renal disease.[984]

Genus *Comamonas*. *Comamonas* species are all motile, oxidase positive, and produce alkaline reactions in OF sugars. They phenotypically resemble *Delftia* species, *P. alacaligenes, P. pseudoalcaligenes,* and *Brevundimonas* species from which they must be differentiated. The phenotypic characteristics for this group of organisms are given in Table 7-7.

Comamonas testosteroni. *C. testosteroni* is an uncommon isolate in the clinical laboratory, despite its wide environmental distribution. Eighteen *C. testosteroni* infections have been reviewed by Barbaro and colleagues.[63] They report that the organism was most often found in association with anatomic abnormalities of the gastrointestinal tract, with perforation of the appendix being the most common. Other previously published reports describe *C. testosteroni* as a cause of sepsis,[52,410,1005,1085] central venous catheter-related infection,[626,791] endocarditis,[229] pseudo-bacteremia traced to contaminated disinfectant used for disinfecting blood-culture bottles before use,[988] pneumonia in a patient with AIDS,[334] endophthalmitis,[887] and meningitis in a patient with recurrent cholesteatoma.[40] Coenye reported isolation of *C. testosteroni* from the respiratory secretions of two patients with CF.[212]

Comamonas terrigena. *C. terrigena* actually comprises three genotypically separate groups. *C. terrigena* DNA group 1 retains the name *C. terrigena*, while *C. terrigena* DNA group 2 (containing *Aquaspirillum aquaticum* and some E. Falsen [EF] group 10 strains) has been renamed *C. aquatica* and *C. terrigena* DNA group 3

(containing some former EF group 10 strains) has been renamed *C. kerstersii*.[1160] *C. terrigena* is not considered a human pathogen, although Sonnenwirth reported the isolation of *C. terrigena* from two blood cultures of a patient with endocarditis; however, the role of the organism as a pathogen in this patient was uncertain.[1014]

Comamonas kerstersia. *C. kerstersia* can be differentiated from other *Comamonas* species by its ability to grow at 42°C. Other phenotypic characteristics are shown in Table 7-7. *C. kerstersia* has been reported to cause intra-abdominal infections[16,97] and bacteremia in a patient with diverticulosis.[804]

Facilis–Delafieldii Group

Genus *Acidovorax*. Willems and colleagues proposed a new genus, *Acidovorax*, which contained three species: *Acidovorax facilis* (formerly *Pseudomonas facilis*), *Acidovorax delafieldii* (formerly *Pseudomonas delafieldii*), and *Acidovorax temperans* (for several former *Pseudomonas* and *Alcaligenes* strains).[1177] These three species form a separate group within the rRNA group III complex. Two of the species, *A. delafieldii* and *A. temperans*, have been isolated from clinical specimens; however, no information regarding the clinical significance of these organisms is available.[368,1177] *A. delafieldii* is probably the same as CDC group WO-1 and *A. temperans* is probably the same as CDC group WO-1A.[1172] Subsequently, the genus has been expanded and now includes 18 species and subspecies that have been isolated from plants and the environment. Three new species have been recovered from human clinical specimens. *A. avenae* and *A. oryzae* have both been reported in patients with catheter-associated bloodstream infection[685,805,980] and *A. wautersii* has been reported in two human infections although the clinical context was not clearly described.[1120] *Acidovorax* species are oxidase positive, motile and utilize carbohydrates oxidatively. For additional differentiating features, see Table 7-3 and the published report by Willems and colleagues.[1177]

Family Caulobacteraceae—rRNA Group IV

All species in rRNA group IV have been transferred to the new genus *Brevundimonas* (meaning bacterium with short-wavelength flagella).[967]

Genus *Brevundimonas*. The genus *Brevundimonas* includes 25 species of which only two, *B. diminuta* and *B. vesicularis,* are found in human clinical specimens. They are grouped with the alkaline pseudomonads because they are nonreactive or only weakly reactive in most carbohydrates (Table 7-7). This group is characterized by the presence of a single, tightly coiled (wavelength of 0.6 to 1.0 mm) polar flagellum. *B. vesicularis* is slow-growing and usually requires 48 hours of incubation for colonies to

be observed on sheep blood agar, producing dark-yellow to orange-pigmented colonies (Image Plate 7-3C). Most strains fail to grow on MacConkey agar. *B. vesicularis* can be separated from all other species of alkaline pseudomonads by virtue of a strong esculin hydrolysis reaction. We have isolated *B. vesicularis* from peritoneal dialysate fluid, a renal dialysis machine, an oral abscess and a scalp wound. Others have reported isolating this species from cervical specimens,[807] blood,[185,365,620,796,858,1123] and a patient with botryomycosis, a rare, chronic suppurative, granulomatous condition affecting the skin.[143] *B. vesicularis* has also been recovered from hospital environmental samples including a shower hose[584] and a hydrotherapy pool.[51] *B. diminuta* has been isolated in patients with bacteremia,[185,620] keratitis,[819] and pleuritis.[669] *B. diminuta* has also been isolated from a sputum of a patient with CF and pneumonia.[725] An extensively drug-resistant VIM-2 MBL-producing *B. diminuta* has been isolated from a thigh biopsy specimen.[13] *B. vesicularis* can be separated from *B. diminuta* by hydrolysis of starch and esculin (both positive with *B. vesicularis*). Moss and Kaltenbach have reported that glutaric acid is produced by *B. diminuta* but not by *B. vesicularis* when the organisms are grown on trypticase soy agar.[755]

Family Xanthomonadaceae—rRNA Group V

Genus *Stenotrophomonas.* The genus *Stenotrophomonas* was created in 1993 to accommodate *Xanthomonas maltophilia* (formerly *Pseudomonas maltophilia*).[815] Several recent studies have shown that there is considerable genetic diversity within *S. maltophilia* and that this species consists of at least nine genomic groups.[223,426] In addition, 12 new *Stenotrophomonas* species have been described that have been recovered from sewage, sludge and soil. (http://www.bacterio.net/stenotrophomonas.html). *S. africana* was proposed for a single isolate recovered from CSF. Phenotypically, it was determined to be almost identical to *S. maltophilia,* except for

TABLE 7-11 Key Characteristics of *S. maltophilia* and *B. cepacia* complex[a]

Test	*S. maltophilia*	*B. cepacia* Complex
Oxidase	–	+ (93)

Motility	+	+
Growth on MacConkey	+	+
OF glucose	A or Wk	A
OF maltose	A	A
OF lactose	V (86)	A
OF mannitol	–	A
NO_3 reduction	V (42)	V (37)
NO_3 to gas	–	–
Arginine	–	–
Lysine	+	V
Esculin hydrolysis	+	V (67)
ONPG	+ (93)	V (79)
DNase	+	–
Polymyxin B	S	R
Pigment	gray, slight yellow, lavender	gray, chartreuse, yellow

a Data from reference 1172.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; A, acid reaction; Wk, weak acid; S, susceptible; R, resistant; NA, not available. Numbers in parentheses are percentage of strains giving positive reaction.

the property of being *cis*-aconitate assimilation negative.[301] However, based on extensive molecular analyses, Coenye and colleagues have determined that *S. africana* is a strain of the species *S. maltophilia;* therefore, the species name *S. africana* is disallowed.[222] Of the remaining species, only *S. maltophilia* is associated with human infection.

Stenotrophomonas maltophilia. *S. maltophilia* is a motile rod, possesses polar

multitrichous flagella, and can be easily distinguished from other pseudomonads by virtue of being lysine and DNase positive, but oxidase negative (Table 7- 11). Most strains of *S. maltophilia* are susceptible to colistin and polymyxin B. This property can be used to distinguish *S. maltophilia* from *B. cepacia,* which is also lysine positive but is resistant to colistin and polymyxin B and DNase negative. *S. maltophilia* vigorously utilizes OF maltose, but is usually negative or only weakly positive in OF glucose in 24 hours. Colonies may appear pale yellow or lavender gray on blood agar medium (Image Plate 7-3D). We have noted rare strains of *S. maltophilia* that will be slowly oxidase positive but have all the other biochemical features characteristic of *S. maltophilia.* Carmody and colleagues reported that 20% of *S. maltophilia* isolates derived primarily from cultures of respiratory specimens from persons with CF were oxidase positive.[150] Laboratorians should be aware that rare strains possessing aberrant characteristics may be recovered from clinical specimens.

S. maltophilia is ubiquitous and can be recovered from almost any clinical site. It occasionally causes opportunistic infections and is emerging as an important hospital-acquired pathogen.[559,707,760] The most common site for recovery of *S. maltophilia* is the respiratory tract, although in most patients these isolates do not appear to be clinically significant. An increasing incidence of *S. maltophilia* has been reported in some CF centers in recent years,[61,138,377,542,1094] and an association between *S. maltophilia* colonization and lung damage has been observed.[61,542] The use of selective media increases the isolation rates of *S. maltophilia* from the sputa of patients with CF.[276,551] In patients who do not have CF, *S. maltophilia* has been reported to cause a wide spectrum of disease, including pneumonia,[859] bacteremia,[34,339,578,594,732,759,859,971] endocarditis,[412] catheter-related infections,[312] cholangitis,[822] urinary tract infection,[1130] meningitis,[823,859] and serious wound infections, particularly in patients with cancer.[1131] Morrison and colleagues[750] have studied the spectrum of clinical disease in patients with hospital-acquired *S. maltophilia* infections and report both an increasing rate of nosocomial isolation and a crude mortality rate of 43% in all patients from whom the organism was cultured. Risk factors associated with death for patients with an *S. maltophilia* isolate included the following: patient in intensive care unit, age older than 40 years, and a pulmonary source for the *S. maltophilia* isolate.[750] A comprehensive review of infections associated with *S. maltophilia* has been written by Denton and Kerr.[277]

Another important feature in the rising incidence of *S. maltophilia* infections may be the unique antibiotic susceptibility profile of the organism. *S. maltophilia* is inherently resistant to most of the commonly used antipseudomonal drugs, including aminoglycosides and many β-lactam agents, including those effective against *P. aeruginosa*.[781,820,1129] Thus, colonization may be favored by the use of broad-

spectrum antipseudomonal therapy. Interestingly, *S. maltophilia* is inherently susceptible to trimethoprim-sulfamethoxazole, a drug that has no activity against *P. aeruginosa* or most other *Pseudomonas* species.[193,326] In addition to the problem of inherent resistance, there are problems related to susceptibility testing of *S. maltophilia*. Some automated methods (i.e., Vitek Legacy; bioMérieux, Hazelwood, MO) have programmed software that will prevent the reporting of susceptibility results if the test organism is known to be *S. maltophilia*. Trailing end points can be observed in agar dilution and microdilution tests, and false-susceptible readings with disk diffusion assays have occurred with aminoglycosides (i.e., these should be uniformly resistant) and ciprofloxacin.[448,820] Studies on the use of the Etest (discussed in Chapter 17) for antimicrobial susceptibility testing of *S. maltophilia* have noted the presence of tiny microcolonies or a haze of translucent growth within the area of inhibition that if missed could lead to false susceptibility results.[820,1206] To learn more, the reader is referred to the review written by Robin and Janda.[911]

Genus *Wohlfahrtiimonas.* The genus *Wohlfahrtiimonas* consists of two species *W. chitiniclastica* and *W. larvae*. Both species were first isolated from the larvae of parasitic flies. *Wohlfahrtiimonas* derives its name from the fact that the

first isolates were recovered from the 3rd stage fly larvae of the parasitic fly *Wohlfahrtia magnifica*.[1076] *W. chitiniclastica* has been reported to be the cause of fulminant sepsis in two elderly patients in France and Argentina.[18,885] Both patients were homeless, had a history of alcoholism and were in poor sanitary condition. The source of the infection in both patients was attributed to flies. One patient was described as being covered with thousands of body and hair lice, with dozens of insect larvae in her hair.[885] A third reported patient was a 64-year-old man from the Czech Republic with a 4-year history of gangrene in the distal part of the legs and amputations of the toes on both feet, which was associated with this organism.[585] *Wohlfahrtiimonas* are straight, short, gram-negative rods that are nonmotile, non–glucose-fermenting, strictly aerobic, and both catalase and oxidase positive. Complete phenotypic characteristics for *W. chitiniclastica* have been described by Toth et al.[1076] The organism is not in the databases for the commercially available phenotypic identification systems but can be identified using 16S rRNA sequencing and MALDI-TOF MS.[585] *W. chitiniclastica* was reported to be susceptible to all antibiotics tested in three separate reports.[18,585,885]

Family Acetobacteraceae

At the time of this writing the family *Acetobacteraceae* included 33 genera of Acetic Acid Bacteria (AAB) (http://www.bacterio.net/-classifgenerafamilies.html#Acetobacteraceae). A typical feature of most species of AAB is the ability to oxidize ethanol and sugars to acetic acid. AAB are found throughout the world and have been isolated from diverse vegetables, fruits, and flowers. The AAB have many industrial applications, including the production of vinegar and various drugs.[940,1203] The first report of AAB in humans was in 2004, when Snyder et al.[1010] reported a patient with peritonitis associated with *Asaia borgorensis*; the patient had a peritoneal dialysis catheter. This was followed 2 years later with a second AAB infection due to *Granulibacter bethesdensis* in two patients from separate locations with chronic granulomatous disease (CGD).[402,403] Since then, AAB have been increasingly reported as agents of disease in humans. Only those genera known to cause human infection, namely *Acetobacter, Acidomonas, Asaia, Gluconobacter, Granulibacter,* and *Roseomonas,* will be covered further in this chapter. Two genera of AAB produce pink colonies on solid agar, namely, *Asaia,* and *Roseomonas,* and are covered under the heading Pink-Pigmented Group. The remaining species associated with human disease are described below.

Genus *Acetobacter*. *Acetobacter* species are ellipsoidal to rod shaped, straight or slight curved gram-negative bacilli. Motility is variable; if motile, they are peritrichously flagellated. They are obligately aerobic and form pale colonies that are nonpigmented on solid media.[990] A minority of strains produce brown water-soluble pigments or show pink colonies because of the production of porphyrins. They are usually catalase positive and oxidase negative. Optimal growth temperature is 30°C. They are negative for gelatin hydrolysis, indole, and H_2S production.[990] They occur naturally in a variety of flowers and fruits. *Acetobacter* bacteremia has been reported in a 40-year-old HIV-positive man who for 1 year had been receiving chronic hemodialysis for end-stage renal disease. He was admitted because fever and a large inflammatory skin lesion that followed the course of an arteriovenous fistula. Two blood samples drawn through a sublavian catheter and the arteriovenous fistula both grew a very small polymorphic gram-variable rod on blood and chocolate agars that was identified by 16S rRNA sequencing as *Acetobacter cibinongensis*.[388] *A. indonesiensis* was isolated from successive sputum cultures of a 31-year-old man with CF following a lung transplant.[99]

Genus *Acidomonas*

Acidomonas methanolica. The genus *Acidomonas* is phylogenetically located within the *Acetobacteraceae* and contains a single species, *A. methanolica*, which

shares characteristics with AAB; however, methanol utilization and a unique ecologic niche are characteristics unique to this species.[1205] Cells are gram-negative, non–spore-forming, and rod-shaped, occurring singly or rarely in pairs. They are obligately aerobic and grow best at 30°C.[991] Colonies on peptone-yeast extract–malt extract (PYM) nutrient medium are shinny, smooth, entire and beige to pink after 5 days at 30°C,[1205] although other authors have described the colonies as white to yellow on PYM medium.[991] Cells are usually motile by a single polar flagellum (or occasionally polar tuft).[1205] Strains produce catalase but are negative for oxidase, nitrate reduction, and urease.[991,1205] Other phenotypic characteristics can be found in the reference by Yamashita et al.[1205] The natural habitat for *A. methanolica* is activated sludge which differs from other Acetobacteraceae that are isolated from fruits and vegetables. *A. methanolica*-associated necrotizing lymphadenitis has been reported in a 10-year-old patient with CGD.[173]

Genus *Gluconobacter*. *Gluconobacter* species are gram-negative, ellipsoidal to rod-shaped cells occurring singly and/or in pairs, rarely in chains. They may be motile or nonmotile; if motile, they produce three to eight polar flagella. They are obligately aerobic and grow optimally at 25°C to 30°C.[992] They are catalase positive, but oxidase negative. They are negative for nitrate reduction, gelatin hydrolysis, indole, and H_2S production.[992] These bacteria are found naturally occurring in sugar-rich environments such as fruits and flowers. Alauzet et al. have reported isolating *Gluconobacter* species from three patients. One was an HIV-negative man and known to be an intravenous drug abuser who was hospitalized with decreased visual acuity and suspected fungal endophthalmitis. A central venous catheter (CVC) was in place for amphotericin B administration. Fever appeared after 3 days and two peripheral blood samples yielded growth of a gram-negative rod that grew as tiny colonies after 48 hours of incubation at 37°C. The isolate was catalase positive, oxidase negative, and was identified as *Gluconobacter* species by molecular means.[10] Two other patients, a 2-year-old and a 3-year-old, with CF both had positive sputum cultures for *Gluconobacter*, which was recovered on *Burkholderia cepacia*-selective medium.[10] *Gluconobacter* was misidentified in both incidence as either *Acinetobacter* species (low discrimination) using API 20 E, or *Shigella* species (good identification) using the Vitek2 GN system.[10] A *Gluconobacter* bloodstream infection associated with endocarditis was reported in a patient with a history of

intravenous drug abuse.[74] Laboratories are advised that none of the current

commercial systems for bacterial identification are able to recognize AAB. Organisms that show unusual growth characteristics should be further evaluated using molecular testing methods.

Genus *Granulibacter*

Granulibacter bethesdensis. *G. bethesdensis* is an emerging pathogen in patients with CGD and is characterized by frequent infections that cause fever and necrotizing lymphadenitis, and can relapse after periods of clinical quiescence.[404] CGD is a rare inherited disease caused by mutations in genes encoding any of the four subunits that make up nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) that leads to defective production of superoxide and hydrogen peroxide.[966] Patients suffer from recurrent life-threatening infections with catalase-producing organisms and also develop tissue granulomas.[1179] A novel gram-negative rod was isolated from cervical and supraclavicular lymph nodes of a CGD patient in 2003 and from the same patient, three more times through 2005. A similar organism was isolated from two other CGD patients in 2005 and 2006. Phenotypic and geotypic analyses determined that the novel isolates represented a previous undescribed AAB, which was named *Granulibacter bethesdensis*.[402,403] Additional isoates of *G. bethesdensis* have been reported in patients with CGD from North and Central America[404] and Spain.[662] Cells are gram negative, nonmotile, and coccobacillus to rod-shaped. They produce a yellow pigment on solid media and grow optimally at 35°C to 37°C.[403] They are strictly aerobic, catalase positive, oxidase negative, and urease variable. Acid is produced from glucose but not from mannitol, sorbitol, dulcitol, lactose, sucrose, maltose, or xylose.[403] Biochemical differentiation of *G. bethesdensis* from similar nonfermenting species that appear as coccobacili and are oxidase-negative and nonmotile can be found in Table 7-18. Laboratorians are advised that isolation of *G. bethesdensis* is time-consuming, growth is consistently sparse, and definitive identification requires sequencing the 16S rRNA gene.[404] Strains of *G. bethesdensis* are reported to be multidrug resistant, and therapy requires surgery and combination antimicrobial drugs, including long-term ceftriaxone.[404] Antibiotic susceptibility data are provided in the report by Greenberg et al.[402]

Yellow-Pigmented Group

Family Pseudomonadaceae

Genus *Pseudomonas*

Pseudomonas luteola. This species has been previously known as

Chromobacterium typhiflavum, CDC group Ve-1, *Pseudomonas luteola*, *Pseudomonas polytricha,* and *Chryseomonas luteola*.[472] The taxonomic placement was resolved in 1997 when the species was placed back into the genus *Pseudomonas*.[32] *P. luteola* is motile by means of multitrichous polar flagella, is oxidase negative, and grows on both MacConkey and blood agar media, producing yellow-pigmented colonies that are often wrinkled and become adherent to the agar (Image Plate 7-3B). The biochemical features that differentiate *P. luteola* from other yellow-pigmented pseudomonads are given in Table 7-12. It is a rare clinical isolate and has been recovered from a variety of clinical specimens, including wound, cervix, urine, and throat specimens.[368] It is often isolated with other organisms and judged not to be clinically significant. In one study, only 14 strains of *P. luteola* were found among 565 clinical isolates of nonfermenters over a 2-year period.[829] Reports of serious infections caused by *P. luteola* include bacteremia,[80,89,188,238,314,337,808,809,874,878,883,1167] endocarditis,[153,799] meningitis,[188,587] leg ulceration,[1083] osteomyelitis,[874] brain abscess,[356] and peritonitis.[226,346,874,1039]

Pseudomonas oryzihabitans. This organism has been described formerly as *Chromobacterium typhiflavum*, *Pseudomonas oryzihabitans*, CDC group Ve-2 and *Flavimonas oryzihabitans*.[472] The taxonomic placement was resolved in 1997 when the species was placed back into the genus *Pseudomonas*.[32] *P. oryzihabitans* has characteristics similar to *Chryseomonas luteola* in that these organisms are also motile and oxidase negative and form yellow-pigmented colonies on blood agar medium. Like *P. luteola*, colonies of *P. oryzihabitans* form rough or wrinkled colonies. This organism can be differentiated from *P. luteola* by negative reactions for esculin hydrolysis and orthonitrophenyl-β-D-galactopyranoside (ONPG) and the feature of having a single polar flagellum. Additional differential characteristics are given in Table 7-12. *P. oryzihabitans* has been recovered from a variety of clinical sites, including wounds, sputum, ear, eye, urine, peritoneal fluid, inhalation-therapy equipment, and blood.[95,337,370,572,624,641,642,855,874,1082] More recently, infections with this organism have been related to the presence of an intravascular catheter in immunocompromised patients.[671, 698,918,1133] In our clinical laboratory, this organism has been recovered from sputum, urine, prostatic secretion, skin, and blood. *P. oryzihabitans* also appears to be an emerging pathogen in peritonitis related to continuous ambulatory peritoneal dialysis, with several cases now reported in the literature.[24,87,318,319,633,824,994] Other predisposing factors for *P. oryzihabitans* infections include indwelling intravascular catheters, artificial grafts, intravenous drug abuse, severe head trauma requiring surgery, and bone marrow transplantation.[174]

Family Sphingomonadaceae[586]

Genus *Sphingomonas*. The genus *Sphingomonas* was described by Yabuuchi et al. in 1990[1200] and was emended by Takeuchi et al. in 1993.[1053] Organisms in this genus are gram-negative, non–spore-forming rods that have a single polar flagellum when they are motile. They are yellow, obligately aerobic, and produce catalase. Bacteria belonging to the genus *Sphingomonas* are ubiquitous in soil, water, and sediments, and strains isolated from these environments are known to be decomposers of aromatic compounds and, therefore, are expected to be used for bioremediation of the environment.[60] It is now known that members of the genus *Sphingomonas* can be divided into four phylogenetic groups, each representing a different genus. Consequently, three new genera, *Sphingobium*, *Novosphingobium,* and *Sphingopyxis*, in addition to the genus *Sphingomonas* have been created to accommodate the four phylogenetic

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TABLE 7-12 Key Characteristics of Yellow-Pigmented Pseudomonads

	Pseudomonas		*Sphingomonas*		*"Agrobacterium"*	*Balneatrix*	*Massilia*	CDC Groups	
Test	***P. luteola***[a]	***P. oryzihabitans***[a]	***S. paucimobilis***[a]	***S. parapaucimobilis***[b]	**Yellow Group**[c]	***B. alpica***[d]	***M. timonae***[e]	**O-1**[c]	**O-2**[c]
Oxidase	–	–	+ (94)	+	+	+	+	V (77)	+
Motility	+	+	+ (92)	+	+	+	+	+	V (20)
Growth on MacConkey agar	+	+	– (10)	–	V (50)	–	+	V (40)	– (10)
OF glucose	A	A	A	A	A	A	Wk	Wk	V (84)
OF xylose	A	A	A	A	A	NA	A	–	–
OF maltose	A	A	A	A	A	A	A	–	A
OF mannitol	A	A	–	–	–	A	–	–	–
Indole	–	–	–	–	–	+	–	–	–
Esculin	+	–	+	+	+	–	+	+	V (64)
ONPG	+	–	+	+	V (30)	–	NA	NA	NA
DNase	–	–	+	–	–	–	+	NA	NA
Citrate	+	+	–	+	–	NA	+	–	–
H_2S lead acetate	V (12)	+	–	+	–	–	NA	+	+
3-Ketolactonate	NA	NA	–	NA	+	NA	NA	NA	NA
Lipase	NA	NA	+	+	–	NA	NA	NA	NA
Polymyxin B	S	S	S (89)	V	NA	S	S	NA	NA
Pigment	Dull yellow	Dull yellow	Deep yellow	Deep yellow	Yellow	Yellow	Straw	Yellow	Yellow
Flagella	Multitrichous	Single polar	Single polar	Single polar	Single polar	Single polar	Single polar	1–2 polar	1–2 polar

[a]Data from reference 367.
[b]Data from references 586 and 1200.
[c]Data from references 1047 and 1172.
[d]Data from reference 255.
[e]Data from reference 645.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; S, susceptible; A, acid reaction; NA, results not available. Numbers in parentheses are percentage of strains giving positive reaction.

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groups.[1052] The emended genus *Sphingomonas* contains at least 90 species, of which

only *S. paucimobilis*, designated the type species, and *S. parapaucimobilis* are thought to be important clinically.

Sphingomonas paucimobilis. This organism, formerly known as *Pseudomonas paucimobilis* and as CDC group IIk-1, is the most common species found in human clinical specimens. It is a gram-negative, motile rod with a polar flagellum. However, few cells are actively motile in broth culture, thus making motility a difficult characteristic to demonstrate. Motility occurs at 18°C to 22°C, but not at 37°C.[814] The oxidase reaction is weak positive or negative. Colonies grown on blood agar medium are yellow-pigmented; however, this species is slow-growing, and only small colonies may be observed after 24 hours of incubation. Growth occurs at 37°C, but not at 42°C, with optimal growth occurring at 30°C.[814] Isolates are strongly esculin hydrolysis positive and produce a zone of inhibited growth around a vancomycin disk (30 mg) placed on BAP (Schreckenberger P, personal observation). Additional biochemical features are given in Table 7-12. *S. paucimobilis* has been isolated from a variety of clinical specimens, including blood, CSF, urine, wounds, vagina, and cervix and from the hospital environment.[460,890,1051] Community-acquired bacteremia and peritonitis have also been reported in patients receiving long-term ambulatory peritoneal dialysis.[751]There have been a few reports of nosocomially acquired *S. paucimobilis* infections from contamination of hemodialysis fluids,[144] contamination of a hospital water system,[840] contamination during *in vitro* processing of bone marrow for transplantation[614]and catheter-related sepsis.[266,489,946,947] There has also been a report of *S. paucimobilis* bacteremia that was accompanied by septic shock in a burn patient.[152] Toh and colleagues reviewed the risk factors associated with *S. paucimobilis* infection and found that community-acquired infections, diabetes mellitus and alcoholism were significant risk factors for primary bacteremia.[1074] Bayram et al.[81] reviewed the clinical features of *S. paucimobilis* infections in children. Most strains are susceptible to tetracycline, chloramphenicol, trimethoprim–sulfamethoxazole, and aminoglycosides; their susceptibility to other antimicrobial agents, including fluoroquinolones, varies.[324,489,890]

The cellular and colonial characteristics of *S. parapaucimobilis* are similar to those of *S. paucimobilis*. It is differentiated from *S. paucimobilis* by blackening of lead acetate paper suspended over KIA, ability to grow and alkalinize Simmons' citrate medium and a negative extracellular deoxyribonuclease reaction.[1200] Clinical isolates have been obtained from the sputum, urine, and vaginal specimens.[1200]

Family Oceanospirillaceae

Genus *Balneatrix*

Balneatrix alpaca. *Balneatrix* is a new genus consisting of a single species, *B.*

alpica.[255] This bacterium was first isolated in 1987 during an outbreak of pneumonia and meningitis among persons who attended a hot (37°C) spring spa in Southern France.[154,255,492] Thirty-five patients with pneumonia and two with meningitis occurred. Isolates from eight patients were recovered from blood, CSF, and sputum; one was also recovered from water. The bacterium is described as gram-negative, straight or curved rods, motile by a single polar flagellum, strictly aerobic, and growing at a wide range of temperatures (20°C to 46°C). Colonies are 2 to 3 mm in diameter, convex, and smooth. The center of the colonies is pale yellow after 2 to 3 days and pale brown after 4 days. The organism grows on chocolate and tryptic soy agars, but not on MacConkey agar. It is oxidase positive and nonfermentative but oxidatively utilizes glucose, mannose, fructose, maltose, sorbitol, mannitol, glycerol, and inositol. Indole is produced, and nitrate is reduced to nitrite. Gelatin is weakly hydrolyzed, and lecithinase is positive. The following substrates are not utilized: arginine, lysine, ornithine, urease, esculin, acetamide, starch, and ONPG (Table 7-12).[154,255] *B. alpica* is reported to be susceptible to penicillin G and to all other β-lactam antibiotics and to all aminoglycosides, chloramphenicol, tetracycline, erythromycin, sulfonamides, trimethoprim, ofloxacin, and nalidixic acid. It is resistant to clindamycin and vancomycin.[154]

Family Oxalobacteraceae

Genus *Massilia*. The genus *Massilia* consists of 25 species isolated mainly from sewage, sludge and soil. The species of the genera *Massilia* and *Naxibacter* are often grouped together on the basis of 16S rRNA gene sequence comparisons. For this reason Kampfer and colleagues proposed the transfer of all species of the genus *Naxibacter* to the genus *Massilia*.[534]

Massilia timonae. *M. timonae* is the most frequently reported species to cause human infection. *M. timonae* is an actively motile (with lateral as well as a single polar flagellum), strictly aerobic gram-negative rod. The original description by La Scola and colleagues[608] described the type strain as being oxidase negative and arginine dihydrolase positive; however, Lindquist et al.[645] reported that the type strain as well as four additional human isolates tested oxidase positive and arginine negative. Colonies appear pale yellow and are distinctly tenacious on agar media and have a tendency to form pellicles on the surface of liquid medium.[608] Acid is produced oxidatively from some carbohydrates. Lindquist and colleagues reported that when grown in OF medium without added carbohydrate, the strains produced a distinctly alkaline reaction. Consequently, a neutral reaction in a tube with carbohydrate was interpreted as weakly positive for acid production.[645] Additional biochemical reactions are given in Table 7-12. Isolates have been recovered from a surgical wound, CSF, the

femur of a 29-year-old with osteomyelitis, biopsy of a cervical lymph node in a patient with lymphadenopathy, and from the blood of three other patients.[608,645,1000,1097] *M. timonae* is susceptible to most antibiotics, with resistance reported to ampicillin, amoxicillin-clavulanate, cefuroxime, cephalothin, and aztreonam.[608,1000,1097]

Massilia oculi. This organism was described in 2012 as a novel species isolated from the eye of a patient with endophthalmitis.[535] The organism was described as producing colonies that are beige, translucent, and shiny with entire edges within 24 hours on blood agar and nutrient agar. They have an oxidative metabolism and are positive for esculin hydrolysis, but negative for nitrate reduction, urease, and oxidase.[535]

Massilia haematophilus. *M. haematophilus* (formerly *Naxibacter haematophilus*) is described as nonmotile, oxidase-positive, gram-negative rods with an oxidative metabolism. Colonies are beige, translucent, and shiny with entire

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edges and are negative for esculin and ONPG.[533] The type strain was isolated from human blood of a 23-year-old Swedish man with multiple health problems. *M. haematophilus* has also been isolated from a neck vertebrae bone of an Israeli patient who had vertebral osteomyelitis. Identification was confirmed with 16S rRNA sequencing (Orna Schwartz, personal communication).

Unnamed Species

CDC Groups O-1, O-2. CDC groups O-1 and O-2 are phenotypically similar, motile, and usually oxidase-positive, gram-negative rods. Both groups are yellow-pigmented and most closely resemble *Agrobacterium* yellow group and *Sphingomonas* species (Table 7-12). These organisms grow poorly or not at all on MacConkey agar, usually hydrolyze esculin, but are otherwise inactive. They are motile, although motility may be difficult to demonstrate. On Gram's stain, O-1 appears as uniformly short gram-negative rods, and O-2 appears as slightly pleomorphic rods, with some cells appearing thin in the central portion with thickened ends. Isolates have been from a variety of clinical sources. One patient with group O-1-associated pneumonia complicated by bronchopulmonary fistula and bacteremia has been reported.[872]

***Agrobacterium* Yellow Group.** Organisms in this group are represented by slender, medium-to-long gram-negative rods that produce a yellow insoluble growth pigment and most closely resemble *Sphingomonas paucimobilis* and CDC group O-1 and O-2 organisms. Growth on MacConkey agar is variable, motility occurs via a single polar flagellum, and oxidase and catalase are positive; glucose, xylose, lactose, sucrose, and maltose are oxidized, but not mannitol. Carbohydrate reactions may be weak or late.[1172] A positive 3-ketolactonate reaction and negative lipase and DNase reactions differentiate this organism from *S. paucimobilis* (Table 7-12). Isolates have been from blood and peritoneal fluid.[166,1047,1172]

Curved Rod Group

Family Caulobacteraceae

Genus *Caulobacter*. The genus *Caulobacter* is composed of dimorphic, prosthecate bacteria that at the time of this writing includes16 species. Reproduction in *Caulobacter* results in the separation of two cells that are morphologically different from each other. One cell is nonmotile, sessile by virtue of adhesive material and prosthecate, possessing at least one elongated cylindrical appendage (called a prostheca). The other cell is flagellated, bearing one polar flagellum, by which it is motile.[4] *Caulobacter* species stain gram negative and appear distinctly curved, but may be straight in some isolates and have a fusiform shape. The cells are arranged frequently in typical rosettes in which the stalks originate from a common point or region and the cells become arranged symmetrically around this point. The rosettes are especially numerous and large in preparations from agar colonies.[124] In the laboratory,

rosette formation is often observed in wet mounts.[529] A picture of rosette formation from a wet mount of *Caulobacter* can be seen in a photo appearing in the publication by Justesen et al.[529] The organism grows well on most common culture media at temperatures between 15°C to 35°C with optimal temperature of 20°C to 25°C for most isolates.[4] Colonies are circular, convex and glistening. and may be colorless or yellow upon aging.[355] The organism is reported to be oxidase positive and catalase negative.[529] Phenotypic identification is usually carried out using carbon source utilization studies.[355] In one report, biochemical identification with API ID 32 GN and Vitek 2 GN systems (bioMèrieux, Marcy l'Etoile, France) resulted in incorrect identifications as *Brevundimonas vesicularis* (probability, 90.6%) and *Sphingomonas paucimobilis* (probability, 97.24%), respectively.[529] *Caulobacter* are ubiquitous in water and have been isolated from distilled, tap, and commercially bottles drinking water; river, canal, and pond water and sewage (activated slidge during secondary treatment).[4,124,676] There have been three reports of of *Caulobacter* causing human infection. Bridger et al.[129] reported a pediatric patient with *Caulobacter* meningitis following neurosurgery. Justesen et al.[529] described a 64-year-old man who developed peritonitis because of *Caulobacter* while undergoing intermittent peritoneal dialysis for chronic renal insufficiency. Drancourt et al.[300] conducted 16S rRNA sequencing on clinical isolates that could not be identified by conventional phenotypic methods and found one isolate of *Caulobacter intermedius,* although no details were provided about the clinical significance of the isolate. Laboratorians are advised to suspect *Caulobacter* species when cresent shaped bacteria with rosette formation are observed in microscopic examination. Final identification would be confirmed by 16S rRNA sequencing.

Family Oxalobacteraceae

Genus *Herbaspirillum*. This genus consists of small spiral-shaped bacteria from herbaceous seed-bearing plants. *Herbaspirillum* species are gram negative, generally curved and sometimes helical bacilli. Individual cells are 0.6 to 0.7 mm wide and 1.5 to 5.0 mm in length and have one to three or more flagella on one or both poles.[59] A group of clinical isolates previously described as EF-1, has been shown by molecular hybridization to belong to the genus *Herbaspirillum* and is designated as a new unnamed species, *Herbaspirillum* species 3.[59] The organism is oxidase and urease positive; catalase is weak or variable. Other reactions are given in Table 7-13. Isolates in the original description were recovered from the respiratory tract, feces, urine, ear, eye, and wound sites, but clinical significance was not reported.[59] Subsequently, clinical isolates have been recovered from sputum of patients with CF.[212,1021] It has

also been recovered from the blood of one patient with CF who had a chronic (3-year) respiratory tract infection,[1021] two patients with leukemia,[177,1218] and one patient with cellulitis and bacteremia who had jumped into a freshwater canal in central Florida.[1056] *Herbaspirillum* species has also been found in the arterial walls of aortic aneurysms.[704] Antibiotic susceptibility data are not available.

Family Neisseriaceae

Genus *Laribacter*

Laribacter hongkongensis. Based on phylogenetic affiliation, this bacterium belongs to the *Neisseriaceae* family and

TABLE 7-13 Curved Pseudomonads

Test	*Laribacter hongkongensis*[a]	CDC Group O-3[b]	*Herbaspirillum* species 3[c]
Oxidase	+	+	+
Catalase	+	+ or weak	NA
Motility	+	+	+
Growth on MacConkey	+	V (38)	NA
OF glucose	–	A	A
OF xylose	–	A	A
OF mannitol	–	–	A
Arginine	+	–	NA
Urea	+	–	NA
NO_3 to NO_2	+	V (8)	NA
Indole	–	–	NA
Esculin	–	+	–
Citrate	–	–	+
Pigment	–	–	–
Flagella	Bipolar	Single polar	1–3 Polar

[a]Data from reference 1185.
[b]Data from reference 248.
[c]Data from reference 59.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; A, acid reaction; NA, results not available. Numbers in parentheses are percentage of strains giving positive reaction.

is a facultatively anaerobic, nonsporulating, nonfermentative bacillus. On Gram's stain, the organisms appear as gram-negative, seagull-shaped or spiral rods (Image Plate 7-5D). Good growth occurs on sheep blood agar as nonhemolytic, gray colonies 1 mm in diameter after 24 hours of incubation at 37°C in ambient air. On MacConkey agar,

colonies are small and lactose negative, but take on a cornflower-blue appearance similar to colonies of *Acinetobacter* (Schreckenberger, personal observation; Image Plate 7-5E). Most strains are motile with bipolar flagella. All strains are oxidase, catalase, urease and arginine dihydrolase positive; these organisms reduce nitrate, but do not ferment, oxidize, or assimilate any carbohydrates (Table 7-13).[1185,1212] *L. hongkongensis* was first discovered in Hong Kong from the blood and empyema fluid of a 54-year-old patient with cirrhosis[1212] and from the stools of patients with community-acquired diarrhea.[611,1185,1186] Two additional patients with *L. hongkongensis* bacteremia including one fatal case have been reported from Korea[564] and Hong Kong.[1084] *L. hongkongensis* has been found in drinking water reservoirs in Hong Kong[610] and in up to 60% of intestines from commonly consumed freshwater fish of the carp family.[1063,1186] Woo and colleagues reported an association between *L. hongkongensis* and community-acquired diarrhea, eating fish, and travel[1186]; however, a causative role was not shown.[322] Isolation of *L. hongkongensis* from stool was subsequently reported from countries in Asia (China and Japan), Europe (Switzerland), Africa (Tunisia), and Central America (Cuba), suggesting that the bacterium is found worldwide.[783,1186] Beilfuss and colleagues recently reported the first isolation of *L. hongkongensis* from the stool of a patient with no travel outside of the United States.[83] A review of the microbiologic characteristics and pathogenicity of *L. hongkongensis* has been published by Raja and Ghosh.[875]

Isolation of *Laribacter* from stool specimens requires the use of selective media. *L. hongkongensis* grows poorly or not at all on *Campylobacter* BAP (CAMPY BAP) and Hektoen agars (Schreckenberger, personal observation). Others have reported isolation of *L. hongkongensis* from stools using Charcoal Cefoperazone Deoxycholate Agar (CCDA) under microaerophilic conditions[611,1185] or Cefoperazone MacConkey agar in ambient air.[327,611] Growth also occurs on MacConkey agar in a microaerophilic or anaerobic environment, and at 25°C and 42°C, but not a 4°C, 44°C, or 50°C. No enhancement of growth is observed with 5% CO_2.[1212] The failure to recognize *L. hongkongensis* from human stools is likely due to a combination of the organisms not being sought, or misidentification by phenotypic methods. Commercial identification kits do not include *L. hongkongensis* in their databases, and isolates set up on these systems are either given a low probability ID or are misidentified as *Acinetobacter lwoffii*.[83,564] *L. hongkongensis* can be identified correctly by MALDI-TOF MS using the Bruker Biotyper 3.1 and reference library v3.1.2.0 software (Bruker Daltonik, Billerica, MA).[83,1057,1084]

L. hongkongensis is generally resistant to β-lactam antibiotics, including broad-spectrum penicillins and cephalosporins, but is susceptible to carbapenems,

amoxicillin-clavulanate, quinolones, and aminoglycosides.[609, 611,1185,1186,1212] Lau and colleagues have demonstrated that all isolates of *L. hongkongensis* possess a chromosomally encoded AmpC β-lactamase which they have named LHK-5 (gene, *bla* LHK-5),[609] Antibiotic treatment is usually not necessary in patients with *Laribacter* gastroenteritis; however, for serious infections, susceptibility testing of individual isolates is recommended.

CDC Group O-3

CDC group O-3 cells are motile, oxidase positive, gram-negative rods. They grow poorly or not at all on MacConkey agar, usually hydrolyze esculin, oxidize OF glucose and OF xylose, but are otherwise inactive (Table 7-13). Unlike Groups O-1 and O-2, they do not produce a yellow pigment. On Gram's stain, O-3 cells appear as thin, medium to slightly long curved rods with tapered ends (sickle-like) that sometimes form rosettes.[248,1172] Most isolates of O-3 grow well on CAMPY CVA (*Campylobacter* agar with cefoperazone, vancomycin, and amphotericin B) plates under microaerophilic conditions, thus creating the potential for misidentification of O-3 organisms as *Campylobacter*.[248] Isolates have been recovered from a variety of clinical sources. Group O-3 isolates have been reported to be susceptible to the aminoglycosides, trimethoprim–sulfamethoxazole, and imipenem. Resistance was noted to most β-lactams, and variable susceptibility was noted for chloramphenicol, tetracycline, ciprofloxacin, and amoxicillin-clavulanate.[248]

Halophilic and/or Hydrogen Sulfide-Positive Pseudomonads

Family Shewanellaceae

Genus *Shewanella*. In 1985, MacDonell and Colwell proposed the new genus *Shewanella,* composed of three species: *S. putrefaciens* (formerly *Pseudomonas putrefaciens*, *Alteromonas putrefaciens,* and CDC group Ib), *S. hanedai,* and *S. benthica*.[673] Currently, there are at least 62 species included in the genus *Shewanella* most of which are associated with aquatic and marine habitats; however, the type species, *S. putrefaciens*, has been recovered from human clinical specimens. The CDC recognizes two biotypes of *S. putrefaciens* based on the requirement of NaCl for growth, oxidation of sucrose and maltose, and the ability to grow on *Salmonella*–

Shigella (SS) agar.[1172] Owen et al.[810] have shown that organisms identified as *S. putrefaciens* comprise at least four clearly separated genomic groups (I–IV). Based on the taxonomic proposals of Nozue and colleagues[790] and Simidu et al.,[995] strains belonging to Owen's genomic group IV (synonymous with CDC biotype 2) should be identified as *S. alga* (corrected to *S. algae*).[1081] Khashe and Janda[560] have reported that *S. algae* is the predominant human clinical isolate (77%), while *S. putrefaciens* (CDC biotype 1) represents the majority of nonhuman isolates (89%). *S. algae* requires NaCl for growth, while *S. putrefaciens* does not (Table 7-14). Strains of *S. putrefaciens* and *S. algae* are oxidase positive and motile by means of polar flagella. They are easily distinguished because they are the only nonfermenters that produce hydrogen sulfide in KIA and TSI media. All strains are positive for ornithine decarboxylase, nitrate reductase, and DNase. Colonies produce an orange-tan pigment on blood agar medium. Although they are infrequent clinical isolates, *S. putrefaciens* and *S. algae* have been associated with skin ulcers,[35,182,264,271,292,812,1208] ear infections,[366, 474,1150] eye infections,[140] arthritis and osteomyelitis,[634,864] bacteremia,[130,292,500,565,702,812,955,1113] infective endocarditis,[283] and peritonitis in patients undergoing continuous ambulatory peritoneal dialysis.[241] Many of the infections reported to be caused by *S. putrefaciens* were probably caused by *S. algae*.[292,1143] A review of human infection with *S. putrefaciens* and *S. algae* has been provided by Vignier et al.[1136] Shewanellae are generally susceptible to most antimicrobial agents effective against gram-negative rods, except penicillin and cephalothin.[324,1146] Recent investigations have noted that the mean MICs of *S. algae* to penicillin, ampicillin and tetracycline were higher than the corresponding MICs of *S. putrefaciens*.[560,1143] For a review of the epidemiology, disease spectrum, and identification of *Shewanella* species, see the review by Janda and Abbott.[504]

Family Alteromonadaceae—Halophilic Group

Genus *Alishewanella*

Alishewanella fetalis. *A. fetalis* is a halophilic gram-negative rod that grows at temperatures between 25°C and 42°C, with optimal growth at 37°C. NaCl is required for growth. It can withstand NaCl concentrations up to 8%, but it does not grow at 10% NaCl, which helps differentiate this species from *S. algae,* which can grow in 10% NaCl.[1144] Also, unlike *S. algae,* it is esculin hydrolysis positive. It is oxidase and catalase positive and asaccharolytic. It does not produce H_2S in the butt of TSI and KIA. Other reactions are given in Table 7-14. It has been isolated from a human fetus at autopsy; however, its association with clinical infection is unknown.[1144]

Family Halomonadaceae

Genus *Halomonas.* *Halomonas* species are halophilic and alkaliphilic microbes belonging to the family *Halomonadaceae*, that are typically isolated from high-salt environments. *Halomonas venusta* was originally described as *Alcaligenes venustus*,[77] but later transferred to the new genus *Deleya*, as *Deleya venusta*, by Baumann and colleagues.[78] In 1996 Dobson and Franzmann proposed combining the genus *Deleya* into a more broadly defined genus, *Halomonas*.[287] von Graevenitz and colleagues were the first to report a human infection caused by *H. venusta* in a wound that originated from a fish bite.[1147] They reported that the organism grew on BAP and MacConkey agars and appeared as mucoid, colorless colonies. Positive reactions occurred with nitrate, urea, and esculin. Additional biochemical reactions are given in Table 7-14. *H. venusta* are reported to be susceptible to most antibiotics.[1147]

CDC Halophilic Nonfermenter Group 1 consists of six phenotypically similar isolates received by the CDC between 1971 and 1998 that were similar to *H. venusta,* except for

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TABLE 7-14 Halophilic and/or Hydrogen Sulfide-Positive Pseudomonads

Test	*Shewanella*[a]		*Alishewanella*[b]	*Halomonas*[c]	CDC[d]
	S. putrefaciens	*S. algae*	*A. fetalis*	*H. venusta*	Halophilic Nonfermenter Group 1
Oxidase	+	+	+	+	+
Motility	+ p. 1–2	+ p. 1–2	–	+ pe	+ pe
Growth on MacConkey	+	+	+	+	+
Growth in 6.5% NaCl	–	+	+	+	+
H_2S (TSI butt)	+ (93)	+ (100)	–	NA	–
Growth at 42°C	–	+	+	NA	V (17)
OF glucose	Wk	–	–	+	V (83)
OF maltose	–	–	–	+	V (67)
OF lactose	–	–	–	–	–
OF mannitol	–	–	–	V (80)	V (67)
NO_3 reduction	V (80)	+	+	+	V (33)
NO_3 to gas	–	–	NA	NA	–
Ornithine	+	+	NA	–	–
Esculin hydrolysis	–	–	+	+	–
Gelatin hydrolysis	+	+	+	–	–
DNase	+	+	NA	NA	NA

[a]Data from references 367, 560, and 1172.
[b]Data from reference 1144.
[c]Data from reference 1147.
[d]Unpublished data from the Centers for Disease Control.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; A, acid reaction; Wk, weak acid; S, susceptible; R, resistant; NA, not available; p, polar flagella; pe, peritrichous flagella. Numbers in parentheses are percentage of strains giving positive reaction.

esculin hydrolysis and cellular fatty acid composition. Five of these were from human blood cultures, the sixth was from a hip wound culture (CDC, unpublished data). Three novel species of *Halomonas* were reported by Stevens and colleagues to cause bacteremia in two patients undergoing renal dialysis at a renal care center. A subsequent investigation of the renal dialysis equipment resulted in the isolation of three novel species of *Halomonas* traced to contaminated bicarbonate used to prepare dialysis fluid.[1030]

Pink-Pigmented Group

The pink-pigmented, nonfermentive, gram-negative bacilli associated with human infection include *Methylobacterium* species, *Rosemonas* species, *Azospirillum brasilense,* and *Asaia bogorensis*. These bacteria are slow-growing and present multiple diagnostic challenges to the microbiology laboratory, including culture, isolation, and identification to the species level. A review of the disease spectrum,

taxonomy, and laboratory identification of the pink-pigmented group of NFBs has been published by Hogue and colleagues.[447]

Family Methylobacteriaceae

Genus *Methylobacterium*. *Methylobacterium* species are gram-negative, pink-pigmented bacteria that have the ability to facultatively utilize methane.[399] Fifty-one species of *Methylobacterium* are currently recognized and additional unassigned biovars are recognized on the basis of carbon assimilation type, electrophoretic type, and DNA–DNA homology grouping.[354,399,400,1090] *M. mesophilicum*, formerly classified as *Pseudomonas mesophilica* and *Vibrio extorquens*, is the species most often isolated from human clinical specimens. Isolates are reported to be oxidase positive and motile; however, the oxidase reaction may be weak, and motility may be difficult to demonstrate. In our experience, all isolates seen have appeared nonmotile. Other key reactions include positive tests for catalase, urease, and amylase (Table 7-15). Additional differentiating characteristics can be found in the report by Urakami and colleagues.[1090]Isolates are slow-growing on ordinary media, with the best growth occurring on Sabouraud's agar, buffered charcoal–yeast extract agar, or Middlebrook 7H11 agar.[368] Optimal growth occurs from 25°C to 30°C. Colonies are dry and appear pink or coral in incandescent light (Image Plate 7-3E). Under UV

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TABLE 7-15 Key Characteristics of Pink-Pigmented Pseudomonads

Test	*Methylobacterium* spp.[a]	*Roseomonas* spp.[a]	*Azospirillum brasilense*[a] (*Roseomonas fauriae*)	*Asaia* spp.[b]
Oxidase	+[b]	V	+	–
Motility	+	V	+	+
Growth on MacConkey	V (15)	+	+	V (13)
OF fructose	V (50)	+	–	+
OF glucose	V (40)	–[c]	V (20)	+
OF xylose	V (89)	V	V (80)	+
OF mannitol	–	V	–	+
OF methanol	+	–	–	–
NO_3 reduction	V (25)	V	+	–
Starch hydrolysis	+	+	NA	NA
Urea	+	+	+	–
Colonies appear dark when exposed to long-wave UV light	+	–	–	–
Colonial morphology	Dry, coral	Mucoid, pink	Mucoid, pink	Pink-yellowish white, shiny, smooth
Gram's stain morphology	Vacuolated rods	Coccoid rods	Coccobacillus and rod	Rod

[a]Data from references 367, 447, and 1172.
[b]Data from references 252 and 1202.
[c]Except for some *R. gilardii* strains.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; NA, not available. Numbers in parentheses are percentage of strains giving positive reaction.

light, colonies appear dark, owing to the absorption of UV light. Although classified as a gram-negative rod, this species often does not stain well, or it may show variable results on Gram's staining. Individual cells contain large, nonstaining vacuoles that give this organism a unique microscopic appearance[1009] (Image Plate 7-3F). *M. mesophilicum* has been reported to cause chronic skin ulcers,[603] central catheter infection,[949] bacteremia in immunocompromised patients,[134,369,372,547,1009] synovitis[654] and peritonitis in a patient undergoing continuous ambulatory peritoneal dialysis.[934] Isolates have also been reported from bronchial washings[331] and from the cornea of a patient receiving corticosteroids.[310] One patient with meningitis caused by *M. mesophilicum* has been reported.[1219] Bloodstream infections caused by *M. radiotolerans, M. thiocyanatum, M. aminovorans, M. lusitanum,* and *M. zatmanii* have also been reported.[479,599] Tap water has been implicated as a possible mode of transmission for methylobacteria in the hospital environment because they have been reported to exhibit resistance to chlorination.[442,902] To learn more, see the review by Kaye,[547] Sanders,[949] Truant,[1080] and Kovaleva.[588]

Family Acetobacteraceae

Genus *Roseomonas* and Genus *Azospirillum*. *Roseomonas* are pink-pigmented bacteria that phenotypically and genotypically resemble *Methylobacterium* species but are separable from the latter by their inability to oxidize methanol, to assimilate acetamide and by lack of absorption of long-wave UV light.[906] Members of the genus are nonfermentative, weakly staining, gram-negative, plump coccoid rods, appearing in pairs or short chains, to mainly cocci with only an occasional rod (Image Plate 7-3G). They grow on 5% sheep blood agar, chocolate agar, buffered charcoal yeast extract (BCYE) agar, Sabouraud's agar, and almost always (91%) on MacConkey agar. Growth occurs at 25°C, 35°C and usually 42°C. Growth appears as pinpoint, pale-pink, shiny, raised, entire and often mucoid colonies after 2 to 3 days of incubation at 35°C (Image Plate 7-3H). We have noted that the *Roseomonas* grow very well on Sabouraud's agar, producing light pink mucoid (sometimes runny) colonies. The organisms do not appear black when viewed under UV light which helps to differentiate *Roseomonas* from *Methylobacterium* which do appear black under UV light.[906]

All strains are weakly oxidase positive (often after 30 seconds) or oxidase negative, catalase positive, and urease positive (Table 7-15).

The genus *Roseomonas* was first described in 1993 to include three named species, *R. gilardii* (genomospecies 1), *R. cervicalis* (genomospecies 2), and *R. fauriae* (genomospecies 3), and three unnamed genomospecies (genomospecies

4, 5, and 6).[906] Han et al.[420] sequenced the 16S rRNA of all 6 *Roseomonas* genomospecies and reported that *Roseomonas* genomospecies 1, 2, 4, and 5 were valid taxa, but that genomospecies 3 and 6 are not and indicated that the invalid *Roseomonas* species belong to the genus *Azospirillum*. Han and colleagues also proposed a new species, *Roseomonas mucosa*, and a new subspecies, *R. gilardii* subspecies *rosea* (in differentiation from *R. gilardii* subspecies *gilardii*).[420] *Roseomonas* genomospecies 3 (named *R. fauriae*) was then found to be a later synonym of *Azospirillum brasilense* and so the earlier name for this species has taxonomic preference.[433,1060] As of this writing, there are 21 named species in the genus *Roseomonas* and 17 named species in the genus *Azospirillum.*

Roseomonas species have been reported in several single-case reports from blood or catheter-related infections,[11,64,260,699,731,903,985,1040] vertebral osteomyelitis,[768] postoperative endophthalmitis,[178] and spinal epidural abscess.[694] Peritonitis, caused by *R. gilardii,*[950] *R. fauriae* (now *A. brasilense*),[96] and *R. mucosa*[127] in patients undergoing continuous ambulatory peritoneal dialysis have also been reported. In

multiple-case reports, about 60% of the isolates recovered have been from blood, with about 20% from wounds, exudates, and abscesses, and about 10% from genitourinary sites.[260,636,1037] The clinical characteristics of infections caused by *Roseomonas* species and antimicrobial susceptibilities of the isolates have been reviewed by Wang and colleagues.[1156]

Genus *Asaia*. *Asaia* are gram-negative obligately aerobic environmental bacteria belonging to the AAB and are assigned to the family *Acetobacteraceae*. The genus currently contains eight species. The natural habitats of *Asaia* spp. are reported to be in the flowers of the orchid tree, plumbago and fermented glutinous rice, all originating in hot tropical climates, particularly in Indonesia and Thailand.[1202] *Asaia bogorensis* has been reported as a cause of peritonitis in a patient on automated peritoneal dialysis.[1010] Daneshvar and colleagues identified 14 isolates of a novel *Asaia* species, of which 3 were from a dialysis-associated outbreak.[252] An *Asaia* species was isolated from the sputum of a 2-year-old boy with CF using *Burkholderia cepacia*-selective medium in a French hospital.[10] *A. bogorensis* has been recovered from the blood of two unrelated episodes of bacteremia in patients with a history of intravenous drug abuse.[1087,1088] Another species, *A. lannensis,* has been reported to cause bacteremia in two children with idiopathic dilated cardiomyopathy awaiting cardiac transplantation[527] and in a child with cancer and bone marrow transplantation.[2] *Asaia* species have also been isolated in two separate incidences from fruit-flavored bottled water that had spoiled as a result of bacterial overgrowth.[590,745] Isolates characterized at CDC are pink-pigmented, oxidase-negative, motile rods that are oxidative in OF glucose, xylose, and mannitol (Daneshvar MI, personal communication). Additional characteristics are given in Table 7-15. Moore reported that *Asaia* spp. were resistant to ceftazidime, meropenem, imipenem, trimethoprim, amikacin, vancomycin, aztreonam, penicillin, and ampicillin by disk diffusion.[745] The *A. bogorensis* strain reported by Snyder et al.[1010] was susceptible to aminoglycosides (amikacin, tobramycin, gentamicin) and resistant to ceftazidime and meropenem by disk diffusion.

Unnamed Species

Some organisms possessing characteristics of the Pseudomonads have not been officially named. These are included below.

***Pseudomonas*-Like Group 2.** This is an unclassified group of *Pseudomonas*-like organisms that previously had been included in CDC group IVd. Strains are oxidase positive and motile. Colonies on blood agar are reported to have a sticky consistency

and are difficult to remove.[368] Other identifying characteristics include growth on MacConkey agar; oxidation of glucose, xylose, mannitol, and lactose; hydrolysis of urea; and negative reactions for indole, nitrate, esculin hydrolysis, and oxidation of sucrose and maltose.[268] Isolates are similar to *B. gladioli* but do not oxidize dulcitol or inositol.[1172] The majority of strains (66%) are colistin resistant.[367] Human clinical isolates have been recovered from the respiratory tract, blood, spinal fluid, feces, urine, and dialysate.[368,391,567,579]

CDC Group 1c. CDC group 1c is a *Pseudomonas*-like organism that on Gram's stain appears as a gram-negative slender rod of variable length. It is motile with one or two polar flagella, grows on MacConkey agar, and oxidizes glucose and maltose, but not xylose, mannitol, lactose, or sucrose. It reduces nitrate to nitrite without gas formation, is arginine dihydolase positive, but negative for lysine and ornithine decarboxylase, esculin hydrolysis, and gelatin hydrolysis. Urease and citrate are variable. The organism grows well at 25°C, 35°C, and usually 42°C.[1172] Additional reactions are given in Table 7-3. Most isolates have come from human sources, including urine, sputum, blood, bronchial wash, wound, bile, and other sites.[1172] Their ability to cause human infection is not reported and antibiotic susceptibility data are not available.

CDC Group WO-1. This is the designation given to a group of weakly oxidative (WO) gram-negative rods isolated primarily from clinical specimens. They oxidize mannitol and glucose, often weakly and sometimes delayed (3 to 7 days), and reduce nitrate. Most strains are motile, with one or two polar flagella; however, motility is usually delayed in motility medium or is detected only by wet preparation. Strains are usually oxidase and catalase positive. Some strains produce soluble pigment (yellow, tan, amber, olive green, or brown). Other differentiating characteristics can be found in the article by Hollis and colleagues.[452] CDC WO-1 strains that oxidize xylose and are citrate positive are probably *Acidovorax delafieldii*, whereas strains WO-1 that are xylose negative and citrate negative are probably *Acidovorax temperans*.[1172] Isolates characterized at the CDC have been from blood (33%), CSF (10%), urine, lung, wound, and some environmental sources.[452]

OFBA-1. OFBA-1 is an unclassified medium-to-long gram-negative, motile rod with one or two polar flagella that has the unusual property of producing acid in OF base medium without carbohydrate; thus the acronym "OFBA," meaning "OF Base Acid." The organism most closely resembles *P. aeruginosa* biochemically because of β-hemolysis, growth at 42°C, presence of arginine dihydrolase, nitrate reduction

to gas, and utilization of most carbohydrates.[1149,1172] Unlike *P. aeruginosa,* it is negative for pyocyanin and pyoverdin production and acetamide hydrolysis. Isolates have been recovered from blood, leg ulcer, abdominal wound, bronchial wash, and a catheter tunnel infection in a patient on continuous ambulatory peritoneal dialysis.[1149,1172]

Organisms That Are Motile With Peritrichous Flagella

Family Alcaligenaceae

This family includes the clinically relevant genera *Achromobacter*, *Advenella, Alcaligenes*, *Bordetella*, *Kerstersia,* and *Oligella*.[273,354] Members of the family *Alcaligenaceae* are gram-negative rods that are usually oxidase positive, grow on MacConkey agar, and are motile by means of peritrichous flagella. The biochemical features that differentiate the members of this family, as well as certain other nonfermenters with similar biochemical characteristics, are given in Tables 7-16 and 7-17.

Genus *Alcaligenes*. The taxonomy of the genus *Alcaligenes* is closely intertwined with the taxonomy of the genus *Achromobacter*, and several *Alcaligenes* species have now been reclassified as *Achromobacter* species.[1196]

Alcaligenes faecalis. *A. faecalis* is the most frequently isolated member of the *Alcaligenaceae* in the clinical laboratory. Members of this species produce strong alkaline reactions in all carbohydrate media. Most strains form characteristic colonies with a thin, spreading irregular edge (Image Plate 7-4A). Some strains (previously named "*A. odorans*") produce a characteristic fruity odor (sometimes described as the odor of green apples) and cause a greenish discoloration of blood agar medium. A key biochemical feature of this species is its ability to reduce nitrite but not nitrate. *A. faecalis* exists in the soil and water and has been isolated from many types of clinical specimens. It is a rare cause of acute otitis, urinary tract infection and bacteremia.[8,102] Most infections are opportunistic and are acquired from moist items, such as nebulizers, respirators, and lavage fluids. It is often found in mixed cultures, particularly in samples of diabetic ulcers of the feet and lower extremities, and its clinical significance is difficult to determine.

Genus *Achromobacter*. The genus *Achromobacter* was described in 1981 by Yabuuchi and Yano[1199] and originally contained a single species, *Achromobacter*

xylosoxidans. Based on taxonomic studies, Yabuuchi et al.[1196] transferred the species *Alcaligenes ruhlandii* and *Alcaligenes piechaudii* to the genus *Achromobacter* and proposed the transfer of *Alcaligenes denitrificans* to the genus *Achromobacter* as *Achromobacter xylosoxidans* subsp. *denitrificans*, thus automatically creating a second subspecies, *Achromobacter xylosoxidans* subsp. *xylosoxidans*. However, the reclassification of *Alcaligenes denitrificans* as a subspecies of *Achromobacter xylosoxidans* contradicted previous work that showed that the two taxa are distinct species.[1105] Accordingly, Coenye and colleagues have proposed that *Alcaligenes denitrificans* be reclassified as *Achromobacter denitrificans*.[217] In this textbook, we treat these organisms as separate species, referring to them as *Achromobacter xylosoxidans* and *Achromobacter denitrificans*, respectively. For identification purposes, the *Achromobacter* species can be divided into the asaccharolytic and saccharolytic species based on their ability to oxidize OF glucose (Tables 7-16 and 7-17). The in vitro susceptibility of *Achromobacter* spp. and a comparison of disk diffusion, Etest, and agar dilution methods have been provided by Almuzara and colleagues.[17]

Asaccharolytic *Achromobacter* Species. *A. piechaudii* was first described in 1986.[571] It can be distinguished from *Alcaligenes* and other asaccharolytic *Achromobacter* species by its ability to reduce nitrate but not nitrite and grow in 6.5% sodium chloride (Table 7-16). Although this species is reported to have been isolated mainly from human clinical material, there are only rare reports of a possible pathogenic role for this species. One patient with diabetes developed a chronic ear discharge associated with this organism,[830] an immunocompromised elderly man developed recurrent bacteremia in association with an intravenous catheter in,[546] and a patient with previous malignancy but no known immunosuppression developed bacteremia with this organism.[589]

A. denitrificans strains are found predominantly in the soil but can occasionally be found in human clinical specimens. Isolates have been reported from blood collection tubes, blood, ear, CSF, and urine.[1146] Serious infections attributed to *A. denitrificans* include community-acquired pneumonia,[54] peritoneal dialysis-related peritonitis,[147] and a patient with a renal abscess and a nephro-cutaneous fistula.[974] An organism that is biochemically similar to *A. denitrificans*, known as *Alcaligenes*-like group 1, has been recovered from blood, brain abscess, urine, bronchial wash, knee joint and water, suggesting that it may have greater potential to cause human infection. It can be differentiated from *A. denitrificans* by cellular fatty acid composition, failure to grow on SS agar, failure to alkalinize tartrate and acetamide, and a positive urease reaction (Table 7-16).[1172]

Two novel asaccharolytic *Achromobacter* species, *A. insolitus* and *A. spanius,* have

been recently described by Coenye et al.[218] These two species are found rarely in human clinical specimens. They are both oxidase and catalase positive and reduce nitrate without the formation of gas. They are both asaccharolytic and are biochemically difficult to separate from each other and from other asaccharolytic *Achromobacter* species and from *Alcaligenes faecalis*.[218]

Recently Vandamme and colleagues have named four new asaccharolytic species of *Achromobacter* representing different MLST genogroups. The new species are *A. mucicolens* (genogroup 4), *A. animicus* (genogroup 6), *A. spiritinus* (genogroup 10) and *A. pulmonis* (genogroup 11). All have been isolated from human sputum, some from patients with CF.[1111]

Saccharolytic *Achromobacter* Species. *A. xylosoxidans* is easily distinguished from the genus *Alcaligenes* and the asaccharolytic *Achromobacter* species by acidification of OF glucose and xylose (thus, the species name). It has been isolated from many types of specimens, most frequently blood,[8,304,384,1163] but also from CSF, bronchial washings, urine, pus, and wounds.[497,1145,1146] It may be an opportunistic pathogen that has been reported to cause nosocomial infections, including pneumonia, bacteremia, and meningitis,

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TABLE 7-16 Key Characteristics of *Alcaligenes*, Asaccharolytic *Achromobacters, Advenella, Kerstersia, Bordetella*, and related species[a]

Test	*Alcaligenes* *A. faecalis*	*Achromobacter* *A. denitrificans*	*Achromobacter* *A. piechaudii*	CDC *Alcaligenes*-like Group 1[b,c]	*Advenella* *A. incenata*[d]	*Kerstersia* *K. gyiorum*[e]	*Bordetella* *B. trematum*[f]	*Bordetella* *B. hinzii*	*Bordetella* *B. avium*	*Bordetella* *B. bronchi-septica*	*Oligella* *O. ureolytica*	*Cupriavidus* *C. pauculus*	*Cupriavidus* *C. gilardii*[g]
Oxidase	+	+	+	+	+	–	–	+	+	+	+	+	+
Motility	+	+	+	+	V	V	+	+	+	+	V (84)	+	+
Growth on MacConkey	+	+	+	+	NA	+[i]	+	+	+	+	V (79)	+	NA
OF Glucose	–	–	–	–	V	–	–	–	–	–	–	–	–
OF Xylose	–	–	–	–	V	–	–	–	–	–	–	–	–
NO_3 to NO_2	–	+	+	+	–	–	V (66)	–	–	+ (92)[b]	+	V (11)[b]	–
NO_3 to N_2	–	+	–	+	–	–	V (11)	–	–	–	V (58)	–	–
NO_2 to N_2	+	+	–	+	NA	–	NA	–	–	–	V (63)	–	–
Urea	–	V (31)	–	V (75)	V	–	–	–	–	++	++	++	–
PAD	–	–	–	NA	NA	–[i]	NA	V (15)	–	V (25)	+	V (7)	NA
Acetamide	+	V (45)	V (42)	–	–	–	V (89)	+	+	–	V (11)	–	–
6.5% NaCl	+	–	+	V (13)	V	+[i]	NA	–	–	–	–	–	–
Malonate	+	+[h]	V (29)[b]	NA	NA	V[i]	+	+	–	+	NA	NA	NA
Flagella	pe	pe	pe	pe	NA	NA	pe	pe	pe	pe	pe	pe	1–2 p

[a]Unless otherwise stated, data are from reference 367.
[b]Percentage positive derived from reference 1172.
[c]Similar to *A. denitrificans*, can be differentiated by inability to grow on SS agar.
[d]Data from reference 224.
[e]Data from reference 217.
[f]Data from reference 1105.
[g]Data from reference 210.
[h]Data from reference 571.
[i]Schreckenberger P, personal observation.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; ++, strong positive reaction (4 hours); pe, peritrichous flagella; p, polar flagella; NA, results not available. Numbers in parentheses are percentage of strains giving positive reaction.

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TABLE 7-17 Key Characteristics of *Ochrobactrum*, *Rhizobium*, *Agrobacterium*, *Pannonibacter*, and Saccharolytic *Achromobacter* Species[a]

Test	*Ochrobactrum* *O. anthropi* *O. intermedium*[b]	*Rhizobium* *R. radiobacter*	*Agrobacterium* "*Agrobacterium* yellow group"[c]	*Pannonibacter* *P. phragmitetus* Biovar B	*Pannonibacter* *P. phragmitetus* Biovar E	*Achromobacter* Group F	*Achromobacter* *A. xylosoxidans*	OFBA-1[d]
Oxidase	+	+	+	+	+	+	+	+
Motility	+; pe	+; pe	+; p, 1–2[e]	+; p, L	+; p, L	+	+; pe	+; p, 1–2
Growth on MacConkey	+	+	+	+	+	–	+	+
OF glucose	+	+	+	+	+	+	+	+
OF xylose	+	+	+	+	+	+	+	+
OF lactose	–	+	+	–	–	–	–	+
OF mannitol	V (50)	+	–	+	–	+	–	+
OF adonitol	+	+	–	–	–	+	–	NA
OF dulcitol	+	+	–	–	–	+	–	NA
ONPG	–	+	V (40)[f]	+	+	–	–	–
NO_3 to NO_2	+ (98)	V (84)	V (40)[f]	+ (100)[g]	+ (100)[g]	+[f]	+ (99)	+ (100)[g]
NO_3 to N_2	+ (91)	– (8)	NA	+ (100)[g]	+ (100)[g]	NA	V (69)	+ (100)[g]
NO_2 to N_2	+ (99)[f]	V (38)[g]	–[f]	+ (100)[g,h]	+ (100)[g,h,i]	–[f]	V (51)[f]	NA
Urea	+	+	–	+	+	+	–	V (50)
PAD	+	+	–	NA	NA	NA	–	+
Acetamide	–	–	NA	–	–	NA	V (66)	–
Esculin	V (40)	+	+	+	+	+	–	–
Pigment	–	–	Yellow	–	–	–	–	–

[a]Unless otherwise stated, data from reference 367.
[b]Colistin resistance has been suggested to differentiate *O. intermedium* (colistin-resistant) from *O. anthropi* (colistin-sensitive).[1132]
[c]Data from reference 1047.
[d]Data from reference 1149.
[e]Motile at room temperature, nonmotile at 37°C.[1047]
[f]Data from reference 464.
[g]Data from reference 1172.
[h]When tested at 48 hours of incubation, nitrite reduction may only be observed in media containing ≤0.01% nitrite.
[i]Holmes reports nitrite reduction negative for *Achromobacter* group E.[464]
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; pe, peritrichous flagella; p, polar flagella; L, lateral flagella; NA, results not available; PAD, phenylalanine deaminase.
Numbers in parentheses are percentage of strains giving positive reaction.

in patients with underlying disease.[749,898,1018] It has been reported that *A. xylosoxidans* colonizes the respiratory tract of intubated children and patients with CF and that colonization of patients with CF is associated with an exacerbation of pulmonary symptoms.[305,942] *A. xylosoxidans* has been shown to be the predominant *Achromobacter* species isolated from CF patients around the world.[25,68,263,882,1023]

A. ruhlandii was originally described as a soil commensal and not thought to be pathogenic to humans.[553] However, in a study by Spilker and colleagues on the distribution of *Achromobacter* species in CF patients, *A. ruhlandii* was found to be the second most commonly isolated species after *A. xylosoxicans* accounting for 23.5% of the isolates.[1023]An exceptionally resistant clone of *A. ruhlandii*, designated the Danish epidemic strain (DES), spread among CF patients in Denmark in 2006 and could not be stopped despite aggressive infection control measures.[904,905] Species of *Achromobacter* recovered from CF patients are frequently misidentified using biochemical reactions.[904,905] 16S rRNA sequencing is also not able to discriminate

among different *Achromobacter* species.[825,1022] Papalia reported that identification of *A. ruhlandii* can be achieved by sequencing a single OXA gene (gene $bla_{\text{oxa-258}}$).[825]

Four new saccharolytic species of *Achromobacter* representing different MLST genogroups have recently been named. The new species are *A. insuavis* (genogroup 2), *A. aegrifaciens* (genogroup 5), *A. anxifer* (genogroup 7), and *A. dolens* (genogroup 14). All have been isolated from human sputum in patients with and without CF.[1112]

Genus *Advenella.* *Advenella* is in the family *Alcaligenaceae* and consists of four species: *A. faeciporci, A. incenata, A. kashmirensis,* and *A. mimigardefordensis* that are mainly found in soil, sludge, and compost. Only *A. incenata* has been isolated from various human and veterinary clinical samples.[224] *Advenella incenata* is a gram-negative, small (1 to 2 μm), rod-shaped or coccoid bacterium that occurs singly, in pairs, or in short chains.[224] On nutrient agar, colonies are flat or slightly convex with smooth margins and appear light brown in color.[224] They are oxidase and catalase positive. Motility and oxidation of OF glucose are strain dependent. They are uniformly negative for amylase, lysine and ornithine decarboxylase, arginine dihydrolase, gelatinase, DNase, ONPG, esculin hydrolysis, and indole production (Table 7-16). Isolates have been recovered from human blood, sputum, and wound specimens.[224]

Genus *Bordetella.* Currently the genus *Bordetella* comprises eight species, four are motile with peritrichous flagella (*B. avium*, *B. bronchiseptica*, *B. hinzii*, *B. trematum* [Table 7-16]) and four are nonmotile (*B. holmesii*, *B. pertussis*, *B. parapertussis*, *B. petri* [Table 7-18]). The three most common human species, *B. pertussis*, *B. parapertussis,* and *B. bronchiseptica*, cannot be differentiated genotypically through DNA homology studies and are likely subspecies or strains of a single species with different host adaptations.[827] Phenotypically they behave quite differently, however. *B. bronchiseptica* is motile by means of peritrichous flagella and grows readily on ordinary media, whereas *B. pertussis* and *B. parapertussis* are both nonmotile. *B. pertussis* requires special media for growth, whereas *B. parapertussis* will grow on blood, chocolate, and MacConkey agars. *B. pertussis* and *B. parapertussis* are the etiologic agents of whooping cough and are discussed in detail in Chapter 9 with the fastidious gram-negative rods.

Bordetella bronchiseptica. Colonies of *B. bronchiseptica* grow well on blood and MacConkey agar and in 24 hours appear as smooth, translucent, colorless colonies about 1.5 mm in diameter. On Gram's stain, the organisms appear small and coccobacillary. They have the distinguishing biochemical feature of rapidly converting Christensen's urea agar (Image Plate 7-2A). Other distinguishing features are given in

Table 7-16. *B. bronchiseptica* is found in the respiratory tract of domestic and wild mammalian animals including dogs, cats, rabbits, rodents, horses and pigs ("bronchiseptica" is derived from the Greek word *bronchus*, meaning "trachea"). It is an infrequent isolate in the clinical laboratory, and only a few human infections have been reported in the literature.[364] Pedersen and coworkers[829] reported the recovery of only 12 strains of *B. bronchiseptica* from a total of 565 nonfermenters, all of which were from respiratory specimens obtained from patients who were free of infections. Most symptomatic infections have been in animal caretakers who presented with mild pertussis-like symptoms. Ghosh[364] reported fatal *B. bronchiseptica*-induced septicemia and bronchopneumonia in a malnourished patient with alcoholism, which indicates that the organism can be virulent under the right circumstances. Woolfrey and Moody reviewed 25 cases of human *B. bronchiseptica* infection associated with sinusitis, tracheobronchitis, acute pneumonia, pneumonia with septicemia, septicemia, and whooping cough.[1191] For the whooping cough cases, it is likely that *B. bronchiseptica* acted as a colonizer and not as the cause. *B. bronchiseptica* has been reported to cause pneumonia in patients with AIDS,[23,267,272,307,351,664,727,782,1189] acute leukemia,[383] CF,[1154] and thoracic trauma[889] and following bone marrow[79,194] or heart transplantation.[175] There have been two reports of bronchitis caused by *B. bronchiseptica*, one in an elderly woman[841] and one in an immunosuppressed patient.[723] Two patients with meningitis,[84,169] one patient with endocarditis,[359] and one patient with a pancreatic abscess with associated bacteremia[714] due to *B. bronchiseptica* have also been reported. These highlight the fact that *B. bronchiseptica* can be associated with severe disease. A review of the microbiolgic and clinical aspects of respiratory infections associated with *B. bronchiseptica* has been provided by Garcia-de-la-Fuente et al.[349] Most strains of *B. bronchiseptica* are susceptible to most antibiotics, with the exception of ampicillin, cefamandole, and cefoxitin.[385,593]

Bordetella ansorpii. *B. ansorpii* was first described in 2005 and was isolated from a purulent epidermal cyst but its pathogenic significance was unclear.[580] A second report was published in 2007 wherein an 88-year-old man with leukemia developed positive blood cultures.[340] The organism is described as a motile, gram-negative bacillus that grows on both blood agar and MacConkey agar and is oxidase negative. It is also negative for indole production, urease, arginine dihydrolase, esculin and gelatin hydrolysis, ONPG, nitrate reduction, and is asaccharolytic.[580]

Bordetella avium. *B. avium* is a pathogen for birds causing coryza or rhinotracheitis in poultry, especially in turkeys.[554] Similar to the species in the *B. bronchiseptica* complex, *B. avium* exhibits a strong tropism for the ciliated epithelium of the upper respiratory tract.

Bordetella hinzii. *B. hinzii* was formerly referred to as *Alcaligenes faecalis* type II, or *B. avium*–like, and has been isolated from the respiratory tracts of chickens and turkeys in various parts of the world. Human isolates have been recovered from blood,[228,544] and sputum,[347,1109] including repeated isolations from the sputum of a patient with CF.[345] *B. hinzii* has also been isolated from multiple biliary specimens collected over 6 months from a liver-transplant recipient with cholangitis.[45] *B. hinzii* are motile and oxidase positive and must be distinguished from phenotypically similar organisms, as shown in Table 7-16.

Bordetella holmesii. Strains of *B. holmesii* have been recovered mostly from human blood.[405,646,717,747,786,978,1058,1171] Shepard and colleagues at the CDC analyzed the clinical histories of 30 patients with *B. holmesii* bacteremia whose isolates were submitted to CDC for identification. Of the 26 patients for whom data were available, 22 (85%) were anatomically or functionally asplenic. *B. holmesii* was the only organism isolated from the blood samples in 25 (96%) of the 26 patients.[978] Despite initial reports that *B. holmesii* does not cause respiratory disease, patients may be either infected or colonized with *B. holmesii* in their nasopharynx. Scientists from the Massachusetts State Laboratory Institute reported the isolation of *B. holmesii*, but not *B. pertussis*, from 32 nasopharyngeal specimens collected over a 3-year period from patients with pertussis-like symptoms.[717,1207] Several studies have shown that *B. holmesii* can give strong positive results with PCR assays targeting IS *481* of *B. pertussis*, thus confounding the diagnostic reliability of such assays.[659,660,896] Russell and colleagues reported a patient with interstitial and lobar pneumonia with progression to pulmonary fibrosis caused by *B. holmesii*.[933] *B. holmesii* is reported to be susceptible to amikacin, ampicillin, cefazolin, cefotaxime, ceftazidime, chloramphenicol, gentamicin, mezlocillin, trimethoprim–sulfamethoxazole, imipenem, ciprofloxacin, and piperacillin–tazobactam.[1058] *B. holmesii* was formerly classified as CDC Group NO-2 and is nonmotile and oxidase negative, making it phenotypically similar to *Acinetobacter* species and CDC Group NO-1 (Table 7-18). Additional morphologic and phenotypic characteristics are given under the heading "Organisms That Are Nonmotile and Oxidase Negative."

Bordetella petrii. *B. petrii* is nonmotile and oxidase positive. It has been isolated mainly from the environment and is the only member of the genus capable of anaerobic growth.[1152] More recently *B. petrii* has been isolated from clinical specimens linked to sinusitis,[767] septic arthritis and osteomyelitis,[788] mandibular osteomyelitis,[341] mastoiditis,[1027] and chronic pulmonary disease[616] and from respiratory samples and a postmortem spleen sample of a patient presenting with

bronchiectasis and cavitary lung disease associated with a nontuberculous mycobacteria infection.[1215] *B. petrii* has also been reported in respiratory specimens from patients with CF.[737,1020]

Bordetella trematum. *B. trematum* is oxidase negative and motile with peritrichous flagella. Growth occurs on

TABLE 7-18 Key Characteristics of *Acinetobacter, Bordetella*, CDC Groups NO-1 and EO-5, and *Granulibacter bethesdensis*[a]

	Acinetobacter		*Bordetella*		CDC Groups		*Granulibacter*
Test	*A. baumannii*	*A. lwoffii*	*B. holmesii* (NO-2)	*B. parapertussis*[b]	NO-1	EO-5[c]	*G. bethesdensis*[d]
Oxidase	–	–	–	–	–	–	–
Motility	–	–	–	–	–	–	–
Growth on MacConkey agar	+	+	+	+	V (20)	–	NA
Growth at 42°C	+	–	–	V (18)	V (15)	–	–
OF glucose	+	–	–	–	–	+	+
NO_3 reduced	–	–	–	–	+	–	NA
Gelatin	V	V	–	–	–	–	NA
Urea	V	V	–	+	–	+	V
Pigmentation	–	–	Brown, soluble	Brown, soluble	–	Yellow pigment produced by some strains	Yellow

[a]Except where noted, data are from reference 1172.
[b]Grows slowly on blood agar producing minuscule colonies, β-hemolysis, and a brown, water-soluble pigment.[1172]
[c]Data from reference 249.
[d]Data from reference 403.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; NA, results not available. Numbers in parentheses are percentage of strains giving positive reaction.

MacConkey agar and malonate is utilized. Other biochemical reactions are given in Table 7-16. Strains have been isolated from ear infections and wounds of humans, but not from respiratory specimens.[1105] Dorittke et al.[294] had described the isolation of a "*B. avium*-like organism" from a patient with chronic otitis media, whereby the respective isolate (strain LMG 13506) was later reclassified as *B. trematum*.[1105] Daxboeck et al.[259] reported the isolation of *B. trematum* from a diabetic foot ulcer; however, there was no evidence for a causative role of the organism in the foot infection. A summary of *B. trematum* reported cases human infection has been

provided by Almagro-Molto et al.[12]

Genus *Kerstersia*

Kerstersia gyiorum. Coenye and colleagues have described a novel genus, *Kerstersia* gen. nov. with a single species *K. gyiorum* (Greek *gyion,* meaning "limb": referring to the fact that the majority of strains were isolated from human leg wounds).[217] These bacteria appear as gram-negative, small (1 to 2 μm long), coccoid cells that occur singly, in pairs or in short chains. On nutrient agar, colonies are flat or slightly convex, with smooth margins and color ranging from white to light brown. Growth occurs at 28°C and 42°C, motility is strain dependent, and all strains are asaccharolytic. All strains are catalase positive but are negative or slow positive for oxidase. Arginine, lysine, and ornithine decarboxylase, β-galactosidase, gelatinase, urease, DNase, reduction of nitrate and nitrite, esculin, and starch hydrolysis are all negative (Table 7-16). In our experience, *K. gyiorum* grows well on both BAP and MacConkey, producing characteristic dull, flat, spready colonies that give a swarming appearance (Image Plate 7-5F, 7-5G). In the original description, *K. gyiorum* was isolated from various human specimens, including feces, sputum, and leg and ankle wounds; however, the clinical significance was unknown.[217] Serious infections caused by *K. gyiorum* have subsequently been reported and include chronic ear infections,[15,765,836] chronic leg wound[836] and bronchoalveolar lavage fluid from a patient with a chronic tracheostomy.[282] We have isolated three strains at Loyola University Medical Center from urine, leg wound, and tracheal aspirate. Our strains and those from three published reports were unambiguously identified with MALDI-TOF MS using the Bruker BioTyper (software version 3.1).[282,765,836]

Kerstersia similis. A second species of *Kerstersia*, *K. similis*, has been described for a genospecies that biochemically resembles strains of *K. gyiorum*. Identification can only be obtained with genetic typing or carbon assimilation using the Biolog GEN III system.[1101]

Genus *Oligella*

The genus *Oligella* consists of two species: *O. urethralis* (formerly *Moraxella urethralis* and CDC group M-4) and *O. ureolytica* (formerly CDC group IVe).[925] One species is oxidase positive and motile with pertitrichous flagella (*O. ureolytica*), whereas the other species is oxidase positive and nonmotile but will be discussed here for the purpose of continuity.

Oligella ureolytica. Colonies of *O. ureolytica* are first seen as slow-growing on blood agar medium, producing pinpoint colonies after 24 hours but large colonies after 3 days of incubation. Colonies are white, opaque, entire, and nonhemolytic. *O. ureolytica* strains phenotypically resemble asaccharolytic *Achromobacter* species, *Bordetella bronchiseptica* and *Cupriavidus pauculus*, in that they are

nonsaccharolytic, oxidase positive, and motile by means of peritrichous flagella. They differ from *Achromobacter* species by their ability to rapidly hydrolyze urea in Christensen's urea agar. Additional differentiating features are shown in Table 7-16. Most isolates have been obtained from human urine, often in patients with long-term indwelling catheters. *O. ureolytica* bacteremia has been reported in several patients with varying predisposing conditions, including a patient with obstructive uropathy,[913] a patient with AIDS,[689] an 18-month-old child with pneumonia,[615] a newborn infant[275] and a cancer patient.[70] *O. ureolytica* tends to be susceptible to most antibiotics, although there is one report of a highly resistant strain that demonstrated in vitro resistance to the penicillins, cephalosporins, imipenem, meropenem, ciprofloxacin, and trimethoprim–sulfamethoxazole, but was susceptible to the aminoglycosides, tetracycline, and levofloxacin.[615]

Oligella urethralis. *O. urethralis* is similar to *Moraxella* species in that isolates are coccobacillary, oxidase positive, nonmotile, and gram-negative bacteria. Colonies are smaller than those of *M. osloensis* and are opaque to whitish. *O. urethralis* and *M. osloensis* share additional biochemical similarities (e.g., accumulation of poly-β-hydroxybutyric acid and failure to hydrolyze urea), but can be differentiated on the basis of nitrite reduction, growth at 42°C, and alkalinization of formate, itaconate, proline, and threonine (all positive in *O. urethralis*, negative in *M. osloensis*).[853] Cellular fatty acid analysis can also be used to differentiate these two species.[1172] Biochemical features that help differentiate *O. urethralis* from *Moraxella* species are shown in Table 7-21. As the name indicates, *O. urethralis* is most frequently recovered from urethral specimens and is considered a commensal of the genitourinary tract; however, in rare cases it may lead to urosepsis.[871] There has been one report of *O. urethralis* infectious arthritis in a patient wherein the presentation clinically mimicking gonococcal arthritis.[728]

Family Rhodobacteriaceae

Genus *Pannonibacter* and *Achromobacter* Groups A–F. Holmes and colleagues separated achromobacters into six groups (A–F) based on genetic patterns.[457] *Achromobacter* groups A, C, and D constitute a single species and were found to be identical to *Ochrobactrum* anthropi (discussed later in this chapter).[465] *Achromobacter* groups B and E constitute biotypes of a single genus and species.[455,456,459] that was confirmed by Holmes et al.[466] to be identical to a previously described taxon, *Pannonibacter phragmitetus*.[112] *Achromobacter* group F, however, is genetically distinct from groups B and E and is currently unnamed.[455,456] The biochemical tests useful for separation of *Achromobacter* groups A–F from phenotypically similar

bacteria have been reported by Holmes and coworkers.[464] *P. phragmitetus* can be separated from *O. anthropi* by the properties of being ONPG and esculin positive and failing to produce acid from adonitol and dulcitol.[459] *Achromobacter* Group F are similar to *O. anthropi* except that they do not produce gas from nitrite and fail to grow on MacConkey agar (Table 7-17). *P. phragmitetus* biovar B has been isolated from the blood of patients with septicemia[455,458,512] and one patient with

replacement-valve endocarditis.[721] Isolates of *P. phragmitetus* biovars E and F have also been recovered from blood.[455,456]

Family Rhizobiaceae

The only medically important member of the family *Rhizobiaceae* is the genus *Rhizobium*. However, members of the genus *Rhizobium* are phenotypically similar to saccharolytic *Achromobacter* species and *Ochrobactrum anthropi*, from which they must be differentiated. Characteristics that are useful in separating these organisms are given in Table 7-17.

Genus *Rhizobium* (Formerly *Agrobacterium*). The former genus *Agrobacterium* contained several species of plant pathogens occurring worldwide in soils.[552] As a result of a large number of comparative studies, four distinct species of *Agrobacterium* were recognized: *Agrobacterium radiobacter* (formerly *A. tumefaciens* and CDC group Vd-3), *Agrobacterium rhizogenes* (subsequently transferred to the genus *Sphingomonas* as *S. rosa*),[1054] *Agrobacterium vitis*,[803] and *Agrobacterium rubi*.[952] The separation of the phenotypically indistinguishable species *A. tumefaciens* and *A. radiobacter* was based on the presence of a plant tumor-inducing plasmid, present in *A. tumefaciens* and absent in *A. radiobacter*. Genetic studies showed that the two species were the same, and a proposal was made to reject the name *A. tumefaciens* and to designate *A. radiobacter* as the type species for the genus *Agrobacterium*.[952] Young and colleagues[1210,1211] proposed an emended description of the genus *Rhizobium* to include all species of *Agrobacterium*. Following this proposal, the new combinations are *Rhizobium radiobacter, R. rhizogenes, R. rubi,* and *R. vitis*.[1210] Farrand and colleagues have presented evidence from classical and molecular comparisons that support the conclusion that agrobacteria biovars 1 and 3 are sufficiently different from members of the genus *Rhizobium* to warrant retention of the genus *Agrobacterium*.[323] No doubt, the final chapter on the classification of *Agrobacterium radiobacter* has not been written, and the controversy is likely to

continue for some years; however, for the purposes of this textbook, we have decided to accept the arguments set forth by Young and colleague[1211] and will refer to this bacterium as *Rhizobium radiobacter*.

Rhizobium radiobacter. The key biochemical tests used in differentiating *R. radiobacter* from the closely related species *Ochrobactrum anthropi* are given in Table 7-17. Key features for this group of organisms are a rapid urease reaction and a positive test for phenylalanine deaminase. Colonies of *R. radiobacter* grow optimally at 25°C to 28°C, but will grow at 35°C as well. They appear circular, convex, smooth, and nonpigmented to light beige on blood agar. Colonies may appear wet and become extremely mucoid and pink on MacConkey agar with prolonged incubation (Image Plate 7-4B). *R. radiobacter* is occasionally isolated from clinical specimens but only rarely linked with human infection. In reports of human infection, *Rhizobium* has been most frequently isolated from blood,[105,142,306,309,311,336,418,687,860,867,916,1178] followed by peritoneal dialysate,[425,914,916] urine,[21,916] and ascitic fluid.[879] The majority of infections have occurred in patients with transcutaneous catheters or implanted biomedical prostheses, and effective treatment often requires removal of the device.[105,306,309,311,336,418,860,867,916,1178] Lai and colleagues examined the medical records of 13 patients with *R. radiobacter* infections during a 7-year period. Ten (76%) had underlying hematologic malignancy or solid-organ cancer. Six (46%) had febrile neutropenia during the course of their infection. Fifty-four percent of the infections were catheter-related bacteremia, and 92% were hospital-acquired.[601] Two cases of *R. radiobacter* endophthalmitis subsequent to cataract extraction have also been reported.[736,769] Antimicrobial susceptibility is variable, and requires testing of individual isolates. Lai et al.[601] reported that all isolates tested in their series were susceptible to cefepime, piperacillin–tazobactam, carbapenems, and ciprofloxacin.

Family Brucellaceae

Genus *Ochrobactrum*

Ochrobactrum anthropi. *O. anthropi* is the name given to the urease-positive "*Achromobacter*" species formerly designated CDC group Vd-1 and Vd-2 and *Achromobacter* groups A, C, and D of Holmes et al.[464,465] Subsequent studies have shown, however, that biogroup C and some strains belonging to biogroup A constitute a homogeneous DNA–DNA hybridization group separate from *O. anthropi* that has been given the new species designation of *Ochrobactrum intermedium*.[1132] Both *Ochrobactrum* species are closely related to *Brucella* spp., with *Ochrobactrum intermedium* occupying a phylogenetic position that is intermediate between *O. anthropi* and *Brucella*.[1132] The two species share identical phenotypic properties. They are oxidase positive, saccharolytic and motile by means of peritrichous flagella.

Good growth is observed on routine media in 24 hours. Colonies are about 1 mm in diameter and appear circular, low convex, smooth, shining, and entire. Isolates we have observed grow readily on MacConkey agar, and colonies appear mucoid. Key tests useful in distinguishing *O. anthropi* from related organisms include their ability to hydrolyze urea, inability to hydrolyze esculin, and a negative ONPG test. Additional biochemical tests useful in differentiating *O. anthropi* from *Rhizobium* species and saccharolytic *Achromobacter* species are shown in Table 7-17.

All strains of *O. anthropi* have thus far been recovered from human clinical specimens (*anthropi* is derived from Greek and means "of a human being"). Strains have been isolated predominantly from blood, wounds, urogenital tracts or urine, respiratory tracts, ears, feces, an eye, and CSF.[36,69,133,167,465,511,686,1124] Of particular concern are recent reports of CVC-related sepsis caused by *O. anthropi*.[20,198,393,415,550,573,937,1031] Isolation of this organism from blood should raise suspicion of CVC-related infection. Opportunistic infections and nosocomial outbreaks of *O. anthropi* are being increasingly reported. There has been one report of a patient who developed septic shock following peripheral venous infusion of a solution contaminated with *O. anthropi*,[555]and another of nosocomial bacteremia in five organ-transplant recipients following infusion of contaminated anti-thymocyte globulin.[321] Patients with infective endocarditis due to *O. anthropi* have also been reported.[683,919] The clinical characteristics of *O. anthropi* bacteremia have been reviwed by Hagiya and colleagues.[416] A review of *O. anthropi* infection in immunocompetent hosts has been provided by Vaidya et al.[1093]

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O. anthropi is reported to be susceptible to aminoglycosides, carbenicillin, fluoroquinolones, imipenem, tetracycline, and trimethoprim–sulfamethoxazole, but resistant to other antimicrobial agents.[101,367,550,1146]

Ochrobactrum intermedium. There are no biochemical tests currently available that separate *O. intermedium* from *O. anthropi*; however, it has been suggested that colistin (polymyxin E) and polymyxin B resistance can be used to separate *O. intermedium* (resistant) from *O. anthropi* (susceptible).[1132] One patient with a pyogenic liver infection due to *O. intermedium* has been reported[741]; however, because of the close phenotypic similarity between *O. anthropi* and *O. intermedium*, it is possible that certain infections thought to be caused by *O. anthropi* were actually caused by *O. intermedium*.

Organisms That Are Nonmotile and Oxidase Positive

Family Flavobacteriaceae

Vandamme and colleagues have reported that none of the established species of flavobacteria were closely related to the type species *F. aquatile*; therefore, they proposed that the generically misclassified organisms *F. balustinum*, *F. gleum*, *F. indologenes*, *F. indoltheticum*, *F. meningosepticum,* and *F. scophthalmum* be included in a new genus, *Chryseobacterium,* with *C. gleum* as the type species.[1098] These same authors reported that *Flavobacterium breve* represented a distinct genetic taxon and proposed the name *Empedobacter brevis* for this species.[1098] Furthermore, *Flavobacterium odoratum* was found to comprise two distinct species, which have been renamed *Myroides odoratus* and *Myroides odoratimimus*.[1096] In 2005, Kim et al.[566] proposed the transfer of *C. meningosepticum* and *C. miricola* to a new genus, *Elizabethkingia* gen. nov., with the names *Elizabethkingia meningoseptica* and *Elizaethkingia miricola*. An outline of the current classification and nomenclature for the medically important members of the family *Flavobacteriaceae* is given in Box 7-10.

Interestingly, none of the remaining *Flavobacterium* species are found in human clinical specimens, and none are indole positive—a feature that has been synonymous with the genus *Flavobacterium* in the past. The key differentiating features of the clinically significant members of the family *Flavobacteriaceae* and related bacteria are given in Tables 7-19 and 17-20. Most species produce yellow-pigmented colonies on blood agar medium, and all are oxidase positive. All species are nonmotile and negative for nitrate reduction, and most species fail to grow on MacConkey agar. Most species (except *Weeksella virosa*) are polymyxin-resistant, a property they share with the pseudomallei group discussed elsewhere in this chapter. Only the clinically significant members of the *Flavobacteriaceae* will be discussed further in this chapter. The taxonomic dilemma of *Flavobacterium* and *Sphingobacterium* has been reviewed in detail elsewhere.[92,981,1098,1195]

Genus *Chryseobacterium*. These former *Flavobacterium* species occur naturally in soil, water, plants and foodstuffs. In the hospital environment they exist in water systems and on wet surfaces. They are readily distinguished from other nonfermenters by their ability to produce indole in tryptophan broth (Image Plate 7-2C). Often the indole reaction is weak and difficult to demonstrate; therefore, the more sensitive Ehrlich method (discussed earlier in this chapter) should be used. *C. indologenes* is easily recognized by the production of dark-yellow colonies (Image Plate 7-4C). *E. meningoseptica*, in contrast, produces colonies with a very pale yellow pigment that

may not be evident on initial examination of colonies at 24 hours. Colonies of *Empedobacter brevis* are pale yellow. Pigment production may be augmented by incubating the culture for an additional 24 hours at room temperature. The *Chryseobacterium* species generally grow poorly, or not at all, on MacConkey agar and are considered to be glucose oxidizers, although most strains will slowly ferment glucose after prolonged incubation. Microscopically, cells of *E. meningoseptica,*

BOX 7-10

Description of Medically Important Members of the Family *Flavobacteriaceae*

Genus: *Flavobacterium*

Type genus consisting of 121 species isolated from aquatic environments. Not associated with human infection

Genus: *Bergeyella*

B. zoohelcum
B. cardium

Genus: *Capnocytophaga*

C. canimorsus
C. cynodegmi
C. gingivalis
C. granulosa
C. haemolytica
C. leadbetteri
C. ochracea
C. sputigena

Genus: *Chryseobacterium*

87 species described but only two associated with human infection
C. gleum
C. indologenes

Genus: *Elizabethkingia*

E. anophelis
E. meningoseptica
E. miricola

Genus: *Empedobacter*

E. brevis

E. falsenii

Genus: *Myroides*

Eight species described but only two associated with human infection

M. odoratus

M. odoratimimus

Genus: *Weeksella*

W. virosa

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TABLE 7-19 Characteristics of the Indole-Positive Nonfermenters[a]

Test	*Elizabethkingia*			*Chryseobacterium*		CDC Groups						*Weeksella*	*Bergeyella*	*Empedobacter*		
	E. meningoseptica[b]	*E. miricola*[c]	*E. anophelis*[d]	*C. gleum*[b]	*C. indologenes*[b]	IIb[e]	IIc[f]	IIe[e]	IIg[e]	IIh[b]	IIi[b]	W. virosa[b] (IIf)	*B. zoohelcum*[b] (IIj)	*E. falsenii*[g] GV 1	*E. falsenii*[g] GV 2	*E. brevis*[b]
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth on MacConkey agar	V (26)	+	–	V (50)	–	V (54)	–	–	+	–	–	V (79)	+	+	+	+
OF glucose	+	+	+	+	+	+	+	D[h]	–	+	+	–	–	+	+	V (80)
OF mannitol	+	+	+	–	V (10)	V (10)	–	–	–	–	–	–	–	–	–	–
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NO_3 to NO_2	–	–	NA	V (67)	V (14)	V (22)	+ (90)	–	–	–	–	–	–	–	–	–
NO_2 to N_2	NA	–	NA	+	–	V (20)	+ (90)	–	+	–	–	–	–	–	–	–
Gelatin	+	+	–	+	+	V (78)	V (20)	–	–	D[i]	–	–	–	+	Weak+	+
Starch	V (8)	–	–	+	+	+	NA	+	NA	+	V (14)	–	–	+	+	V (40)
Esculin	+	+	+	+	+	V (70)	+	–	–	+	+	NA	NA	+	V (8)	–
ONPG	+	+	+	+	V (41)	V (57)	NA	NA	NA	–	+	NA	NA	–	V (75)	–
DNase	+	+	–	V (17)	V (7)	NA	NA	–	NA	V (78)	–	V (13)	–	NA	NA	+
Urea	–	+	–	+	V (10)	V (14)	–	–	–	–	–	–	+	+	+	–
Penicillin	R	NA	R	R	12% S	NA	NA	S	NA	67% S	57% S	S	S	NA	NA	R
Polymyxin	R	NA	NA	R	3% S	NA	NA	S	NA	22% S	R	S	R	R	R	R
Pigment	Pale yellow	Pale white-pale yellow	Pale yellow	Yellow-orange	Yellow-orange	Yellow-orange	Tan to buff	–	–	–	–, or yellow	Butterscotch	–	Yellow	Yellow	Pale yellow

[a]Unless otherwise noted, data from references 367 and 1172.
[b]Percentage positive derived from reference 367.
[c]Data from references 566 and 639.
[d]Data from reference 536.
[e]Percentage positive derived from reference 1172.
[f]Data from reference 451.
[g]Data from references 532 and 1216.
[h]Gilardi[367] reports glucose-negative, Weyant[1172] reports glucose-positive or delayed positive.
[i]Gilardi[367] reports gelatin positive, Weyant[1172] reports gelatin negative.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; pe, peritrichous flagella; p, polar flagella; GV, genomovar; S, susceptible; R, resistant; NA, results not available; D, different results reported. Numbers in parentheses are percentage of strains giving positive reaction.

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TABLE 7-20 Key Characteristics of *Myroides* and *Sphingobacterium*[a]

Test	*Myroides*	*Sphingobacterium*			
	M. odoratus, M. odoratimimus	*S. multivorum*	*S. spiritivorum*	*S. thalpophilum*	*S. mizutaii*
Oxidase	+	+	+	+	+
Motility	–	–	–	–	–
Growth on MacConkey[b]	V (91)	+	V (46)	+	–
OF glucose	–	+	+	+	+
OF mannitol	–	–	+	–	–
Indole	–	–	–	–	–[c]
NO_3 to NO_2	–	–	–	+	–
NO_2 to N_2	V (46)	NA	NA	NA	+
Gelatin	+	–	–	V (86)	–
Starch	–	V (79)	–	+	–
Esculin	–	+	+	+	+
ONPG	NA	+	+	+	+
DNase	+	–	+	+	–
Urea	+	+	+	+	–
Penicillin	19% S	R	R	R	R
Polymyxin	R	R	R	R	R
Pigment	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Yellow

[a]Data from references 367 and 1172.
[b]Growth on MacConkey from reference 1172.
[c]Very weak pink color may be observed in the xylene layer.[1172]
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; S, susceptible; R, resistant; NA, results not available. Numbers in parentheses are percentage of strains giving positive reaction.

C. indologenes, and groups IIe, IIh, and IIi are thinner in their central region than in their peripheral portions and include filamentous forms; IIh cells are significantly smaller than those of other species. It should be emphasized that test results (e.g., DNase, indole, urea, starch hydrolysis) in this group are dependent on the choice of medium, reagents, and length of incubation.[847] *Chryseobacterium indologenes, C. gleum*, and CDC Group IIb are tabulated individually in Table 7-19. Group IIb is genetically heterogeneous and includes strains of *C. indologenes, C. gleum*, and probably additional genomospecies. Additional DNA–DNA hybridization studies will be required to resolve this issue. Phenotypic separation between *C. indologenes* and *C. gleum* has been difficult; however, acid production from xylose and growth at 41°C are consistently positive in DNA groups clustering around the type strain of *C. gleum*.[1091] Additional characteristics separating the members of this group of organisms can be found in Table 7-19.

Chryseobacterium indologenes. *C. indologenes* (formerly *F. indologenes* and CDC group IIb) is the most frequent human isolate, although it rarely has clinical significance.[1146] It has been documented to cause bacteremia in hospitalized patients

with severe underlying disease, although the mortality is relatively low even among patients who were administered antibiotics without activity against *C. indologenes*.[484] Nosocomial infections due to *C. indologenes* have been linked to the use of indwelling devices during a hospital stay.[487,490,779,793] Rare instances of pneumonia,[487,490,779] meningitis and central nervous system infection,[315,435,798] wound infection,[487,490] keratitis,[670] urinary infection,[5,487] peritonitis,[6] and bacteremia[56,317,397,484,487,490,720,793] have been reported.

Chryseobacterium hominis. *C. hominis* is a new species of *Chryseobacterium* proposed in 2007 for a collection of clinical isolates that were characterized biochemically as being similar to CDC groups IIh and IIc.[1121] All strains are nonmotile, oxidase, and catalase positive. Growth occurs at 30°C and 37°C with optimal growth at 30°C. No growth occurs on MacConkey agar, cetrimide agar, or 3% NaCl agar. Colonies are circular and mucoid; some are also sticky. Some strains exhibit a pale yellow or tan pigment. Acid is produced in OF glucose and OF maltose, but not OF mannitol. All strains are positive for indole production, hydrolysis of esculin, starch, and gelatin. Acid production from sucrose and reduction of nitrate and nitrite are variable.

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All strains are negative for growth at 42°C, urease, H_2S on Kligler's agar, ornithine and lysine decarbosylase, arginine dihydrolase, alkalinization of Simmons' citrate, L-phenylalanine deaminase, and ONPG.[1121] Clinical isolates in the initial report were recovered from blood, dialysis fluid, pus, infraorbital drain, eye, and aortic valve. The clinical significance of these isolates was not reported.[1121] A 58-year-old man with Parkinson's disease who presented to the emergency room at Loyola University Chicago Medical Center with a 2-day history of nausea and vomiting was found to have *C. hominis* bacteremia. He reported having intermittent abdominal pain localized to the epigastric region. He was dizzy upon standing or sitting up, and his serum lactate was elevated. Review of systems was otherwise normal. Blood cultures from the day of admission grew coagulase-negative *Staphylococcus* (likely contaminant) and an indole-positive, glucose nonfermenting gram-negative rod. The isolate was identified by MALDI-TOF MS as *C. hominis* (top rank) with a value score of 1.695 using the Bruker Daltonics MicroFlex LT instrument, biotyper software 3.1. The identification was confirmed by 16S rRNA sequencing as *C. hominis* (Schreckenberger P, personal communication).

Genus *Elizabethkingia*

Elizabethkingia meningoseptica. *E. meningoseptica* (formerly *Flavobacterium*

meningosepticum and CDC group IIa)[566] is the species most often associated with significant disease in humans. In adults, it has been reported to cause pneumonia, endocarditis, wound infections, postoperative bacteremia, and meningitis, usually in patients with severe underlying illness.[50,104,190,233,342,424,443,526,617,640,688,801,811,908,979,1066,1168] It is highly pathogenic for premature infants and has been associated with neonatal meningitis.[190,233,358,427,462,568,677,861,908,1072] Although neonatal meningitis is only rarely encountered, it is important to diagnose the disease accurately because epidemics may occur in nurseries, and a mortality rate as high as 55% has been reported.[1146] *E. meningoseptica* has increasingly been reported as a cause of healthcare-associated infections. A cluster of four patients with ventilator-associated pneumonia was reported in an intensive care unit traced to contaminated humidifier water.[176] *E. meningoseptica* has also been reported to cause infections in patients receiving hemodialysis[837] and healthcare-associated bacteriuria primarily in elderly patients with diabetes.[1220] Jean et al.[508] have reviewed the incidence, predisposing factors, clinical features, and measures for the prevention and control of infections caused by *E. meningoseptica.*

Elizabethkingia miricola. *E. miricola* is a nonmotile, nonfermenting rod that was first described in 2003 when it was isolated from condensation water on the Russian space station Mir.[639] It was initially named *Chryseobacterium miricola* but was reclassified as a species of *Elizabethkingia* in 2005.[566] The only confirmed clinical isolate of *E. miricola* was from the sputum and blood of a man with mantle cell lymphoma who had undergone stem cell transplant and chemotherapy and required ventilator support.[398] Good growth is observed on MacConkey agar. Colonies are very sticky on solid medium. Indole is produced and urea is hydrolyzed. Acid is produced from fructose, glucose, lactose, maltose, mannitol, and trehalose, but not from arabinose, cellobiose, raffinose, sucrose, salicin, or xylose.[566] *E. miricola* has a biochemical profile similar to *E. meningoseptica*, but can be differentiated by a positive urease reaction. (Table 7-19) Several authors have reported misidentification of *E. meningoseptica* as *E. miricola* when performing MALDI-TOF MS using the Bruker Daltonics MicroFlex LT instrument, biotyper software 3.0 and 3.1.[14,724,960]

Elizabethkingia anophelis. *E. anophelis* is a recently discovered bacterium isolated from the midgut of the *Anopheles gambiae* mosquito.[536] It is a gram-negative, nonmotile, non–spore-forming rod. Oxidase and catalase are positive. Colonies on nutrient agar are smooth, yellowish, translucent, and shiny. No growth occurs on MacConkey agar. Acid is produced from glucose, lactose (weak), mannitol, maltose, rhamnose, sucrose, trehalose, and cellobiose (weak). Esculin, indole, and ONPG are positive. Urease, hydrolysis of gelatin, starch, DNA, and tyrosine are negative. Arginine dihydrolase, lysine and ornithine decarboxylase, and utilization of malonate

are negative.[536] (Table 7-19) *E. anophelis* has been reported in three patients with sepsis involving two neonates who had meningitis and one neonate's mother who had chorioamnionitis.[612] It has also been reported to cause meningitis in an 8-day-old girl delivered by cesarean section in the Central African Republic.[333] A nosocomial outbreak of *E. anophelis* was reported in an intensive care unit in Singapore.[1065] The outbreak involved five patients who were ventilated via tracheostomy and had CVCs. Isolates recovered from the sputum and blood specimens of the infected patients were related to strains isolated from hand hygiene sink aerators in the cardiothoracic and surgical ICUs where the patients were housed.[1065]

The appropriate choice of effective antimicrobial agents for treatment of *Chryseobacterium* and *Elizabethkingia* infections is difficult. Species in this group are inherently resistant to many antimicrobial agents commonly used to treat infections caused by gram-negative bacteria (aminoglycosides, β-lactam antibiotics, tetracyclines, chloramphenicol), but are often susceptible to agents generally used for treating infections caused by gram-positive bacteria (rifampin, clindamycin, erythromycin, sparfloxacin, trimethoprim-sulfamethoxazole, and vancomycin).[324,1017,1146] Although early investigators recommended vancomycin for treating serious infection with *E. meningoseptica*,[427,861] recent studies have shown greater in vitro activity of minocycline, rifampin, trimethoprim–sulfamethoxazole, and quinolones.[104,335,1017] Further complicating the choice of appropriate antimicrobial therapy is the fact that MIC breakpoints for resistance and susceptibility of this group have not been established by the Clinical Laboratory Standards Institute and the results of disk diffusion testing have been shown to be unreliable in predicting antimicrobial susceptibility to *Chryseobacterium* and *Elizabethkingia* species.[3,168,335,1148] The Etest has been shown to be a possible alternative to the standard agar dilution method for testing cefotaxime, ceftazidime, amikacin, minocycline, ofloxacin, and ciprofloxacin, but not piperacillin.[483] Definitive therapy for clinically significant isolates should be guided by individual susceptibility patterns determined by an overnight MIC method.

Genus *Empedobacter.* The genus *Empedobacter* includes two species, *Empedobacter brevis* (formerly *Flavobacterium*

breve) and *Empedobacter falsenii* (formerly *Wautersiella falsenii*) containing two distinct genomovars.[532,1098,1216,1217] These two species are rarely recovered from human clinical specimens and little is known about their capacity to cause disease.

Empedobacter brevis. Janknecht et al.[505] reported an outbreak of *E. brevis* endophthalmitis in a series of patients who had cataract extractions performed on the same day by the same surgeon, suggesting that the source of infection may be anything from the lens to the sterilization process. *E. breve* was also recovered from a foot lesion in a patient with anaphylactoid purpura,[785] and from the blood of a patient following right knee cellulitis.[877] All strains produce yellow colonies on nutrient agar. Some strains are pale yellow or opaque gray and nonhemolytic on blood agar.[467] All strains are nonmotile, oxidase, catalase, DNase, and gelatin positive. All strains produce indole, but a sensitive method (utilizing Ehrlich reagent) is necessary to demonstrate the presence of indole.[467] Most strains oxidize glucose and maltose but not mannitol. Additional biochemical reactions are shown in Table 7-19.

Empedobacter (Wautersiella) falsenii. The clinical origins of *E. falsenii* isolates used in the original species description were from blood, ear, oral cavity, pleural fluid, pus, respiratory tract, surgical wound, and vaginal swab, but the clinical significance was not provided.[532] There is one report of *E. falsenii* isolated from the urine of an infant with a complicated urinary tract infection.[1114] *E. falsenii* has also been found in a respiratory sample from a CF patient with unknown clinical significance.[696] *E. falsenii* can be separated into two distinct clusters or genomovars based on molecular characterization and biochemical reactions. All strains are gram-negative, nonmotile, nonfermenting rods, and grow aerobically at 20°C, 30°C, and 37°C on blood agar. Some strains display yellow-pigmented colonies after prolonged incubation. All strains are oxidase and catalase positive and produce acid from glucose and maltose.[532] All strains produce indole and urease. Nitrate reduction is negative and nitrite reduction is variable. All genomovar 1 isolates rapidly hydrolyze gelatin and esculin, but are ONPG negative. All genomovar 2 isolates but one are esculin negative, ONPG positive, and weak gelatin positive.[532] Additional reactions are given in Table 7-19.

Genus *Weeksella* and Genus *Bergeyella.* The genus *Weeksella* as originally proposed contained two species, *W. virosa* (formerly CDC group IIf) and *W. zoohelcum* (formerly CDC group IIj).[470,471] Vandamme and colleagues have shown that these two species represent separate genetic taxa and thus proposed the reclassification of one of these species, *W. zoohelcum*, as *Bergeyella zoohelcum*.[1098] Both species are oxidase positive, usually grow on MacConkey agar, are nonpigmented, nonsaccharolytic, and indole positive. Both species have the unusual feature of being susceptible to penicillin, which allows them to be easily differentiated from the related genera (Table 7-19).

Weeksella virosa. *W. virosa* (derived from the Latin word for "slimy") forms mucoid, sticky colonies that are difficult to remove from agar. Colonies are at first observed to be nonpigmented but further incubation may result in a butterscotch

pigmentation. *W. virosa* is urease negative and polymyxin B susceptible. It has been recovered primarily from the urogenital tract of women, but there is little evidence that it can play a pathogenic role.[470,697,892,893] There have been published reports of spontaneous peritonitis[108] and septicemia caused by *W. virosa*.[1001] A review of infections caused by *W. virosa* along with in vitro susceptibility data has been provided by Slenker et al.[1001]

Bergeyella zoohelcum. A key differentiating feature of *B. zoohelcum* is the production of an intense urease reaction in Christensen's urea agar. *B. zoohelcum* forms sticky colonies that are at first nonpigmented, but may form tan to yellow-colored colonies with prolonged incubation. It is penicillin susceptible, but polymyxin B resistant. *B. zoohelcum* is part of the normal oral and nasal flora of dogs and cats.[951] It is not surprising, therefore, that the majority of human isolates have been the result of dog or cat bites,[128,471,744,891,951,986,1172] or in patients who had significant exposure to dogs or cats.[408,643,787] It has been a cause of septicemia in elderly patients with severe skin infections,[577,643,787] and in two cases, the patients reported that they allowed their cat to sleep on top of their legs,[577,787] *B. zoohelcum* has also been reported to have caused pneumonia in a patient exposed to a dog that was a carrier of the organism,[408] and to have caused bacteremia in another patient after ingestion of a dish prepared with goat blood.[85] There is one report of meningitis owing to *B. zoohelcum* following multiple dog bites.[128]

Bergeyella cardium. Recently, a new species of *Bergeyella, B. cardium*, was reported as a causative agent of infective endocarditis in two patients from two differen hospitals in Korea.[1013] The organism was reported to grow slowly on blood and chocolate agars at 37°C and 41°C, but did not grow at 24°C and 30°C. 16S rRNA gene sequencing of the two strains demonstrated sequence similarity of 94.9% to *B. zoohelcum* strongly suggesting that the isolates belonged to a novel bacterial species on the genus *Bergeyella*.[1013] Additional biochemical properties of the isolates were not reported. The organisms were reported susceptible to many commonly used antibiotics incuding penicillin.[1013]

Genus *Myroides*. Vancanneyt et al.[1096] determined that the organism formerly classified as *Flavobacterium odoratum* consisted of a heterogeneous group that comprised two distinct species for which they proposed the names *Myroides odoratus* and *Myroides odoratimimus*. Cells of both species are gram-negative rods 0.5 µm in diameter and 1 to 2 µm long. Various colony types may occur, but most colonies are yellow pigmented and form effuse, spreading colonies that may be confused with the colony morphology of a *Bacillus* species (Image Plate 7-4D). A characteristic fruity odor (similar to that of *A. faecalis*) is produced by most strains. *Myroides* grow on

most media, including MacConkey agar. Growth occurs at 18°C to 37°C, but not at 42°C. They are asaccharolytic but are oxidase, catalase, urease, and gelatinase positive. Indole is not produced, and nitrite (but not nitrate) is reduced (Table 7-20). There are no routine phenotypic tests for differentiating the two *Myroides* species, their differences being confined to assimilation tests and cellular fatty acids.[1096] Organisms identified as *M. odoratus* have been reported mostly from urine but have also been found in wound, sputum, blood and ear specimens.[468,1201] Clinical infection with *Myroides* spp. is exceedingly rare; however, patients with rapidly progressive necrotizing fasciitis and

bacteremia have been reported,[236,491] as well as individuals with recurrent cellulitis with bacteremia.[57,396] A review of skin and soft tissue infections caused by *M. odoratus* has been provided by Crum-Cianflone and colleagues.[236] Most strains are resistant to penicillins, cephalosporins, aminoglycosides, aztreonam, and carbapenems.[468]

Unnamed CDC Groups IIc, IIe, IIg, IIh, and IIi

The unnamed CDC groups IIc, IIe, IIg, IIh, and IIi are phenotypically related indole-positive glucose nonfermenting rods.[1172] Holmes et al. performed DNA–DNA hybridization and 16S rRNA gene sequence analysis of 182 phenotypically similar isolates that phenotypically resembled species from *Chryseobacterium, Elizabethkingia, Empedobacter* or belonged to CDC groups IIc, IIe, IIh, and IIi. They reported that there was little correlation with genomic data and members of the CDC groups since members of each group belonged to at least two different DNA–DNA hybridization groups (three groups in the case of CDC Goup IIi), although one group was comprised almost entirely of strains of CDC Group IIe.[469] In another study, Wauters et al.[1161]reported that CDC group IIi strains belong predominantly to *Sphingobacterium mizutaii*, and in yet another study, Vaneechoutte et al.[1121] reported that CDC groups IIh and IIc belong predominantly to *Chryseobacterium hominis*. The disposition of the various groups identified by these researchers and their correlation to the CDC phenotypic groups remains unresolved. For laboratory identification purposes, the authors have chosen to maintain the CDC group designations in the current edition of this text (Table 7-19) The CDC groups are rarely recovered from clinical material, and little is known about their involvement in clinical disease. One patient with meningitis caused by CDC Group IIe has been reported,[1157] and the phenotypic characteristics of several clinical isolates of CDC groups IIc and

IIg have also been reported.[449,451] Group IIg isolates are easily distinguished from the other CDC groups by their ability to grow on MacConkey agar and failure to produce acid in OF glucose[449] Phenotypic characteristics useful for separating the CDC groups are shown in Table 7-19.

Family Sphingobacteriaceae

Genus *Sphingobacterium*. The sphingobacteria are yellow pigmented, oxidase positive, and nonmotile. They are differentiated from chryseobacteria and weeksellae by their failure to produce indole from tryptophan, and are separated from *Myroides* species by their ability to produce acid in OF glucose. As of this writing, there are 34 species in the genus *Sphingobacterium. S. multivorum* (formerly *Flavobacterium multivorum*, CDC group IIk-2), and *S. spiritivorum* (includes species formerly designated *Flavobacterium spiritivorum, F. yabuuchiae*, and CDC group IIk-3), are the two species most frequently recovered from human clinical specimens. They can be distinguished from the similar organism *Sphingomonas paucimobilis* (formerly IIk-1) by lack of motility and resistance to polymyxin B. Additional differentiating features of these bacteria are given in Table 7-20. *S. multivorum* has been isolated from various clinical specimens, but has only rarely been associated with serious infections, including peritonitis and septicemia.[42,284,338,463,865] Blood and urine have been the most common sources for the isolation of *S. spiritivorum*.[461,700,1079] *S. thalpophilum* has been recovered from wounds, blood, eye, abscess, and an abdominal incision.[1172] A positive nitrate test and growth at 42°C differentiates *S. thalpophilum* from other *Sphingobacterium* species. *Sphingobacterium mizutaii* (formerly *S. mizutae*, and *Flavobacterium mizutaii*)[191,473,1161,1195] has been isolated from blood, CSF, and wound specimens and can be differentiated from other *Sphingobacterium* species by its failure to grow on MacConkey agar and its usual lack of urease activity.[1172] *Sphingobacterium* species are generally resistant to aminoglycosides and polymyxin B, while they are susceptible in vitro to the quinolones and trimethoprim–sulfamethoxazole. Susceptibility to β-lactam antibiotics is variable, requiring testing of individual isolates.[1017]

Family Moraxellaceae

The family *Moraxellaceae,* contains three genera of clinical importance: *Moraxella*, *Acinetobacter*, and *Psychrobacter*.[842]

Genus *Moraxella*. Several key features make one suspect that an unknown nonfermenter may belong to the genus *Moraxella*. After 24 hours on blood agar, the colonies tend to be small and pinpoint (usually less than 0.5 mm in diameter), with

poor or no growth on MacConkey agar. The bacterial cells appear as tiny, gram-negative diplococci or diplobacilli in Gram-stained preparations, and have a tendency to resist decolorization.[254] Both the cytochrome oxidase and catalase reactions are positive (the former rules out *Acinetobacter* species; the latter rules out *Kingella* species). The inability of *Moraxella* species to form acid from carbohydrates also eliminates most *Neisseria* species from consideration. Most *Moraxella* species are extremely susceptible to low concentrations of penicillin. Examination of Gram-stained smears prepared from the outer zone of inhibition around the penicillin susceptibility disk can be used to distinguish *Neisseria* species (which retain their coccal morphology) from *Moraxella* species (which produce elongated, pleomorphic forms) (Image Plates 7-4E and F).[159] All *Moraxella* species are nonmotile.

The *Moraxella* species of medical importance are *M. lacunata*, *M. nonliquefaciens*, *M. osloensis*, *M. atlantae* (CDC group M-3), and *M. catarrhalis*. *M. catarrhalis* is covered along with the pathogenic *Neisseria* species in Chapter 11. CDC groups M-2 and M-4 have been named *Psychrobacter phenylpyruvicus* and *Oligella urethralis*, respectively, and are covered later in this section. CDC groups M-5 and M-6 have both been placed in the genus *Neisseria*, even though their microscopic appearance is that of gram-negative rods. Group M-5 has been designated *Neisseria weaveri*,[454] and group M-6 has been named *Neisseria elongata* subsp. *nitroreducens*.[394] These species, along with other recently described *Moraxella* species such as *M. canis*[506] and *M. lincolnii*,[1102] are difficult to distinguish from the established species of *Moraxella*. Animal species include *M. bovis*, isolated from healthy cattle and other animals, including horses, *M. boevrei* and *M. caprae* (goats), *M. caviae* (guinea pigs), *M. cuniculi* (rabbits), and *M. ovis* (sheep).

Table 7-21 provides some useful differential tests for identification of the medically important *Moraxella* species. *M. atlantae*, *M. lacunata,* and *M. nonliquefaciens* are similar in many of their features. Growth of *M. atlantae* is stimulated by bile salts and sodium desoxycholate, whereas the growth of *M. lacunata* and *M. nonliquefaciens* is not. Only *M. lacunata* liquefies gelatin, whereas both *M. lacunata* and *M. nonliquefaciens* reduce nitrate to nitrite.[122,850] Separation of *M. lacunata* and nonspreading *M. nonliquefaciens* may prove difficult, because gelatin hydrolysis (with any method) and liquefaction of Loeffler slants may take more than 1week. In some instances, fatty acid analysis may help determine the species.[1172] Because many strains of *Moraxella* are somewhat fastidious and biochemical reactions are often negative or equivocal, many laboratories choose to simply report members of this group as "*Moraxella* species."

TABLE 7-21 Key Characteristics of *Haematobacter, Moraxella, Neisseria, Oligella urethralis, Psychrobacter phenylpyruvicus*, and Gilardi Rod Group 1

	Moraxella							*Oligella*	*Psychrobacter*	*Haematobacter*	*Neisseria*		
Test	***M. atlantae***[a]	***M. canis***[a,b,c]	***M. catarrhalis***[a]	***M. lacunata***[a]	***M. lincolnii***[a,d]	***M. nonliquefaciens***[a]	***M. osloensis***[a]	***O. urethralis***[a]	***P. phenylpyruvicus***[a]	***Haematobacter* sp.**[e]	***N. weaveri***[a,f]	***N. elongata subsp. nitroreducens***[a,g,h]	**Gilardi Rod Group 1**[a,i]
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	–	+
Growth on MacConkey	+	+	–	–	–	V(10)	V(70)	+	V(86)	V(54)	V(45)	V(54)	+
Motility	–	–	–	–	–	–	–	–	–	–	–	–	–
OF glucose	–	–	–	–	–	–	–	–	–	–	–	V(23)	–
Urea	–	–	–	–	–	–	–	–	+	+	–	–	–
PAD	–	–	V(68)	V(17)	NA	–	V(14)	+	+	+	V(71)	–	+
Gelatin	–	–	–	V(42)	–	–	–	–	–	–	–	–	–
NO_3 reduced	–	+	+(92)	+	–	+	V(24)	–	V(68)	–	–	+	–
NO_2 reduced	–	V	V(86)	–	V	–	–	+	–	–	+	+	–
DNase	–	+	+	–	–	–	–	–	–	NA	–	–	–
Penicillin	S	S	R	S	S	S	92% S	S	73% S	NA	S	S	S
Cell shape	CB	C	C	CB	CB	CB	CB	CB	CB	R serpentine, filamentous	R	R	R

[a]Data from reference 1172.
[b]Data from reference 506.
[c]*M. canis* produces a brown pigment when grown on starch-containing Mueller–Hinton agar.[506]
[d]Data from reference 1102.
[e]Data from reference 434.
[f]Data from references 28 and 454.
[g]Data from reference 394.
[h]*N. elongata* subsp. elongata is catalase, glucose, and nitrate negative and nitrite positive. *N. elongata* subsp. glycolytica is catalase positive, weakly glucose positive or glucose negative, nitrate negative, and nitrite positive. *N. elongata* subsp. nitroreducens is catalase negative and weakly glucose positive or glucose negative and reduces nitrate to nitrite.[28,454]
[i]Data from reference 754.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; S, susceptible; R, resistant; NA, not available; C, coccus; CB, coccobacillus; R, rod; PAD, phenylalanine deaminase. Numbers in parentheses are percentage of strains giving positive reaction.

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Moraxella species are normal flora on mucosal surfaces and are considered to have low pathogenic potential. They occur most frequently in the respiratory tract and less commonly in the genital tract and occasionally may cause systemic infection. *M. lacunata*, which has been known for over a century to cause conjunctivitis, is fastidious and requires either enriched nonpeptone media or the addition of oleic acid or rabbit serum to counteract a toxic proteolytic effect. In addition to conjunctivitis, this species has been reported to cause keratitis, chronic sinusitis, and endocarditis.[766,907,1075] *M. nonliquefaciens* also may require serum supplements for optimal growth. It is part of the normal flora in the human upper respiratory tract and is frequently isolated from the nasal cavity. It has been isolated from the blood, eye, CSF, lower respiratory tract, and other local sites[121,392,1075] and has been associated with endophthalmitis[308,613,658] and septic arthritis.[518] Atypical mucoid strains of *M. nonliquefaciens* have been recovered from sputum samples of three patients with chronic lung disease.[257] *M. osloensis* is commonly recovered from clinical specimens

and does not require growth supplements. It is usually not pathogenic when isolated from humans; however, isolated cases of sinusitis, conjunctivitis, bronchitis, septic arthritis, osteomyelitis, peritonitis, meningitis, endocarditis, CVC infection, and septicemia have been reported.[136,325,421,922,975,1042] *M. atlantae* grows slowly in culture medium, producing colonies with a tendency to form a spreading zone after 48 hours of incubation.[122] They are a rare cause of bacteremia in immunocompromised patients.[135,261]

M. canis is a new species that resides in the upper respiratory tracts of dogs and cats. Human isolates have been from blood,[506] lymph node[1116] and a dog bite wound.[506] *M. canis, M. catarrhalis*, *M. cuniculi*, *M. caviae,* and *M. ovis* belong to the coccoid moraxellae, which in contrast to the bacillary moraxellae, all exhibit DNase activity. Isolates of *M. canis* resemble *M. catarrhalis* on Gram's stain; however, their colony morphology on sheep blood agar more closely resembles that of members of the *Enterobacteriaceae* (large, smooth colonies).[506] Some isolates may also produce very slimy colonies resembling colonies of *Klebsiella pneumoniae*.[506] The production of a brown pigment on starch-containing Mueller-Hinton agar is also typical of most *M. canis* strains.[506] *M. lincolnii* has been isolated mainly from the respiratory tract of humans.[1102]

Most *Moraxella* strains are susceptible to penicillin and its derivatives, cephalosporins, tetracyclines, quinolones and aminoglycosides.[324,923,1017] Production of β-lactamase has been reported only rarely in *Moraxella* species other than *M. catarrhalis*.[518,766,923] Because of the fastidious nature of many *Moraxella* species and the predictability of the antibiotic profile, antibiotic susceptibility testing, except for β-lactamase testing, is usually not performed on clinical isolates.

Genus *Psychrobacter.* The *Psychrobacter* species that are important clinically are *P. immobilis* and *P. phenylpyruvicus* (formerly *Moraxella phenylpyruvica*).[125]

Psychrobacter phenylpyruvicus. *P. phenylpyruvicus* is both urea and phenylalanine deaminase positive, features that help to distinguish it from *Moraxella* species and *Oligella urethralis* (Table 7-21). Students and laboratorians should be aware that *P. phenylpyruvicus* can phenotypically resemble *Brucella* species, and there have been several reports of *Brucella* species misidentified as *P. phenylpyruvicus* in commercial identification systems.[65,75,833] The differentiation of *P. phenylpyruvicus* and *Brucella* spp. requires microscopy (*Brucella* are tiny coccobacilli) and tests for acidification of xylose and glucose.[849,852] *P. phenylpyruvicus* is asaccharolytic, whereas *Brucella* spp. utilize xylose and usually glucose when a sufficiently sensitive method for detecting acidification of glucose is used.[849] *P. phenylpyruvicus* is a rare cause of infections in humans, but has been reported to cause bacteremia,[630] endocarditis,[413,1078]

peritonitis[165] and a fungating lesion of the foot.[563]

Psychrobacter immobilis. Most strains of *P. immobilis* grow slightly or not at all at 35°C and grow best at 20°C.[493] *P. immobilis* is divided into saccharolytic and asaccharolytic strains. Saccharolytic *P. immobilis* strains share all of the characteristics of the asaccharolytic strains (Table 7-22), except that glucose, xylose and lactose, but not sucrose and maltose are oxidized. Asaccharolytic strains are phenotypically similar to *P. phenylpyruvicus*. The diagnosis of *P. immobilis* can be confirmed by transformation studies, cellular fatty acid profile, and optimal growth temperatures <35°C.[756,1172] Many strains of *P. immobilis* have an odor resembling that of phenylethyl alcohol (PEA) agar (i.e., roses)[493] and are resistant to penicillin but susceptible to most other antibiotics.[376,657] *P. immobilis* septicemia has been reported in a 16-year-old boy with CGD and fulminant hepatic failure.[1025] One patient with an ocular infection and one infant with meningitis have also been reported to be caused by *P. immobilis*.[376,657]

Family Neisseriaceae

Genus *Neisseria*. Some *Neisseria* species appear rod-shaped on Gram's stain and as such may not be recognized initially as *Neisseria* species. Two such species are *N. weaveri* and *N. elongata*, both of which must be differentiated from other phenotypically similar NFBs (Table 7-21). The coccoid members of the genus *Neisseria* are covered in Chapter 11.

Neisseria elongata. *N. elongata* subsp. *elongata* and *N. elongata* subsp. *glycolytica* are considered to be transient colonizers of the human upper respiratory tract and urogenital tract; however, *N. elongata* subsp. *elongata* has been reported to cause human endocarditis,[33,771] and *N. elongata* subsp. *glycolytica* has been isolated from human wounds and blood cultures.[29,476] Most human infections are due to *N. elongata* subsp. *nitroreducens*, which has been

TABLE 7-22 Key Characteristics of *Psychrobacter immobilis*, *Paracoccus yeei*, EO-3, EO-4, and *Neisseria zoodegmatis*[1a]

Test	*Psychrobacter* *P. immobilis*	*Paracoccus* *P. yeei* (EO-2)	CDC Groups EO-3	CDC Groups EO-4	*Neisseria* *N. zoodegmatis* (EF-4b)
Oxidase	+	+	+	+	+
Motility	–	–	–	–	–
Growth on MacConkey agar	V (40)	V (64)	+	V (67)	V (65)
OF glucose	–[b]	+	+	+	+
OF xylose	–[b]	+	+	+	–
NO_3 to NO_2	V (40)	+	–	–	+
Growth at					
25°C	+	V (73)	+	+	V (88)
35°C	V (40)	+	+	+	+
42°C	V (20)	V (36)	V (14)	–	V (69)
O-shaped cells	–	+	–	–	–
Yellow pigment	–	–	+	V (83)	–
Odor	Rose-like	–	–	–	Popcorn

[a]Data from reference 1172.
[b]Saccharolytic *P. immobilis* strains share all of the characteristics of the asaccharolytic strains listed here except that glucose, xylose, and lactose, but not sucrose and maltose are oxidized.[1172]
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; NA, results not available. Numbers in parentheses are percentage of strains giving positive reaction.

associated with a variety of human infections, but diseases most commonly produced include bacteremia and endocarditis,[291,394,414,446,498,539,581,729,839,854,920,998,1036,1184] and rarely with osteomyelitis.[353,1184] This organims was first known as CDC group M-6, but was recognized as a unique subspecies of *N. elongata* named "*nitroreducens*" because it reduces nitrates and nitrites to amines without the formation of gas.[394] Other characteristics of the organism include negative catalase, urease, and indole reactions; a positive oxidase reaction; and lack of acid production from carbohydrates (Table 7-21). *N. elongata* subsp. *nitroreducens* is typically susceptible to aminopenicillins, carbenicillin, cephalosporins, aminoglycosides, trimethoprim–sulfamethoxazole, and polymyxin and it is variably susceptible to penicillin.[291]

Neisseria weaveri. *N. weaveri* is found as normal oral flora in dogs and is associated with human wound infections resulting from dog bites.[28,67,392,454] It has also been isolated from blood,[28,149] sputum, and bronchial washings,[28,817] and from eye, peritoneal, and chest fluids.[28] There is one report of a wound infection in a child after being bitten by a white Siberian tiger.[148] *N. weaveri* appears as aerobic, gram-negative, broad, plump, medium-to-large, straight rods of varying length, with a tendency to grow in chains or longer rods in broth cultures.[28] Growth occurs at 25°C and 35°C, and most strains grow at 42°C. Colonies are gray-white, with an entire

border, flat, somewhat glistening, and smooth. *N. weaveri* is nonmotile, strongly positive for oxidase and catalase, and negative for carbohydrate utilization. Nitrite is reduced, but not nitrate, and it is weakly positive for phenylalanine deaminase (Table 7-21).[28] It is susceptible to penicillin, colistin, and vancomycin.

***Neisseria zoodegmatis* (EF-4b).** *Neisseria animaloris* and *Neisseria zoodegmatis* are the new names for the CDC Group Eugonic Fermenters (EF)-4a and EF-4b, respectively.[1106] *N. zoodegmatis* (EF-4b) does not ferment glucose, does not hydrolyze arginine, and does not produce gas from nitrate, which separates it from the glucose-fermenting strains now designated *N. animaloris* (EF-4a) which is covered in Chapter 9 of this text. *N. zoodegmatis* strains are coccoid to short rods that are nonmotile, and oxidase and catalase positive. Colonies on culture plates are nonpigmented and reported to smell like popcorn. Additional characteristics are given in Table 7-22. Most isolates have been recovered from human infections following dog and cat bites.[1172]

Unnamed Species

Gilardi Rod Group 1. This group consists of nonfastidious, nonoxidative, gram-negative, oval to medium-length, and sometimes pleomorphic rods. The cultural and biochemical characteristics of these organisms are most similar to CDC group M-5, now called *Neisseria weaveri*. All Gilardi rod group 1 strains are strongly positive in the phenylalanine deaminase reaction, producing a deep green color in the agar slant, whereas N. weaveri isolates, when positive, give a weak to moderate reaction. All N. weaveri reduce 0.01% nitrite,

whereas all Gilardi rod group-1 isolates are negative.[367,754] Other differentiating characteristics are given in Table 7-21. Isolates of Gilardi rod group-1 have been recovered from a variety of human sources, including leg, arm, and foot wounds; an oral lesion; urine; and blood; however, their pathogenic potential has yet to be determined.[754]

Family Rhodobacteriaceae

Genus *Paracoccus* and CDC Eugonic Oxidizer (EO) Groups

***Paracoccus yeei* (EO-2) and CDC Groups EO-3 and EO-4.** Members of this group are aerobic, gram-negative (sometimes appearing gram-variable), coccoid to short, thick or slightly thick rods that grow, sometimes poorly, on MacConkey agar. All are strongly oxidase positive, nonmotile, and indole negative and utilize glucose,

xylose, and lactose[756] (Table 7-22). Daneshvar et al.[251] proposed the name *Paracoccus yeeii* (later changed to *yeei* in accordance with the international code for bacterial nomenclature)[320] for the former CDC group EO-2. CDC groups EO-3 and EO-4 remain unnamed. *P. yeei* grows on both BAP and MacConkey agars and appears as mucoid to very mucoid, nonpigmented colonies after 48 hours incubation (Image Plate 7-4G). *P. yeei* also has a distinctive O-shaped cellular morphology on Gram's stain examination owing to the presence of vacuolated or peripherally stained cells (Image Plate 7-4H).[756] This O-shaped morphology is not observed with *P. immobilis* or EO-3 or EO-4 strains. EO-3 and EO-4 strains have a definite yellow, nondiffusible pigment that is not observed with either *P. immobilis* or *P. yeei*.[756] *P. yeei* has been isolated from various human wound infections and blood.[251,343] It has also been reported to cause keratitis in a contact lens wearer,[232] corneal graft rejection in a patient following corneal transplant,[537] myocarditis in a transplanted heart,[961] and peritonitis in an ambulatory peritoneal dialysis patient.[1153] EO-3 has been reported to cause peritonitis in a patient on continuous peritoneal dialysis.[239] EO-4 has been recovered from blood, urine, and a nasal sinus, but the clinical significance of these isolates is unknown.[1170]

Genus *Haematobacter*. *Haematobacter* is a new genus of aerobic gram-negative rods that phenotypically most closely resembles *Psychrobacter phenylpyruvicus*. The genus consists of two named species, *H. massiliensis* (formerly *Rhodobacter massiliensis*) and *H. missouriensis*, and one unnamed species, *Haematobacter* genomospecies 1.[434] They are obligately aerobic, nonmotile rods and grow at 25°C and 35°C, but not at 42°C. Microscopically, cells appear as gram-negative serpentine pleomorphic rods with filaments. The Gram stain appearance can be viewed in the publication by Helsel et al.[434] *Haematobacter* species are nonhemolytic and nonpigmented on blood-containing media. Some strains produce green discoloration under areas of moderate to heavy growth on BAPs.[434] Some strains grow on MacConkey agar. All strains are asaccharolytic. They produce catalase, oxidase, urease, phenylalanine deaminase, and H_2S (lead acetate paper), but do not produce indole, reduce nitrate or nitrite, or hydrolyze gelatin or esculin. ONPG is weakly positive.[434] Additional phenotypic properties are given in Table 7-21. Strains received at CDC were mainly recovered from blood cultures, but the clinical significance is unknown.[434] One additional isolate *H. massiliensis* was recovered from the blood of a patient in France.[299] There is also a report of the isolation of a *Haematobacter*-like species from the blood of a 65-year-old man who suffered from probable aortic valve endocarditis.[139] *Haematobacter* strains have low MICs values for amoxicillin, fluoroquinolones, aminoglycosides,

carbapenems, but variable MICs for cephalosporins, monobactams, and piperacillin.[434]

Organisms That Are Nonmotile and Oxidase Negative

Family Moraxellaceae

Genus *Acinetobacter*

Taxonomy. The genus *Acinetobacter* is currently classified in the family *Moraxellaceae* and consists of bacteria that are nonmotile, oxidase-negative, gram-negative coccobacilli.[354,926] In 1986, Bouvet and Grimont provided a new classification that distinguished 12 different groups (genomospecies) within the genus *Acinetobacter* based on DNA–DNA hybridization and nutritional characteristics.[117] In 1989, Tjernberg and Ursing[1073] described three additional DNA groups coded 13 through 15; concurrently, Bouvet and Jeanjean[119] described five DNA groups of proteolytic *Acinetobacter* species that they number 13 through 17. However, two of the DNA groups described by Tjernberg and Ursing differ phenotypically from the DNA groups described by Bouvet and Jeanjean. Thus, different DNA groups have the same number, which adds to the confusion surrounding the present subdivision of the genus. There are also two provisional genomospecies designated, "Between 1 and 3" and "Close to 13TU."[362] The name *Acinetobacter seifertii* has recently been proposed for the "Close to 13TU" strains.[776] At the time of this writing there were 41 named species described within the genus *Acinetobacter* (http://www.bacterio.net/acinetobacter.html).[496,772,773,1016] Many of the original *Acinetobacter* genomospecies have been named and are delineated in Box 7-11.[117,362,592,776–,784,1073] The remaining genomospecies are unnamed. A scheme of phenotypic tests useful for identifying *Acinetobacter* genomospecies 1 through 12 has been described.[117]

One initial clue that a nonfermenter isolate may belong to the genus *Acinetobacter* is the Gram stain morphology: gram-negative coccobacillary cells often appearing as diplococci (Image Plate 7-5A). This similarity in appearance to *Neisseria gonorrhoeae* led to the archaic taxonomic genus designation "*Mima*" (to mimic). Students and laboratory workers should also be aware of the fact that *Acinetobacter* species may initially appear as gram-positive cocci in direct smears of clinical specimens and in smears prepared from positive blood-culture bottles.[254,423] After 24 hours of growth on blood agar, the colonies are between 0.5 and 2 mm in diameter, translucent to

opaque (never pigmented), convex and entire. Most strains grow well on MacConkey agar and produce a faint pink tint (Image Plate 7-5B). Certain glucose-oxidizing acinetobacters may also cause a unique brown

BOX 7-11

Names Assigned to the *Acinetobacter* Genomospecies

Genomospecies[a]	Current Designation
1	*A. calcoaceticus*
2	*A. baumannii*
3	*A. pittii*
4	*A. haemolyticus*
5	*A. junii*
6	Unnamed
7	*A. johnsonii*
8/9	*A. lwoffii*
10	*A. bereziniae*
11	*A. guillouiae*
12	*A. radioresistens*
13TU	*A. nosocomialis*
13BJ, 14TU	Unnamed
14BJ	Unnamed
15BJ	Unnamed
15TU	*A. variabilis*
16	Unnamed
17	Unnamed
Close to 13TU	*A. seifertii*
Between 1 and 3	Unnamed

[a] Genomic species delineation is according to Bouvet and Grimont,[117] Bouvet and Jeanjean,[119] and Tjernberg and Ursing.[1073]
BJ, Bouvet and Jeanjean; TU, Tjernberg and Ursing.

discoloration of heart infusion agar with tyrosine or blood agar into which glucose is incorporated.[987,1172] We have also observed this phenomenon on MacConkey and Mueller–Hinton agars with a clinical isolate of *A. baumannii* (Image Plate 7-5C). Differential and selective media have been described for isolation of *Acinetobacter* spp. from contaminated specimens.[475,507] Presumptive identification of *Acinetobacter* species can be made on the basis of the lack of cytochrome oxidase activity, lack of motility and resistance to penicillin.

A variety of human infections are caused by *Acinetobacter* species including pneumonia (most often related to endotracheal tubes or tracheostomies),[141] endocarditis, meningitis, skin and wound infections, peritonitis (in patients receiving peritoneal dialysis) and urinary tract infections. Sporadic cases of conjunctivitis, osteomyelitis, and synovitis have also been reported.[379] It is now recognized that *Acinetobacter* spp. play a significant role in the colonization and infection of hospitalized patients. They have been implicated in a variety of nosocomial infections, including bacteremia, urinary tract infection, and secondary meningitis, but their predominant role is as agents of nosocomial pneumonia, particularly ventilator-associated pneumonia in patients confined to hospital intensive care units.[90,834,1137,1180] Comprehensive reviews of *Acinetobacter* infections have been provided by Bergogne-Berezin and Towner[90] and Munoz-Price and Weinstein.[761]

***Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) Complex.** The ACB complex consists of five phenotypically and genotypically related species with valid names: *A. baumannii, A. nosocomialis, A. pittii, A. calcoaceticus, A. seifertii*, and a provisional genomospecies called "Between 1 and 3."[776,777] Because of problems in separating the saccharolytic strains belonging to the ACB complex using phenotypic tests,[94,360,363,619] most laboratories have chosen to report members of this group as "*Acinetobacter calcoaceticus–A. baumannii* complex."[363,834] Although *A. baumannii* is the most clinically important species and is frequently involved in nosocomial infections,[332,350,834,1140,1181] including outbreaks associated with multidrug- and pandrug-resistant strains,[107,285,486,522,591,628,762,775,1044] there are an increasing number of reports of non- *A. baumannii* species of the ACB complex causing nosocomial infections.[93,265,481,540,1077,1115,1181] These species differ from *A. baumannii* in characteristics regarding infectious potential, antimicrobial susceptibility and mortality rates, which dictates the need for precise identification to guide therapy and improve clinical outcome.[600,1016] Therefore, it may be important in the future for laboratories to adopt nonphenotypic-based test methods for use in identifying members of the ACB complex. In this regard, a number of genotypic methods have been proposed for *Acinetobacter* identification.[285] The most widely used approaches include PCR, amplified rRNA gene restriction analysis (ANDRA),[1119] amplification

and sequencing of species-specific DNA regions, such as the intrinsic oxacillinases from *Acinetobacter* species,[1086] partial *rpoB* gene sequence analysis,[411] Fourier transform infrared spectroscopy (FTIR),[1016] and MALDI-TOF MS. Of these methods, MALDI-TOF MS seems to be the most practical for incorporation into routine use in the clinical microbiology laboratory. Several studies have demonstrated that MALDI-TOF MS can provide rapid and accurate identification of *Acinetobacter* species including those in the ACB complex.[22,316,485,965,1015]

Acinetobacter baumannii. *A. baumannii* is saccharolytic and acidifies most OF carbohydrates; in particular, definitive identification is made by demonstrating the rapid production of acid from lactose (1% and 10% concentrations). Additional identifying characteristics can be found in Table 7-18. *A. baumannii* is the most commonly found species in human clinical specimens, followed by *A. lwoffii, A. haemolyticus, A. johnsonii*, genomospecies 3 and genomospecies 6.[120,531,969,1073] *A. baumannii* is the species most often responsible for hospital-acquired infections,[82,332,350,665,834,1137,1140] and is increasingly being reported as a significant pathogen causing sepsis, wound infections, and pneumonia in both hospitalized patients and in the community setting.[172,179,187,693,735,762,970] *A. baumannii* has also been associated with combat injuries in Iraq and Afghanistan.[44,258,963,964] A fatal outbreak of extensively drug-resistant *A. baumannii* with enhanced virulence (so-called Clade B strains) has been reported in a cohort of relatively immunocompetent patients raising concerns that the low virulence potential of *A. baumannii* may need to be reevaluated.[522] A biotyping system for differentiating 17 biotypes of *A. baumannii* based on the utilization of six substrates has been established and may be useful for epidemiologic studies.[118]

Other *Acinetobacter* Species. *A. johnsonii, A. lwoffii* and *A. radioresistens* are nonsaccharolytic *Acinetobacter* species that occur as natural inhabitants of human skin[120,969] and may be commensals in the oropharynx and vagina.[120]

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A. junii is a rare cause of ocular infection[868] and bacteremia, particularly in pediatric patients.[93,540,644,1077] *A. lwoffii* has been more commonly associated with meningitis than other *Acinetobacter* species.[989] *A. nosocomialis* clinical isolates have been reported from tracheal secretions[265] and blood.[265,1181] A nosocomial outbreak of *A. nosocomialis* infection was reported in a neurosurgical intensive care unit where 5 of 23 patients who became colonized with the outbreak strain developed an infection.[1115] *A. pittii* has been reported to cause infections in a neonatal intensive care unit,[481] and

nosocomial bloodstream infections.[743,1181] In one study, the clinical profile of patients colonized or infected with *A. pittii* was noticeably different from that observed for patients with *A. baumannii* infections. *A. pittii* was more frequently found in skin and soft tissue infections, including surgical wound infections, while colonization and infection of the respiratory tract was less frequent than observed for *A. baumannii*.[740] *A. radioresistens* has been reported in an HIV-positive patient with community-acquired bacteremia.[1141] *A. schindleri* has been recovered from a variety of human specimens (vaginal, cervical, throat, nasal, ear, conjunctiva, and urine), but are mostly regarded as clinically nonsignificant.[296,772,774] *A. ursingii* has been shown to cause bloodstream infections in hospitalized patients.[296,666,772,774]

A. lwoffii and *A. radioresistens* are asaccharolytic. *A. johnsonii* is also asaccharolytic but can be separated from all other *Acinetobacter* species by its failure to grow at 37°C. Additional biochemical characteristics of *A. lwoffii* are given in Table 7-18. Additional differentiating features of the other genomospecies can be found in the article by Bouvet and Grimont.[117]

Acinetobacter species tend to be resistant to a variety of antibiotics, although one species, *A. lwoffii*, tends to be more sensitive than the others. There is almost universal resistance to penicillin, ampicillin, and cephalothin, and most strains are resistant to chloramphenicol.[672,968] Variable susceptibility to second- and third-generation cephalosporins and to trimethoprim-sulfamethoxazole has been reported. We have noticed an increased trend toward aminoglycoside resistance among the *Acinetobacter* species in recent years and multidrug-resistant strains, including carbapenem-resistant *Acinetobacter* species have been reported in nosocomial outbreaks.[107,115,289,486,591,661,690,743,762,775,1138,1188] Antimicrobial susceptibility testing for *Acinetobacter* species is problem-prone. Swenson and colleagues at the CDC have shown that results obtained using standardized microbroth dilution do not agree with results obtained with the standardized disk diffusion method for certain antibiotics. Very major errors were frequent with the β-lactam and β-lactam inhibitor combination antibiotics with the microbroth dilution method typically showing greater resistance.[1048] Additionally, susceptibility testing via Etest for *Acinetobacter* has been reported to give falsely high MIC values for tigecycline.[695] Hope et al.[478] reported that MICs of minocycline, tetracycline and tigecycline were one or two dilutions higher in Mueller–Hinton broth than Mueller–Hinton agar. Thamlikitkul et al.[1068] reported that tigecycline MICs for *Acinetobacter* species determined by the Etest method were usually fourfold higher than those determined by the broth microdilution method and that Etest might not be an accurate method for in vitro susceptibility testing of tigecycline and *Acinetobacter*. Susceptibility breakpoints for tigecycline and *Acinetobacter* have not been published by either the FDA or the CLSI, because the prescription of tigecycline for treating *Acinetobacter* infections remains a nonindicated

use. However, realizing that tigecycline is being used to treat *Acinetobacter* infections when limited therapeutic options are present, Jones et al. tried applying the FDA disk diffusion breakpoints ≥19 mm (susceptible) and ≤14 mm (resistant) listed for *Enterobacteriaceae* to *Acinetobacter* species, but found that this led to an unacceptable error rate of 23.3%; however, an adjustment of the tigecycline disk diffusion breakpoints for testing *Acinetobacter* to ≥16/≤12 mm (susceptible/resistant) reduced errors to an acceptable level.[523] At present, there are no data to indicate which methods for testing susceptibility to *Acinetobacter* provide more clinically relevant information. Students and laboratorians are advised to use caution when performing and interpreting susceptibility testing results with *Acinetobacter* species. A review of the antimicrobial susceptibilities of *Acinetobacter* species and the main mechanisms of antimicrobial resistance has been provided in two separate reviews by Van Looveren et al.[1127] and Perez et al.[838]

Combined treatment with an aminoglycoside and ticarcillin or piperacillin is synergistic and may be effective in serious infections. For multiply-resistant *Acinetobacter* infections, several studies have demonstrated clinical efficacy of sulbactam in combination with ampicillin or cefoperazone.[441,510,515,631,818] Moland et al.[739] showed *in vitro* synergy between tigecycline and amikacin against multidrug-resistant *A. baumannii*. Old drugs like minocycline are also being brought back into use for treating infections caused by multidrug-resistant *A. baumannii*.[157,380] The only other antibacterial agents that have been shown to be active against multidrug-resistant *Acinetobacter* are colistin and polymyxin B.[158,516,543,561,562,632] Castanheira et al.[157] reported that among 17 antimicrobial agents tested and considered as candidates for treating *A. baumannii*, minocycline and colistin were the only 2 agents that had susceptibility rates (as per CLSI criteria) exceeding 50% (79.1% and 98.8%, respectively). Bowers et al.[123] reported that the intracellular concentration and *in vitro* bactericidal effect of minocycline were both enhanced by polymyxin B suggesting the clinical utility of these two drugs when used in combination. Scheetz et al.[953] have shown that polymyxin B, minocycline and tigecycline are the most active antimicrobials *in vitro* against carbapenem-intermediate or resistant *A. baumannii*. Of increasing concern are reports of the emergence of colistin-resistant and heteroresistant strains of *A. baumannii* in association with previous colistin therapy.[428,628,637,862,873,895] Reviews of the currently available antimicrobial agents and data supporting the use of various agents for treatment of *Acinetobacter* infections has been provided by Fishbain and Peleg,[330] and Neonakis et al.[780]

Family Alcaligenaceae

Genus *Bordetella*

***Bordetella holmesii* (CDC Group NO-2).** *B. holmesii* was formerly classified as CDC Group NO-2, and is described as a gram-negative, small coccoid and short rod, with medium-width longer rods occasionally observed. They are

asaccharolytic, oxidase negative, nonmotile, and fastidious, and they produce a brown soluble pigment (Table 7-18).[1171] The lack of oxidase activity and the production of a brown soluble pigment differentiate *B. holmesii* from *B. pertussis, B. bronchiseptica* and *B. avium*; the lack of urease activity differentiates this species from *B. parapertussis*. A negative nitrate reaction differentiates it from NO-1 strains, and the production of a brown soluble pigment differentiates it from *Acinetobacter* species. Information on the clinical significance of *B. holmesii* and its association with disease in humans was presented elsewhere in this chapter under the heading "Organisms That Are Motile With Peritrichous Flagella—Genus *Bordetella*."

Unnamed Species

CDC Group NO-1. An unnamed species of a fastidious, nonoxidative, gram-negative rod, designated CDC Group NO-1 (nonoxidizer-1), has been isolated from human wounds resulting primarily from dog or cat bites.[58,450,530] This organism is nonmotile, oxidase negative, and asaccharolytic. They appear as coccoid to medium-sized gram-negative rods that form small colonies on sheep blood agar. They resemble *Acinetobacter* spp. phenotypically but can be readily separated from *Acinetobacter* by a positive nitrate reduction test. Cellular fatty acids and ubiquinone analysis are also useful in differentiating NO-1 from *Acinetobacter* species.[450] Other phenotypic characteristic are given in Table 7-18. They are susceptible to a variety of antimicrobial agents, including the aminoglycosides, β-lactam antibiotics, tetracyclines, quinolones, and sulfonamides. Fifty percent of isolates are reported to be resistant to trimethoprim.[450]

CDC Group EO-5. CDC EO-5 are glucose-oxidizing gram-negative rods that have a biochemical profile similar to *A. baumannii*.[249] They are nonmotile and oxidase negative, but unlike *Acinetobacter* species, they fail to grow on MacConkey agar. Some strains produce a yellow soluble pigment. Cellular fatty acid analysis is also useful in differentiating EO-5 strains from *Acinetobacter*. Other characteristics are given in Table 7-18. Isolates have been recovered from blood, peritoneal fluid, transtracheal aspirate, gallbladder, and an arm wound.[249] Antibiotic susceptibility data are not available.

PART III: APPROACH TO RECOVERY AND IDENTIFICATION OF NONFERMENTERS

Levels of Service in Identification of Nonfermenters

The level to which species identification of nonfermenters is performed depends on the size and purpose of the individual laboratory. Reference laboratories, or universities and clinics where students and residents are being trained, may be required to identify all clinically relevant nonfermenters to the species level. Laboratories that provide services primarily for the medical community may be prepared to identify only the more frequently encountered species, sending the rare isolates to a reference laboratory. In the analysis of bacteriology specimens circulated by the CDC for their microbiology performance evaluation programs, Griffin and associates[406] found that laboratories for which the volume of specimen testing is small (fewer than 80 samples per week) average about double the error rate of laboratories handling more than 1,200 specimens per week. They conclude that this difference in performance is constant and that a laboratory cannot necessarily correct its performance, for many of the reasons listed in Box 7-12. They recommend that laboratories limit testing to procedures that can be done well and make arrangements with a reference laboratory to provide services for testing specimens that are received infrequently. Factors that contribute to the difficulties in identifying nonfermenters are listed in Box 7-12.

BOX 7-12

Factors Contributing to the Difficulties in Identifying Nonfermenters

1. Most species are only infrequently encountered.
2. Because of this infrequency, laboratory personnel may not be familiar with many of the nonfermenters.
3. Many of the conventional culture media are not suitable for identifying nonfermenters.
4. Many species grow slowly, and biochemical reactivity is weak, requiring considerable experience to interpret equivocal reactions.
5. Quality control of culture media may be difficult, and, because of infrequent use, outdating becomes a problem.
6. Packaged commercial kit systems often have low accuracy in the identification of

the more fastidious strains of nonfermenters, requiring the use of additional media.

Several commercial packaged systems for the identification of nonfermenters (discussed later in this chapter) are currently available[453,576,797,912,1158]; however, because these systems depend on bacterial growth and on the formation of biochemical products in conventional media or in marginally nutritious substrates, only the more biochemically active species can be identified with an acceptable degree of accuracy. The accuracy of performance is also improved with the use of one of several automated or semiautomated bacterial identification systems, primarily because they rely on automatic instrument readings and recording of results, eliminating the subjective bias inherent in the visual interpretation of equivocal end points. These instruments have the advantage of making identifications several hours faster than conventional methods.

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Guidelines for Recovery of Nonfermenters

With the previous discussion in mind, each laboratory director must develop a logical approach to the identification of nonfermenters in his or her laboratory. The following guidelines are helpful in the laboratory approach to the recovery and identification of nonfermentative gram-negative bacilli:

1. Except for *P. aeruginosa* (and the rarely encountered *B. mallei* and *B. pseudomallei*), the nonfermenters have a low degree of virulence and most often cause nosocomial infections in patients who are debilitated or immunocompromised. This narrow niche of infectivity indicates that infections will be uncommon (except for the relatively high incidence of *P. aeruginosa* and *Acinetobacter baumannii*, as discussed earlier). However, because an increasingly higher proportion of hospitalized patients have serious underlying illness, nonfermenters are being recovered with increasing frequency from clinical specimens and must be considered as important agents for many infectious diseases. Specific conditions or diseases predisposing patients to infection with nonfermenters include the following:
   a. Malignancies (particularly of the reticuloendothelial system) and instrumentation and surgery—catheterizations (particularly urinary tract and indwelling intravascular), tracheostomy, lumbar puncture, dialyses, lavages,

and the placement of shunts and prostheses.

b. Prolonged corticosteroid, antibiotic, antimetabolic and anticancer therapy.

c. Underlying metabolic or chronic infectious diseases (e.g., an apparent link exists between CF and infections caused by *B. cepacia*, mucoid *P. aeruginosa* and *A. xylosoxidans*).

d. Burns, open wounds, and various exudative lesions.

2. Most nonfermenters have their natural habitat in several environments that serve as potential reservoirs for human infections:

a. Various water reservoirs that are prevalent in hospitals—humidifiers, mist tents and nebulizers, water baths, disinfectant and irrigation solutions, distilled water lines, hand creams, body lotions, and so on. These solutions often come in direct contact with mucous membranes and other body surfaces in the course of patient treatment.

b. Implements, such as anesthetic equipment, forceps, and thermometers, that may be stored in disinfectant solutions; and mops, sponges, and towels.

c. Moist intertriginous parts of the skin, such as the toe webs, groin, axilla, and antecubital fossa. Infections from these sources tend to be more prevalent in the summer.

d. Various domestic animals predisposing caretakers to infection.

3. Certain nonfermenters have a propensity for causing specific infections, discussed elsewhere in this chapter. Septicemia may be found with virtually any species; pneumonitis or bronchitis, septic arthritis, urinary tract infections, postoperative and posttraumatic wound infections, and conjunctivitis may also be caused by most species of nonfermenters. Some species, particularly of *Pseudomonas*, may produce cytotoxic and lytic toxins that make some of these infections locally severe and potentially life-threatening.

4. Clinical isolates that are gram-negative bacilli on Gram's stain can be suspected of being nonfermenters if they produce small colonies on blood agar, grow poorly or not at all on MacConkey agar, do not produce acid in either the slant or the deep portion of KIA or TSI medium, and are cytochrome oxidase positive.

5. Many species of nonfermenters also tend to have certain patterns of multidrug-resistance to antibiotics. These patterns are learned through experience and may provide an initial clue that one is dealing with one of the nonfermenters, or may point to a specific genus.

Identification of the Most Common Species

The identification of the three most commonly recovered clinical species,

Pseudomonas aeruginosa, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* is addressed first. Most strains can be identified easily on the basis of only a few observations and chemical tests. Not only does the rapid identification of these common isolates provide the physician with immediate information, but it also relieves the laboratory of performing a battery of time-consuming and expensive secondary tests.

Pseudomonas aeruginosa

More than 95% of *P. aeruginosa* strains recovered from clinical specimens can be identified by observing the presence of the following primary characteristics:

- Gram-negative rod
- Oxidase positive (within 10 seconds)
- Typical fruity grapelike odor, or corn tortilla
- Recognizable colony morphology
  - On blood agar or chocolate agar appear as large colonies with metallic sheen; may be mucoid, rough or pigmented (showing pyocyanin), and often β-hemolytic (Image Plate 7-2F)
  - On MacConkey, appear as lactose negative with green pigmentation, or metallic sheen; may be mucoid (Image Plates 7-2G, 7-2H)

When these characteristics are present, a definitive identification of *P. aeruginosa* can be made with no further testing.[208]

Most strains of *P. aeruginosa* produce pyocyanin, a water-soluble green phenazine pigment that imparts a greenish color to the culture medium. In fact, observing the presence of pyocyanin may be the only characteristic required to identify *P. aeruginosa,* because no other nonfermenter synthesizes this pigment. Reyes and coworkers[899] have shown that 98% of the *P. aeruginosa* strains isolated in their laboratory

produced pyocyanin on Tech agar[569] within 48 hours and suggest that the use of Tech agar is a satisfactory alternative to the use of extensive identification schemes when *P. aeruginosa* is suspected (Image Plate 7-1G). They note that some mucoid strains of *P. aeruginosa* from patients with CF may not produce pigment and, therefore, may be misidentified if pigment production is the only criterion used for identification of these aberrant strains. A few strains of *P. aeruginosa* may produce pigments with other colors—pyorubin (red), pyomelanin (brown to black), and pyoverdin (yellow).

Fluorescein pigment can be visualized by observing the growth on certain media

using a long-wavelength ultraviolet light source (e.g., Wood's lamp; Image Plate 7-1H). Media containing proteose peptone 3 (Difco Laboratories, Detroit, MI) and cations, such as magnesium or manganese, enhance fluorescein synthesis. King's medium B, Sellers' medium, and Mueller-Hinton agar are also suitable for demonstrating fluorescence. Fluorescence may be enhanced if cultures are incubated at 20°C to 30°C rather than at 35°C to 37°C. The following additional characteristics are helpful in identifying non–pigment-producing strains of *P. aeruginosa*:

- Growth at 42°C
- Alkalinization of acetamide
- Denitrification of nitrates and nitrites
- Motile with polar, monotrichous flagellum

Variants producing mucoid or dwarf colonies with atypical biochemical reactions may also be encountered, occasionally making identification difficult.

In summary, most strains of *P. aeruginosa* can be identified easily by observing the typical large colonies, with a blue–green discoloration on primary isolation media, and further confirmed by detecting a typical grapelike odor. Demonstration of fluorescein pigment and cytochrome oxidase activity helps to confirm the final identification, and additional tests are usually not required. The typical characteristics by which *P. aeruginosa* are identified are shown in Table 7-5.

Acinetobacter baumannii

A. baumannii is the second most frequent nonfermenter encountered in clinical laboratories, but with only about one-tenth the frequency of *P. aeruginosa*. The following are the characteristics by which a presumptive identification can be made:

- Appears as cocci or coccobacilli on Gram's stain
- Grows well on MacConkey agar (colonies may have a slightly pinkish tint, a helpful characteristic when present)
- Does not produce cytochrome oxidase
- Exhibits rapid utilization of glucose, with production of acid
- Exhibits rapid utilization of 10% lactose, with production of acid
- Are nonmotile
- Are penicillin-resistant

The initial clue is the observation of tiny (1.0 × 0.7 μm) bacilli on Gram stains prepared directly from clinical materials. When Gram stains are prepared from agar or broth cultures, the cells may appear larger and more like coccobacilli (Image Plate 7-5A). *Acinetobacter* species are not pigmented when grown on blood agar, a helpful characteristic in differentiating them from certain other nonfermenters, such as

occasional oxidase-negative, nonmotile strains of *Burkholderia cepacia*. However, colonies growing on MacConkey agar may produce a faint pink tint or a deeper cornflower blue when observed on eosin methylene blue agar (Image Plate 7-5B). Resistance to penicillin helps distinguish *A. baumannii* from the highly penicillin-sensitive *Moraxella* species, which also usually appear as coccobacilli on Gram stain. Most strains of *Moraxella* species are also cytochrome oxidase positive. *A. lwoffii* is nonsaccharolytic and can be differentiated from *A. baumannii* because it produces no acid when grown in media that contain carbohydrates.

Stenotrophomonas maltophilia

S. maltophilia is the third most frequently encountered nonfermenter in clinical laboratories. The following are the characteristics by which a presumptive identification can be made:

- Good growth on blood and MacConkey agars
- Does not produce cytochrome oxidase (or very delayed reaction)
- Produces acid in OF maltose but may be negative in OF glucose
- Lysine decarboxylase positive
- DNase positive
- Some strains have yellow pigment

The antibiotic susceptibility pattern can also be a clue to the identification of *S. maltophilia*, which is typically resistant to most antibiotics, including the aminoglycosides, but is susceptible to trimethoprim-sulfamethoxazole and usually colistin.

Methods for Identification Using Conventional Biochemical Tests

If an unknown nonfermentative gram-negative bacillus is not *Pseudomonas aeruginosa*, *Acinetobacter baumannii,* or *Stenotrophomonas maltophilia*, additional characteristics must be determined to make a species identification. The two most widely used identification schemes based on phenotypic biochemical reactions are those of Weyant and associates (CDC)[1172] and Schreckenberger.[958] Which approach to select is largely one of personal preference, past experience, and the local availability of the culture media required to perform the various tests, providing the criteria listed in Box 7-13 are met.

CDC Scheme—Weyant and Associates

In answer to the problem of how to identify nonfermenters in the clinical laboratory without doing all the tests that are done in a reference laboratory, Weyant and colleagues[1172] have published a three-part guide that includes (1) an identification key for gram-negative aerobes, (2) a set of 12 identification

BOX 7-13

Approach to Selecting a Conventional Test

1. Positive and negative reaction results delineated in the identification table must be based on the procedures and formulas listed in the laboratory procedure manual. That is, the media and procedures must be the same as those used to generate the results in a table of reactions used for identification.
2. All of the reactions listed in the identification table must be at a confidence level of 90% or higher and derived from a database of a sufficiently large number of organisms to be statistically significant.
3. All tests must be performed under standard quality-control surveillance to ensure that the reagents, reactions, and end points are as close as possible to those on which the table was originally based.

tables, and (3) a numerical code book by which derived biotype numbers can be linked to species names. They also include procedures and media formulas for all of the biochemical tests cited in the identification manual. To correctly interpret results from a given identification table, one must use the same procedures on which the reactions are based. The manual is entitled: "Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria," and is available from Lippincott Williams & Wilkins.[1172]

Practical Approach to Identification of Nonfermenters—Schreckenberger

The approach used in this book was devised by Schreckenberger[958] and requires that the various clinically important nonfermentative bacilli be divided into four functional groups based on an immediate assessment of their motility and ability to produce cytochrome oxidase (Table 7-23). Once having accomplished this subgrouping,

definitive identifications can be made by referring to the identification Tables 7-23 through 7-37 and following the instructions given with the tables. The biochemical tests used in this identification guide are all conventional biochemical formulations and are available commercially from most media manufacturers. Note that in working with these tables, a given species of a nonfermenting bacillus may appear in more than one table because a particular organism may not be 100% positive or negative for a given characteristic; therefore, some redundancy is built into the scheme so that an unknown bacillus will be identified regardless of the test result obtained with the variable screening test. By following these tables, the microbiologist should be able to definitively identify over 95% of nonfermentative bacilli that will be recovered from clinical specimens.

Computer-Aided Schemes

ASHEX Web ID Program. When a large battery of tests is performed, it is often difficult to determine the most likely organism identification by using tables or algorithm-based diagrams. For this reason, Schreckenberger has developed an organism identification application called WIP (Web ID Program).[957] WIP is a free online program (developed by Adam Schreckenberger 2014) that can be used to identify bacteria without the use of downloaded software. The WIP application works on any PC, Apple or smartphone system with an active internet connection. Since the program runs within an internet browser, it requires no additional downloads. One merely needs to enter available phenotypic characteristics into the online Web form to calculate a likelihood probability and modal score. If the likelihood score is less than 95%, a list of phenotypic tests that distinguish the top two candidates will be displayed; likewise, if the modal score is poor, a list of input results that do not match well to the top candidate prediction will be shown. The program provides statistical selection of unknown bacterial isolates through comparisons to phenotypic characteristic matrices of known strains. The program has three major functions: (1) the identification of an unknown isolate, (2) the selection of additional tests to distinguish between possible strains if identification is not achieved, and (3) the storage and retrieval of results. An assortment of WIPs, each of which targets a specific subset of gram-negative bacteria, is available on www.pschreck.com by clicking the appropriate WIP tab of choice on the site toolbar. This collection is referred to as the AID (App Identification) Cloud. Each WIP references a specific characteristic matrix that was created either from a published table in the literature or from the author's own data.

The table associated with the Nonfermenter WIP is named ASHEX and consists of 887 isolate entries representing 93 separate species of NFBs with reactions for 42 phenotypic tests. The table can be downloaded from the website as an Excel spreadsheet. This file includes the characteristic matrix as well as the raw phenotypic

reaction results used to compute each probability. The complete table is reproduced in this chapter as Table 7-3. To access the WIP, go to www.pschreck.com and click the WIP tab of your choice in the tool bar and enter your reactions on the worksheet.

Methods for Identification Using Commercial Kit Systems

Packaged kit systems have been designed for, or adapted to, the identification of the nonfermentative bacilli. These kits share many of the attributes of packaged systems in general; that is, they are convenient to use, have a long shelf life, and preclude the need for fresh supplies of media and reagents. The packaged systems also provide standardized techniques that are accurate and give reproducible results equal to or better than conventional procedures, with the exceptions discussed later in this chapter.

Inherent problems in the use of many of the currently available packaged kits for identifying nonfermenters include

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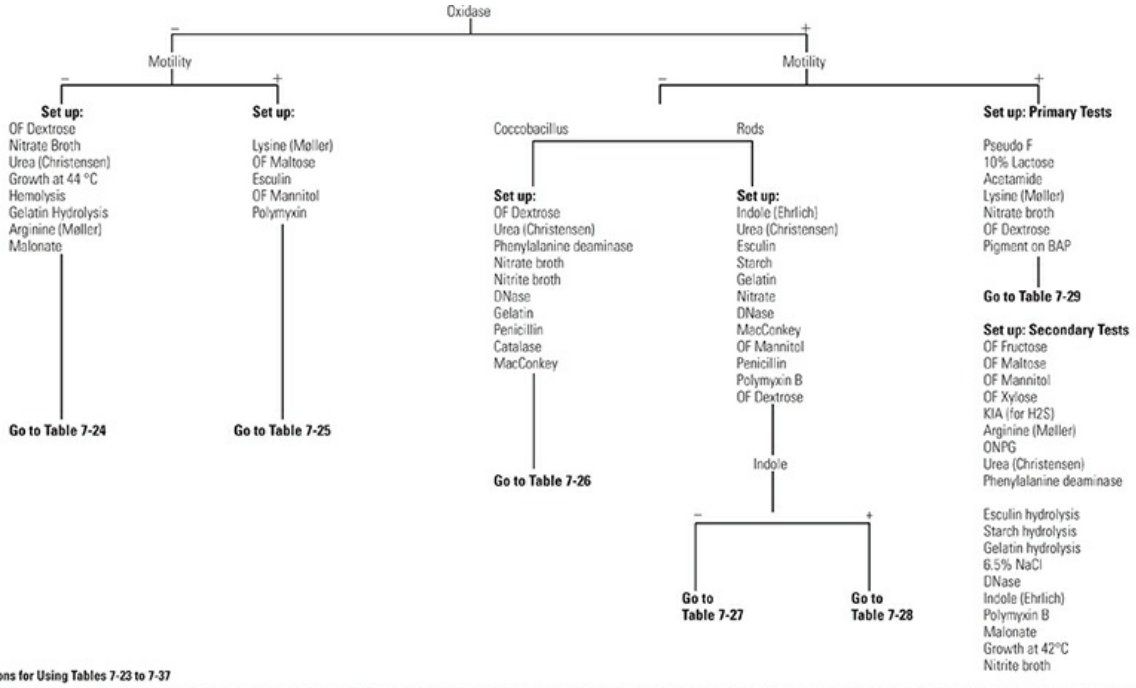
TABLE 7-23 Practical Approach to the Identification of Nonfermenters



Instructions for Using Tables 7-23 to 7-37

This approach to the identification of nonfermenters is designed to minimize the number of biochemical tests needed for identification based on a preliminary assessment of the oxidase and motility reactions of organisms to be identified. Once this information is known, a specific battery of tests is performed to complete the identification of the organism. For those organisms that are both oxidase positive and motile, a two-step approach is used based on the reactions obtained in a primary test battery followed by additional supplemental tests that are specified in the designated Tables. Depending on the needs and resources available, the user of this guide may wish to set up all of the tests included in the primary and secondary batteries whenever a motile, oxidase-positive nonfermenter is encountered in order to obtain a definitive identification in the shortest time possible. As a general rule when working with NFBs, a heavy inoculum should be used and reactions should be held 48 hours before the final reading is taken.

Steps to Follow

1. Determine the motility and oxidase reactions and follow the flow diagram in Table 7-23.
2. Set up the specified biochemical tests and go to the Table indicated to complete the identification.
3. To utilize Tables 7-24 to 7-37, begin with the first biochemical test listed on the left hand side of the Table and locate the shaded box or boxes in the upper left hand corner.
4. If a single box is shaded, and if the reaction given matches the reaction obtained with your specimen, you are done. The organism identification is listed in the same row to the left of the box.
5. If multiple boxes are shaded and the reaction matches that of your specimen, use the reactions to the right of the shaded boxes to determine the correct identification.
6. If the reaction obtained with your specimen does not match that in the shaded box or boxes, proceed to the next column on the right and find the shaded box or boxes in this column. Repeat steps 4 and 5 until you reach a definitive identification.
7. Special consideration must be given to shaded boxes that contain a variable (V) reaction sign. In these rare cases, you must treat the variable reaction in the shaded box as both a match and a nonmatch.

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TABLE 7-24 Oxidase-Negative, Nonmotile Nonfermenters[a]

Organism[b]	Genomospecies	Yellow Pigment	Urease	Nitrate Reduced	Brown Soluble Pigment	Growth at 37°C	Growth at 44°C	Hemolysis Sheep Blood	Gelatin Hydrolysis	OF Dextrose	Arginine	Malonate
CDC EO-5		+[c]	+	–	–	+	–	NA	–	+	–	NA
Bordetella parapertussis		–	+	–	–	+	NA	+	NA	–	NA	NA
CDC NO-1		–	–	+	–	+	NA	–	–	–	–	NA
Bordetella holmesii (NO-2)		–	–	–	+[d]	+	–	–	–	–	–	NA
Acinetobacter johnsonii	7	–	–	–	–	–	–	–	–	–	V (35)	V (13)
Acinetobacter baumannii	2	–	–	–	–	+	+[e]	–	–	+	+	+
Acinetobacter haemolyticus	4	–	–	–		+	–	+	+	V (52)	+	–
Acinetobacter sp.	6	–	–	–	–	+	–	+	+	V (66)	+	–
Acinetobacter bereziniae	10	–	–	–	–	+	–	–	–	+	–	–
Acinetobacter calcoaceticus	1	–	–	–	–	+	–	–	–	+	+	+
Acinetobacter pittii	3	–	–	–	–	+		–	–	+	+	V (87)
Acinetobacter radioresistens	12	–	–	–	–	+	–	–	–	V (33)	+	+
Acinetobacter junii	5	–	–	–	–	+	–	–	–	–	+	–
Acinetobacter lwoffii	8/9	–	–	–	–	+	–	–	–	–	–	–
Acinetobacter guillouiae	11	–	–	–	–	+	–	–	–	–	–	–

[a]Data from references 117, 249, 450, and 1171.
[b]All organisms included in this chart appear as gram-negative coccobacilli on Gram's stain.
[c]Also consider *Granulibacter bethesdensis* (see Table 7-18).
[d]Brown soluble pigment produced when grown at 35°C heart infusion tyrosine agar.
[e]Must also be OF dextrose positive.
+, 90% or more of strains are positive; –, 90% or more of strains are negative; V, 11%–89% strains positive; NA, results not available; numbers in parentheses are percentage of strains giving positive reaction. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

TABLE 7-25 Oxidase-Negative, Motile Nonfermenters[a]

Organism	Lysine Decarboxylase	Esculin	OF Maltose	OF Mannitol	Polymyxin B	Pigment	Additional Characteristics
Stenotrophomonas maltophilia	+	+	+	–	S	Yellow Lavender	DNase positive
Burkholderia cepacia complex	+	V (67)	+	+	R	White or yellow	DNase negative Lipase positive
Sphingomonas paucimobilis	–	+	+	–	V (89)	Deep yellow	Vancomycin sensitive
Pseudomonas luteola	–	+	+	+	S	Dull yellow	
Pseudomonas oryzihabitans	–	–	+	+	S	Dull yellow	
Burkholderia multivorans	V (53)	–	+	+	R	Grayish white	Lipase Pos
Burkholderia gladioli	–	–	–	+	R	Yellow	Lipase Pos
Bordetella trematum	–	–	–	–	NA	Grayish white	Acetamide and malonate Pos
Kerstersia gyiorum	–	–	–	–	NA	White to light brown	Acetamide and malonate Neg
Pandoraea species	–	–	–	–	R	Grayish white	Lipase Neg

[a]Data from references 209, 217, 250, 367, 438, and 1105.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; NA, results not available; numbers in parentheses are percentage of strains giving positive reaction; R, resistant; S, susceptible. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

the (1) tendency for organisms that exhibit weak or delayed biochemical activity to produce false-negative reactions, (2) less than optimal design of many systems for cultivation of certain nonfermenters, and (3) inclusion of some differential tests that may not be applicable to the identification of nonfermenters. Whereas members of the *Enterobacteriaceae* usually grow rapidly and exhibit active enzymatic activity on a variety of substrates that can readily be detected with kit systems, most nonfermenter species are slow-growing and relatively inactive enzymatically. The microbiologist needs considerable experience to interpret some incomplete or weak reactions that may be encountered in the use of these systems.

It is with these perspectives in mind that the following five kit systems are discussed.

- API 20E (BioMérieux, Hazelwood, MO)
- API 20NE (BioMérieux)
- Crystal Enteric/Nonfermenter System (Becton Dickinson Microbiology Systems)
- RapID NF Plus (Remel)
- Biolog System (Biolog, Hayward, CA)

The API 20E System

The API 20E system, originally designed for identification of the *Enterobacteriaceae,* has been extended to include the identification of common nonfermentative bacilli as well. To maximize the use of the API 20E for nonfermenters, six additional tests were added to generate a nine-digit profile number (Box 7-14). Studies performed with nonfermenters have shown that although the API 20E system identifies *P. aeruginosa*, *S. maltophilia,* and *Acinetobacter* species with up to 99% accuracy, particularly after 48 hours of incubation, the performance with other less common nonfermenters was often less than acceptable.[137,298,748,976] Incorrect identifications occurred most often because of false-negative reactions for citrate, gelatin liquefaction, motility, arginine dihydrolase, ONPG, nitrate reduction, and urease tests.[298] In other cases, identifications were not possible because the biotype number derived from the 20E strip was not listed in the API Profile Index.[298,976]

The API 20NE System

The API 20NE is a modification of the API 20E strip. The construction of the plastic strip is the same as in the 20E system; however, the substrates have been changed to include 8 conventional tests and 12 assimilation tests that are based on the observation of microbial growth in the presence of a single source of carbon (Image Plate 7-5H). A description of the operating procedure and substrates included is given in Box 7-14. Positive and negative reactions are converted to a 7-digit biotype number, and

organism identifications are made either from a computerized database or from a profile list provided by the manufacturer. Users of this system should note that the database is constructed on the basis of reactions obtained at an incubation temperature of 30°C (instead of the conventional 35°C to 37°C) with final

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TABLE 7-26 Identification of Oxidase-Positive, Nonmotile Coccobacilli[a]

Organism	Characteristic Odor or Appearance	OF Dextrose	DNase	Urease	Phenylalanine Deaminase	Gelatin Hydrolysis	Nitrate Reduced	Growth on MacConkey	Nitrite Reduced	Catalase	Growth at 35°C
Psychrobacter immobilis (saccharolytic)	Odor of PEA agar (roses)	+	–	+	+	–	V (86)	V	NA	+	–
Paracoccus yeei (CDC EO-2)	O-shaped cells[b]	+	–	+	NA	–	+	V (82)	NA	V (82)	+
CDC EO-3	Yellow colonies (100)	+	–	+	NA	–	–	+	NA	+	+
CDC EO-4	Yellow colonies (83)	+	NA	+	NA	–	–	+	NA	+	+
CDC EF-4b		+	NA	–	NA	–	+	V (65)	V	+	+
M. canis		–	+	–	–	–	+	+	V	+	+
M. catarrhalis		–	+	–	V	–	+	–	+	+	+
Psychrobacter phenylpyruvicus[c]		–	–	+	+	–	V (89)	+	–	Weak	+
O. urethralis	Small coccoid cells	–	–	–	+	–	–	V (83)	+	+	+
M. lacunata		–	–	–	–	+	+	–	–	Weak	+
M. nonliquefaciens		–	–	–	–	–	+	V (17)	–	+	+
M. osloensis		–	–	–	–	–	V (26)	V (49)	–	Weak	+
M. atlantae	Spreading or pitting colonies	–	–	–	–	–	–	+	V (20)	Weak or –	+
M. lincolnii	Coccus-like to plump rods	–	–	–	NA	–	–	–	V	+	+

[a]Data obtained from references 251, 367, 493, 754, 1102, 1170, and 1172.
[b]Gram's stain smears show coccoid to short thick rods that are frequently vacuolated. Cells have unstained centers but are peripherally stained and appear as O's.
[c]*Brucella* species may be misidentified as *Psychrobacter phenyylpyruvicus* in some commercial identification systems.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; NA, results not available; numbers in parentheses are percentage of strains giving positive reaction. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

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TABLE 7-27 Identification of Oxidase-Positive, Nonmotile, Indole-Negative, Rod-Shaped Bacilli[a]

Organism	Characteristic, Odor, or Appearance	OF Mannitol	OF Dextrose	Esculin Hydrolysis	Gelatin Hydrolysis	Urease	Nitrate Reduction	DNase	Growth on MacConkey	Penicillin	Polymyxin
Sphingobacterium spiritivorum (IIk-3)	Pale yellow	+	+	+	–	+	–	+	–	R	R
Sphingobacterium multivorum (IIk-2)	Pale yellow	–	+	+	–	+	–	–	V (17)	R	R
Sphingobacterium thalpophilum	Pale yellow	–	+	+	V (86)	+	+	+	V (14)	R	R
Sphingobacterium mizutaii	Yellow	–	+	+	–	–	–	–	–	R	R
Sphingomonas paucimobilis (IIk-1)	Bright yellow, vancomycin susceptible	–	+	+	–	–	–	–	V (10)	S (33)	S (89)
Ralstonia pickettii (VA-1)	Motile (94) 10% lactose (81) ONPG (0)	–	+	–	V (77)	+ (100)	+ (87)	–	V (77)	R	R
Inquilinus limosus	Nonpigmented	–	– (Delayed)	+	–	V (33)	V (33)	NA	Slight growth 3 days	NA	R
Alishewanella fetalis	Halophilic Grows in 6.5% NaCl	–	–	+	+	–	+	NA	+	NA	NA
Myroides odoratus	Yellow-green fruity odor	–	–	–	+	+	–	+	V (78)	S (19)	R
Haematobacter spp.	PAD +	–	–	–	–	+	–	NA	V	NA	NA
Neisseria elongata subsp. *nitroreducens*	Catalase negative	–	–	–	–	–	+	–	V (20)	S	S
Gilardi rod group 1	PAD strong +	–	–	–	–	–	–	–	+	S	S
Neisseria weaveri	Gray-white colonies	–	–	–	–	–	–	–	V (42)	S	S

[a]Data obtained from references 212, 269, 367, 434, 754, and 1144.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; NA, results not available; numbers in parentheses are percentage of strains giving positive reaction; R, resistant; S, susceptible; PAD, phenylalanine deaminase. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

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TABLE 7-28 Identification of Oxidase-Positive, Nonmotile, Indole-Positive, Rod-Shaped Bacilli[a]

Organism	Characteristic, Color, or Appearance	Urease	Nitrate Reduction	Esculin Hydrolysis	Gelatin Hydrolysis	Starch Hydrolysis	DNase	Growth on MacConkey	OF Mannitol	OF Dextrose	Penicillin	Polymyxin
Bergeyella zoohelcum	No pigment	++	–	–	+	–	–	–	–	–	S	R
CDC Group IIc	No pigment	–	+	+	V	NA	NA	–	–	+	S	S (40)
Weeksella virosa	Colonies stick to agar	–	–	–	+	–	V (13)	–	–	–	S	S
Empedobacter brevis	Pale yellow	–	–	–	+	V (40)	+	+[b]	–	Delayed	R	R
CDC Group IIe	No pigment	–	–	–	–	+	–	–	–	Delayed	S (80)	S (40)
CDC Group IIg	No pigment	–	–	–	–	NA	–	+	–	–	S	S
CDC Group IIh	No pigment	–[b]	–	+	–[b]	+	V (78)	–[b]	–	Delayed	S	R
CDC Group IIi	NA	–	–	+	–	V (14)	–	–	–	Delayed	S (57)	R
Elizabethkingia meningoseptica	Pale yellow	–	–	+	+	– (8)[c]	+ (100)	V (26)	Delayed	Delayed	R	R
Chryseo-bacterium indologenes	Deep yellow	–	–	+	+	+ (100)	– (7)[c]	–	–	Delayed	R	R

[a]Data obtained from references 269, 367, 449, and 1172.
[b]Different results reported; reaction used here is that reported by Dees et al.[269]
[c]Number in parenthesis is percentage positive as reported by Gilardi GL.[367]
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; ++, strong positive reaction (4 hours); NA, results not available; numbers in parentheses are percentage of strains giving positive reaction; R, resistant; S, susceptible; PAD, phenylalanine deaminase. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392
Balneatrix alpaca is pale yellow and indole positive but is excluded from this table because it is motile (see Tables 7-34 and 7-36).

TABLE 7-29 Screening Tests for Species Identification of Oxidase-Positive, Motile Nonfermenters

						–	+	Fluorescence (Pseudo F agar under UV light)
					–	+		10% Lactose
				–	+			Acetamide
			–	+				Lysine (Moeller)
		–	+					Pigment on blood agar
	–	+						Gas from nitrate or nitrite
–	+							OF dextrose
See Table 7-37	See Table 7-36	See Table 7-35	See Table 7-34	See Table 7-33	See Table 7-32	See Table 7-31	See Table 7-30	

TABLE 7-30 Identification of Fluorescent Nonfermenters

	Acetamide	Growth at 42°C	Gelatin
Pseudomonas aeruginosa	+	+	+
P. fluorescens complex	–	–	+
P. putida complex	–	–	–

+, 90% or more of strains are positive; –, 90% or more of strains are negative.

TABLE 7-31 Identification of Strongly Lactose-Positive Nonfermenters[a] (Oxidase +, Motility +, Fluorescein −)

Organism	Arginine	Lysine	OF Mannitol	Urease	ONPG	Polymyxin B
Burkholderia pseudomallei[b]	+	−	+	V (43)	−	R
Burkholderia cepacia complex	−	+	+	V (45)	V (79)	R
Rhizobium radiobacter	−	−	+	+	+	S
Burkholderia multivorans	−	V (53)	+	+ (100)	+ (98)	R
Ralstonia mannitolilytica (Va-3)	−	−	+	+	−	R
Ralstonia pickettii (Va-1)	−	−	−	+	−	R
Sphingomonas paucimobilis	−	−	−	−	+	S (89)

[a]Data obtained from references 367 ,438, and 1172.
[b]Also consider *P. aeruginosa*, *P. fluorescens*, and *P. putida* that are pyocyanin and pyoverdin-negative.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; numbers in parentheses are percentage of strains giving positive reaction; R, resistant; S, susceptible. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392
Acinetobacter baumannii and *Sphingobacterium* species are strongly lactose-positive, but are excluded from this table because they are nonmotile (see Tables 7-24 and 7-27).

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TABLE 7-32 Identification of Acetamide-Positive Nonfermenters[a] (Oxidase +, Motility +, Fluorescein −, 10% Lactose −)

Organism	Arginine	OF Dextrose	OF Fructose	OF Mannitol	Urease	Nitrate to Gas	Nitrate Reduced	Nitrite to Gas	Malonate[b]	Orange Indole
Pseudomonas aeruginosa	+	+	V (89)	V (68)	V (66)	V (60)	V (74)	NA	NA	−
Burkholderia cepacia complex	−	+	+	+	V	−	V (37)	NA	NA	−
Achromobacter xylosoxidans	−	+	V (9)	−	−	V (69)	+	NA	NA	−
Delftia acidovorans	−	−	+	+ (92)	−	−	+	NA	NA	+
Oligella ureolytica	−	−	−	−	++	V (58)	+	V (63)	−	−
Achromobacter denitrificans	−	−	−	−	V (31)	+	+	+	+	−
Achromobacter piechaudii	−	−	−	−	−	−	+	−	+	−
Alcaligenes faecalis	−	−	−	−	−	−	−	+	+	−
Bordetella hinzii	−	−	−	−	−	−	−	−	+	−
Bordetella avium	−	−	−	−	−	−	−	−	−	−

[a]Data obtained from references 367, 438, and 1109.
[b]Data for alkalinization of malonate from reference 62.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; ++, strong positive reaction; NA, results not available; numbers in parentheses are percentage of strains giving positive reaction; R, resistant; S, susceptible. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

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TABLE 7-33 Identification of Lysine-Positive Nonfermenters

	Polymyxin B	DNase	OF Mannitol
Burkholderia cepacia complex[a]	R	–	+
Stenotrophomonas maltophilia[b]	S	+	–

[a]For further separation of the *B. cepacia* complex, see Table 7-9.
[b]Occasional strains of *S. maltophilia* may be oxidase positive.
+, 90% or more of strains are positive; –, 90% or more of strains are negative.

readings taken after 48 hours of incubation. Kiska et al.[575] tested 150 nonfermenters isolated from patients with CF and reported an overall correct identification rate of 57%, including 43% of the *B. cepacia* isolates. The overall performance of the API 20NE has shown it to be one of the better-performing commercial systems for the identification of nonfermenters.[37,605,709,1151,1158]

The Crystal Enteric/Nonfermenter System

The Crystal Enteric/NonFermenter ID kit was described in Chapter 6. The nonfermenter database includes 24 taxa of nonfermenters representing 10 different genera. Twenty additional taxa are included in a group called "Miscellaneous Gram-Negative Bacilli," that consists of a group of oxidase-positive species that are relatively inactive and indistinguishable from each other in the Crystal E/NF System. Included in this group are some medically relevant species of *Alcaligenes, Burkholderia, Comamonas, Moraxella, Ochrobactrum, Oligella,* and *Pseudomonas*. In a study by Wauters and coworkers,[1158] the overall correct identification of 201 nonfermenters (including 31 different species) was 75.9% with the Crystal E/NF compared with 75.3% for the API 20NE. Of note is that only 36 of 45 *P. aeruginosa* strains were correctly identified by the Crystal E/NF compared with 41 of 45 by the API 20NE. The overall percentage of incorrect identifications for nonfermenters was substantially higher for the API (13.8%) than for the Crystal (6.3%). The authors noted that an advantage of Crystal over the API system is that both fermenters and nonfermenters can be tested in the same panel. In addition, the API 20NE may require 48 hours of incubation, whereas the Crystal E/NF requires only 18 hours of incubation. Robinson and colleagues[912] studied 131 nonenteric bacilli on the Crystal E/NF including 11 species of nonfermenters; however, three species (*P. aeruginosa*, *A. baumannii*, *S. maltophilia*) accounted for 90% of the nonfermenters tested. The Crystal system correctly identified all of the *P. aeruginosa*, *A. baumannii,* and *S. maltophilia* species tested, but correctly identified only 8 of the 13 (61.5%) remaining nonfermenter species tested.

The RapID NF Plus System

The RapID NF Plus System (Remel) is a micromethod using conventional and chromogenic substrates for the identification of medically important glucose nonfermenting gram-negative bacteria and other selected glucose-fermenting gram-negative bacteria not belonging to the family *Enterobacteriaceae*. The tests used in the RapID NF Plus System are based on the microbial degradation of specific substrates detected by various indicator systems. The reactions used are a combination of conventional tests and single-substrate chromogenic tests. The system is described in Box 7-15. In the study by Kitch and colleagues,[576] 90.1% of all strains were identified correctly to species without additional tests. Kiska and associates[575] showed that the NF Plus correctly identified 80% of the NFBs recovered from patients with CF, including 81% of the *B. cepacia* isolates. This was the highest overall identification rate of the four commercial systems included in the study.[575] The RapID NF Plus system is currently the only nonautomated 4-hour test system available for the identification of nonfermenters.

The Biolog System

The Biolog System (Biolog, Hayward, CA) was described in Chapter 6. The newest version of the Biolog system available for manual testing is the GEN III MicroLog M System without computer (MLM). Biolog's latest generation redox chemistry enables testing and microbial identification of aerobic gram-negative and gram-positive bacteria in the same test panel. Gram stain and other pretests are no longer needed. The setup protocol is simple and can be performed in about 1 minute. The Biolog Microbial ID System can rapidly identify over 2,500 species of aerobic and anaerobic bacteria, yeasts, and fungi. Unfortunately, this product is not FDA approved for human in vitro diagnostic use, but can be a valuable resource for use in veterinary microbiology, research, or reference laboratory testing.[256,453,1214]

Methods for Identification Using Automated Identification Systems

The Vitek Legacy System

The Vitek Legacy System (bioMérieux), described in Chapter 6, has also been used with success in the identification of the nonfermenters most frequently encountered in the clinical laboratory. Pfaller and coworkers[844] tested 91 NFBs, with 90.1% identified correctly. Fifteen percent were identified at 4 hours, an additional 45% were identified 5 to 8 hours, and an additional 40% were identified at 9 to 18 hours. Colonna and associates[225] tested 142 NFBs and found 79.6% agreement with the API NFT. Kiska

and colleagues[575] evaluated four identification systems, including the Vitek

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TABLE 7-34 Identification of Pigmented Nonfermenters[a] (Oxidase +, Motility +, Fluorescein −, 10% Lactose −, Acetamide −, Lysine −)

Organism	Characteristic, Color, or Appearance	KIA/H_2S	OF Fructose	Arginine	Gas from Nitrate	Indole	OF Mannitol	OF Xylose	Esculin Hydrolysis	Growth in 6.5% NaCl
Roseomonas sp.[b]	Pink, mucoid	−	+	−	−	−	V	V	−	−
Methylobacterium sp.[b]	Coral, dry	−	V (50)	−	−	−	−	V	−	−
Shewanella alga	Tan	+	−	−	NA	−	−	−	−	+
Shewanella putrefaciens	Tan	+	V	−	−	−	−	−	−	−
Massilia timonae	DNase Pos, Pale yellow	−	-	−	−	−	−	+	+	−
Brevundimonas vesicularis	Tan/orange Vancomycin susceptible	−	-	−	−	−	−	V (11)	+	−
Pseudomonas mendocina (Vb-2)[c]	Pale yellow	−	+	+	+	−	−	+	−	+
CDC Vb-3[c]	Pale yellow	−	+	+	+	−	V (88)	+ (94)	−	+
Pseudomonas stutzeri (Vb-1)	Yellow, wrinkled	−	+	−	+	−	V (70)	+ (94)	−	+
Balneatrix alpica	Pale yellow	−	+	−	−	+	+	NA	−	NA
Burkholderia cepacia complex	Yellow	−	+	−	−	−	+	V	V (67)	−
Sphingomonas paucimobilis	Yellow	−	+	−	−	−	−	+	+	−
CDC group O-1[d]	Yellow, urea−	−	+	− (4)	−	−	−	−	+ (95)	−
CDC group O-2[d]	Yellow, urea V (12)	−	+	V (22)	−	−	−	−	V (64)	−

[a]Data obtained from references 255, 367, 400, 560, 645, 906, and 1172.
[b]*Methylobacterium* species appear as dark colonies under long-wave UV light due to absorption of UV light. *Roseomonas* species do not absorb UV light and do not appear dark.
[c]*P. mendocina* is starch hydrolysis negative, CDC Vb-3 is starch hydrolysis positive.
[d]CDC Group O-1 is OF glucose weak or delayed, OF maltose-negative; Group O-2 is OF maltose-, sucrose- and usually glucose-positive.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; numbers in parentheses are percentage of strains giving positive reaction; NA, results not available; KIA, Kligler's Iron Agar.
Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392
All of these pigments develop only as the culture ages. Most strains of *Chryseobacterium* sp. and *Sphingobacterium* sp. are also pigmented (yellow); however, these organisms are excluded from this table because they are nonmotile (see Tables 7-27 and 7-28).

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TABLE 7-35 Identification of Denitrifying Nonfermenters[a] (Oxidase +, Motility +, Fluorescein −, 10% Lactose −, Acetamide −, Lysine −, Pigment −)

Organism	OF Dextrose	Polymyxin B	ONPG	6.5% NaCl	Phenylalanine Deaminase	Arginine	OF Xylose	OF Maltose	Urease
Oligella ureolytica	–	S	–	–	+	–	–	–	++
Pseudomonas sp. CDC group 1[b]	–	S	–	–	–	V (50)	–	–	–
A. denitrificans[b]	–	S (83)	–	–	–	–	–	–	V (31)
Ochrobactrum intermedium	+	R	–	–	+	V (36)	+	V (50)	+
R. pickettii (Va-1)	+	R	–	–	–	–	+	+	+
R. pickettii (Va-2)	+	R	–	–	V (40)	–	+	–	+
Rhizobium radiobacter	+	S	+	–	+	–	+	+	+
P. stutzeri (Vb-1)	+	S	–	+	V (55)	–	+	+	V (17)
P. mendocina (Vb-2)	+	S	–	+	V (50)	+	+	–	V (50)
CDC Vb-3	+	S	–	+	V (56)	+	+	V (88)	V (31)
A. xylosoxidans	+	S	–	V[c]	–	–	+ (100)	–	–
Ochrobactrum anthropi	+	S	–	–	+	V (36)	+	V (50)	+ (100)
P. aeruginosa	+	S	–	–	–	+ (100)	V (85)	V (12)	V (66)
Acidovorax temperans	+	S	–	–	–	–	–	–	– (Weak)

[a]Data obtained from references 367, 1132, and 1172.
[b]*A. denitrificans* can be separated from *Pseudomonas* sp. CDC Group 1 on the basis of flagellar morphology. *Achromobacter* has peritrichous flagella, *Pseudomonas* group 1 has a single polar flagellum.
[c]Growth in 6.5% NaCl may be weak or delayed.
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; ++, strong positive reaction (4 hours) numbers in parentheses are percentage of strains giving positive reaction; R, resistant; S, susceptible. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

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TABLE 7-36 Identification of Dextrose-Positive Nonfermenters[a] (Oxidase +, Motility +, Fluorescein −, 10% Lactose −, Acetamide −, Lysine −, Pigment −, Denitrification −)

Organism	Characteristics, Color	KIA/H_2S	Indole	ONPG	OF Fructose	OF Xylose	Growth in 6.5% NaCl	Phenylalanine Deaminase	Esculin Hydrolysis	Arginine	Growth at 42°C	Gelatin Hydrolysis
Methylobacterium spp.	Coral, dry	–	–	–	V	V	–	–	–	–	–	–
Shewanella putrefaciens	–	+	–	–	V	–	–	–	–	–	V	V
Balneatrix alpica	Pale yellow	–	+	–	+	NA	–	NA	–	+	+	Weak +
Rhizobium radiobacter	–	–	–	+	+	+	–	+	+	–	V (13)	–
Pseudomonas-like group 2	–	–	–	+	+	+	–	+	–	–	–	–
A. xylosoxidans	–	–	–	–	–	+	–	–	–	–	V (86)	–
Brevundimonas diminuta	–	–	–	–	–	–	–	V (16)	–	–	V (19)	V (58)
Pandoraea sp.	Poly B resistant	–	–	–	–	–	–	NA	–	–	V (77)	–
Acidovorax temperans	–	–	–	–	+	–	–	–	–	–	V	–
P. pseudoalcaligenes	–	–	–	–	+	–	–	V (21)	–	V (36)	V (75)	–
CDC Vb-3	–	–	–	–	+	+	+	V (56)	–	+	V (75)	–
O. anthropi/ intermedium[b]	–	–	–	–	+	+	–	+	V (40)	V (36)	V (10)	–
CDC group O-3	–	–	–	NA	+	+	–	NA	+	–	V (40)	–
R. pickettii (VA-1)	–	–	–	–	+	+	–	–	–	–	V (26)	V (77)
P. aeruginosa	–	–	–	–	+	V (85)	–	–	–	+	+	V (46)
P. fluorescens complex	PYR V (23)[c]	–	–	–	+	+	–	–	–	+	–	+
P. putida complex	PYR –[c]	–	–	–	+	+	–	–	–	+	–	–
Acidovorax delafieldii	PYR +[c]	–	–	–	+	+	–	–	–	+	–	V (50)

[a]Data obtained from references 209, 248, 255, 367, 400, 560, 1132, 1172, and 1177.
[b]Colistin and polymyxin B resistance can be used to separate *O. anthropi* (susceptible) from *O. intermedium* (resistant).[1132]
[c]PYR data obtained from Schreckenberger (see Table 7-3).
+, 90% or more strains positive; –, 90% or more strains negative; V, 11%–89% of strains positive; PYR, pyrrolidonyl arylamidase. Numbers in parentheses are percentage of strains giving positive reaction. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

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TABLE 7-37 Identification of Dextrose-Negative Nonfermenters[a] (Oxidase +, Motility +, Fluorescein −, 10% Lactose −, Acetamide −, Lysine −, Pigment −, Denitrification −, Dextrose −)

Organism	Characteristics, Color, Appearance	KIA/H_2S	OF Fructose	Urease	Arginine	Poly B	NO_3 to NO_2	NO_2 to Gas	Phenylalanine Deaminase	DNase	Flagellar Arrangement
Methylobacterium[b]	Coral, dry	–	V	+	–	S (35)	V	NA	–	–	Polar monotrichous
Roseomonas[b]	Pink, mucoid	–	+	+	–	S (50)	–	NA	V (17)	–	Variable
Shewanella putrefaciens	No growth in 6.5% NaCl	+	V	V	–	S	+	–	–	+	Polar, 1–2
Shewanella alga	Growth in 6.5% NaCl	+	–	V	–	S	+	–	–	+	Polar, 1–2
P. pseudoalcaligenes	–	–	+	–	V (36)	S	+	NA	V (21)	–	Polar, 1–2
O. ureolytica	–	–	–	++	–	S	+ (100)	V (63)	+ (100)	–	Peritrichous
B. bronchiseptica	–	–	–	++	–	S	+ (100)	–	V (25)	–	Peritrichous
Cupriavidus pauculus	–	–	–	++	–	S	–	–	–	–	Peritrichous
Laribacter hongkongensis	Curved rods	–	–	–	+	S	+	–	NA	NA	Bipolar
Inquilinus limosus	Lipase Pos	–	NA	V (25)	–	R	–	–	NA	NA	Nonmotile or polar, 1–2
Pandoraea sp.	Lipase Neg	–	–	V (63)	–	R	V (11)	–	NA	–	Polar, 1–8
Brevundimonas diminuta	–	–	–	–	–	S (46)	–	NA	V (16)	V (12)	Polar monotrichous
B. hinzii	–	–	–	–	–	S	–	–	V (15)	–	Peritrichous
P. alcaligenes	–	–	–	V (21)	V (7)	S	V (61)	V (10)	V (20)	–	Polar monotrichous
C. testosteroni	–	–	–	–	–	S	+	V (11)	V (30)	–	Polar multitrichous
A. piechaudii	Acetamide V (42)	–	–	–	–	S	+	–	–	–	Peritrichous

[a]Data obtained from references 209, 212, 367, 400, 560, 906, 1109, 1172, and 1212.
[b]*Methylobacterium* species appear as dark colonies under long-wave UV light due to absorption of UV light. *Roseomonas* species do not absorb UV light and do not appear dark.
+, 90% or more strains positive; −, 90% or more strains negative; V, 11%–89% of strains positive; ++, strong positive reaction (4 hours); NA, results not available; numbers in parentheses are percentage of strains giving positive reaction. Instructions for interpreting table using shaded boxes are provided under heading "Steps to Follow" on p.392

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BOX 7-14

API 20E and API 20NE Systems for Identifying Nonfermenters

Functional Design

Each system consists of a plastic strip with 20 miniaturized cupules containing dehydrated substrates and a plastic incubation chamber with a loosely fitting lid (Image Plates 6-6A and 7-5D). Each cupule has a small hole at the top through which the bacterial suspension can be inoculated with a pipette. Bacterial action on the substrates produces turbidity or color changes that are interpreted visually.

Operating Procedure

For both systems: Add 5 mL of tap water to an incubation tray to provide a humid atmosphere during incubation. Place the strip into the incubation tray. Prepare a bacterial suspension of the test organism by suspending the cells from a well-isolated colony in 5 mL of sterile 0.85% saline. The turbidity must be equivalent to a 0.5 McFarland standard.

API 20E: With a Pasteur pipette, fill each cupule with the bacterial suspension through the

inoculating port. Overlay the three decarboxylase and the urease cupules with sterile mineral oil. The unit is incubated at 35°C for 24 or 48 hours before reading results according to the following rules: after 24 hours, if the glucose is positive add reagents, perform an oxidase test, and generate a 7-digit profile number to look up in the white section of the Profile Index; if glucose is negative, but three or more other reactions are positive before adding reagents, proceed as above; if glucose is negative and fewer than three other tests are positive, do not add reagents, reincubate strip for additional 24 hours and inoculate OF glucose, motility medium, and MacConkey agar. After 48 hours, add reagents, perform oxidase test, and generate a 9-digit profile number to look up in the blue section of the Profile Index.

API 20NE: With a Pasteur pipette, fill the tube portion of the first eight cupules (NO_3through PNPG) with the bacterial suspension. Inoculate an ampule of AUX medium with four drops of the same saline suspension. Mix well. With a new sterile pipette inoculate the assimilation tests GLU through PAC (cupules with colored lines) by filling the tube and cupule until a flat liquid surface without a meniscus is obtained. Add mineral oil to the GLU, ADH, and URE cupules. Incubate strip for 24 hours at 29°C–31°C. After 24 hours, add nitrate reagents to NO_3 cupule and TRP reagent to TRP cupule. Read and record reactions. Assimilation tests are recorded as positive if there is visible growth in the cupule portion of the tube. A 7-digit profile number is generated. If a good identification is not obtained or profile number is not found in codebook, the test strip may be incubated for an additional 24 hours. To do so, immediately cover the $NO_3$and TRP cupules with mineral oil. Record the NO_3, TRP and GLU test results after 24 hours; do not read these tests after 48 hours.

Substrates Included

API 20E:

ONPG

Arginine dihydrolase

Lysine decarboxylase

Ornithine decarboxylase

Citrate

Hydrogen sulfide

Urease

Tryptophan deaminase (add 10% $FeCl_3$)

Indole

Voges–Proskauer (add KOH and α-naphthol)

Gelatin

Glucose

Mannitol

Inositol

Sorbitol

Rhamnose

Sucrose

Melibiose

Amygdalin

Arabinose

Supplemental Tests:

Oxidase

NO_2

N_2 gas

Motility

MacConkey

OF glucose-oxidative

OF glucose-fermentative

API 20NE:

Biochemical Tests:

Nitrate reduction

Tryptophanase

Glucose fermentation

Arginine dihydrolase

Urease

Esculin hydrolysis

Gelatinase

β-Galactosidase

Assimilation Tests:

D-Glucose

L-Arabinose

D-Mannose

D-Mannitol

N-Acetyl-D-glucosamine

Maltose

D-Gluconate

Caprate

Adipate

L-Malate

Citrate

Phenylacetate

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BOX 7-15

RapID NF Plus System for Identifying Nonfermenters

Functional Design

The system consists of 10 reaction cavities molded into the periphery of a plastic disposable tray (Image Plate 7-5F). Reaction cavities contain dehydrated reactants, and the tray allows the simultaneous inoculation of each cavity with a predetermined amount of inoculum. When the test inoculum is added to the reaction cavity, the test substrate is rehydrated and the test reaction is initiated. After incubation for 4 hours, each test cavity is examined for reactivity by noting the development of a color. In some cases, reagents must be added to the test cavities to provide a color change. The resulting pattern of positive and negative test reactions is used as the basis for identification of the test isolate by comparison of test results to reactivity of known organisms stored in a computer-generated database.

Operating Procedure

Preparation of Inocula: Test organisms must be grown in pure culture and should be examined by Gram stain and oxidase before use in the RapID NF System. Test organisms may be removed from a variety of selective and nonselective agar growth media. Plates used for inocula preparation should preferably be 18–24 hours old. Using a cotton swab or inoculating loop, remove organisms from agar plate and suspend in RapID Inoculation Fluid to achieve a visual turbidity of at least a 1 but not in excess of a 3 McFarland Turbidity Standard. Suspensions should be mixed thoroughly and used within 15 minutes of preparation.

Inoculation of Panels: Peel back the panel lid over the inoculation port. With a Pasteur pipette, gently transfer the entire contents of the inoculation fluid into the upper right-hand corner of the panel. Reseal the inoculation port by pressing the peel tab back in place. After adding the test suspension, tilt the panel back away from the test cavities at approximately a 45° angle. While tilted back, gently rock the panel from side to side to evenly distribute the inoculum along the rear

baffles. While maintaining a level horizontal position, slowly tilt the panel forward toward the reaction cavities until the inoculum flows along the baffles into the reaction cavities. Incubate panels at 35°C–37°C in a non-CO_2 incubator for 4 hours.

Reading Panels: Place panel on benchtop and peel back the label lid over the reaction cavities. Without the addition of any reagents, read and score cavities 1 through 10, reading from left to right and record on report form. Record color of cavity 10 (GLU) in space provided on report pad. Then add two drops of NF Plus Reagent to cavities 4–8, two drops of Innova Spot Indole Reagent to cavity 9, and two drops of Innova Nitrate A reagent to cavity 10. Allow 30 seconds but no more than 3 minutes for color development. Record results in appropriate boxes on report form. Look up in Code Compendium or computer database.

Substrates Included

Arginine dihydrolase
Aliphatic thiol utilization
Triglyceride hydrolysis
Enzymatic hydrolysis of glycoside or phosphoester-linked nitrophenyl substrates releases yellow *o*- or *p*-nitrophenol:

p-Nitrophenyl-phosphoester

p-Nitrophenyl- *N*-acetyl-β, D-glucosaminide

p-Nitrophenyl-α, D-glucoside

p-Nitrophenyl-β, D-glucoside

o-Nitrophenyl-β, D-galactoside

Urea hydrolysis
Glucose utilization
Enzymatic hydrolysis of substrate linked β-naphthylamide substrates releases free β-naphthylamine which is detected with the RapID NF Plus Reagent

Proline β-naphthylamide

Pyrrolidine β-naphthylamide

γ-Glutamyl-β-naphthylamide

Tryptophan β-naphthylamide

N-Benzyl-arginine-β-naphthylamide

Tryptophan utilization with formation of indole
Sodium nitrate reduction
The above tests together with oxidase provide 18 test parameters.

GNI card, for identification of NFBs from patients with CF. A total of 150 isolates

were tested including 58 *B. cepacia*, 30 *S. maltophilia*, 24 *A. xylosoxidans*, 14 *P. aeruginosa*, and 24 other NFBs. The Vitek correctly identified only 50% of the *B. cepacia* isolates and 60% of the isolates overall. O'Hara and coworkers[797] tested 23 nonfermenters (8 *Acinetobacter*, 10 *P. aeruginosa*, 5 *S. maltophilia*) and reported 100% correct identification with the Vitek GNI card and version R07.1 software. Rhoads and associates[901] tested 80 *A. baumannii* and 39 *P. aeruginosa* and reported correct identification of 100% and 84.6%, respectively, with the Vitek GNI card and version AMS-R08.2 software. Sung and colleagues evaluated the GNI+ for identification of 301 isolates of NFBs representing 25 different species. Correct identification to the species level at initial testing was 71.8%, improving to 92.3% after additional testing was performed as recommended by the manufacturer's protocol.[1045]

The Vitek 2 System

The Vitek 2 System is described in detail in Chapter 6. The original Vitek 2 card for gram-negative bacteria identification has been redesigned to improve the identification of fermenting and nonfermenting bacilli. The new card contains 47 tests (26 that had been included in the previous card and 21 new tests), compared with 41 in the established Vitek 2 ID-GNB card. The database for the new card has been expanded to 159 taxa compared with only 101 for the original Vitek 2 card. In a study performed by Funke et al.,[344] 133 of 144 (92.4%) NFBs were correctly identified with the new Vitek 2 cards.

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The Microscan Walkaway-96, Walkaway-40, and Autoscan-4 Systems

These three systems (manufactured by Beckman Coulter, West Sacramento, CA), described in Chapter 6, all have an extensive database that includes many species of NFBs. Pfaller and colleagues,[844] using WalkAway-96 Rapid Gram Negative Panel, reported that 92.3% of the nonenteric bacilli were identified correctly with a likelihood of more than 85%. Tenover and colleagues[1064] evaluated the Walkaway-96 (formerly called the autoSCAN-W/A) for its ability to identify 310 well-characterized non–glucose-fermenting gram-negative bacilli. In their study, two types of identification panels were tested: the dried colorimetric Neg ID type 2 panel (DCP) and the rapid fluorometric Neg ID panel (RFP). Results with the DCP showed that 41.3% of 286 organisms were identified correctly, with a confidence of more than 85%, whereas 22.4% were misidentified with the same degree of confidence (major errors). Fifteen percent of the organisms were reported as unidentified. Problems in identifying

relatively common nonfermentative bacilli, such as *P. fluorescens*, *P. putida* and *S. maltophilia* were reported with the DCP panel. The researchers reported better results with the RFP panels, in which 77.1% of 239 isolates were correctly identified, whereas 25% were misidentified. The researchers further noted that the results with the RFP panels were available in 2 hours; thus, if an organism cannot be identified, additional biochemical tests can be inoculated on the same day, and little time is lost in identifying the organisms. Colonna and colleagues at UCLA tested 142 NFBs using the 2-hour rapid Neg ID panel and reported 74.6% agreement with the API NFT.[225] O'Hara and colleagues[797] at the CDC tested 23 strains of NFBs, including 8 *Acinetobacter*, 10 *P. aeruginosa*, and 5 *S. maltophilia*, and reported 100% accuracy with the Walkaway Neg combo 3 panel and version 17.02 software. Rhoads and coworkers[901] reported 97.5% and 82.1% correct identification of *A. baumannii* and *P. aeruginosa* isolates, respectively, using the Walkaway-96 system with urine combo 6 and negative combo 16 and version 20.20 software. Sung and colleagues compared the MicroScan Walkaway (W/A) using conventional (overnight) Neg Combo type 12 panels, with the Vitek GNI+ for identification of 301 isolates of NFBs representing 25 different species. The W/A correctly identified 71.4% of the isolates to the species level at initial testing improving to 96.0% after additional testing that was recommended by the manufacturer's protocol.[1045] Saiman et al.[941] evaluated the ability of the MicroScan Autoscan to identify *P. aeruginosa* isolates recovered from patients with CF. Using Negative Combo type 15 panels, read after 20 to 24 hours and again at 48 hours of incubation, they were able to correctly identify 57% (108 of 189) of nonmucoid strains and 40% (24 of 60) of mucoid strains. The most common misidentification was *Pseudomonas fluorescens/putida*.[941]

In 1997, MicroScan updated the Rapid Gram-Negative Identification panels from the Type 2 panel to the Type 3 panel in order to improve identification accuracy and to remove the need for mineral oil overlay for the decarboxylase tests, and to increase the shelf life from 1 year to 2 years. The identification database for the revised panel was updated to include 119 taxa. In a multicenter evaluation of the new type 3 panel 91.3% (63 of 69) NFBs were correctly identified to the species level.[72] In a study conducted by Schreckenberger et al.,[959] 92.2% (71 of 77) NFBs representing 10 species were correctly identified to the species level with the Rapid Gram Negative type 3 panels.

The Sensititre AP80 System

The Sensititre AP80 Identification panels (TREK Diagnostic Systems, Cleveland, OH) can be inoculated and incubated offline and then read in the Sensititre Autoreader, or can be inoculated and placed in the ARIS (Automated Reading and Incubation System) Instrument described in Chapter 6. The AP80 panel permits the identification

of gram-negative bacilli in as little as 5 hours, with the option of additional overnight incubation if needed or desired. Colonna and coworkers[225] tested 142 NFBs using the Sensititre AP80 panels and reported 71.1% agreement with the API NFT. Staneck and colleagues[1026] tested 144 nonenteric isolates including 135 nonfermenters representing eight species. Ninety-three percent of the isolates tested consisted of just three species (68 *P. aeruginosa*, 33 *Acinetobacter*, 25 *S. maltophilia*). Correct identification was obtained for 99.2% of these three species and for 95.1% of all nonenterics tested. The small number of nonfermenter species tested in this study makes it difficult to evaluate the performance of this system for routine clinical laboratory testing of the nonfermenters.

The Phoenix System

The Phoenix Automated Microbiology System (Becton Dickinson Microbiology Systems) is a fully automated, identification and antimicrobial susceptibility test system. Details of the system are presented in Chapter 6. To date, only a few studies have been published that evaluate the system for identification of NFBs. Stefaniuk et al.[1029] tested 54 NFBs (22 *P. aeruginosa*, 17 *Acinetobacter*, 15 *S. maltophilia*) and reported 96.3% (52 of 54) agreement with standard methods. Donay and colleagues[293] tested 56 NFBs representing 7 species and reported 89.3% correct identification with the Phoenix compared to a reference ID.

Methods for Identification Using Molecular Systems

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

MALDI-TOF MS was described in detail in Chapter 6. An overview of this new technology and modern applications in the clinical microbiology laboratory are described in a recent review.[828] Overall performance of MALDI-TOF MS has been reported to be significantly better than commercially available systems for identification of the NFBs although overall performance is still less than satisfactory.[14,270,502,960] Degand et al. evaluated the performance of the Bruker Autoflex spectrometer with flex control software (Bruker Daltonics, Bremen, Germany) using 512 clinical isolates of NFBs (predominantly *P. aeruginosa*) recovered from the

sputum of children with CF plus 47 strains obtained from the Obervatoire National des Cepacia (Toulouse, France) and found 98.2% correctly identified with MALDI-TOF MS versus 91.8% correctly identified by API 20E. The misidentifications by MALDI-TOF MS all occurred with members for the *B. cepacia* complex.[270] Almuzara et al.[14] evaluated the Bruker MicroFlex LT, RUO software version 3.1 and reported 64.65% correct ID to the species level and 28.28% to the genus level (92.95% combined) for 396 clinical isolates of NFB. Jacquier et al.[502] tested 101 NFB clinical isolates excluding *P. aeruginosa*, *A. baumannii* and *S. maltophilia* using the Bruker Autoflex with the flex control software, Vitek-2 and API 20 NE and reported correct ID to the species level of 80.8%, 70.7% and 57.4% respectively. Schreckenberger et al. performed a side-by-side comparison of the Bruker MALDI-TOF MicroFlex LT MS using Biotyper 3.0 software and the Vitek MALDI-TOF MS System using the Vitek MS RUO version 3.5 software and the Vitek MS IVD version 2.0 software. The population studied included 392 less common clinical isolates of NFBs, including 30 genera, 71 species, and 5 unnamed CDC groups of NFBs. Pyocyanin-producing strains of *P. aeruginosa* were excluded. Results obtained with the MALDI-TOF MS systems were compared to results obtained with a 42-test conventional identification procedure as described by Schreckeberger.[958] Discrepancies were refereed with 16S rRNA sequencing or whole genome sequencing (WGS) using Illumina's MiSeq technology. Correct identification to the species level for Bruker RUO, Vitek RUO, and Vitek IVD was 62.1%, 48%, and 54.3%, respectively. Correct identification when genus only IDs and genus and species IDs were combined for the three libraries evaluated were 89.7% for the Bruker RUO, 87.1% for the Vitek MS RUO, 83.3% for the Vitek MS IVD. Both systems gave a low number (<5%) of incorrect IDs; however, the ability to identify NFBs correctly to species level was low for both systems. Improvements are needed in the databases used for identification of NFBs with both systems for accurate identification of NFBs to the species level.[960]

16S rRNA Gene Sequencing

Due to the poor performance of commercially available systems for the identification of NFBs and sometimes less than satisfactory performance of MALDI-TOF MS to identify NFBs at the species level, laboratories have increasingly turned to sequencing methods such as 16S rRNA gene sequencing to determine the identification of clinically relevant isolates. 16S rRNA is a component of the 30S small subunit of prokaryotic ribosomes. The use of the 16S rRNA gene for phylogenetic studies was pioneered by Carl Woese because it is highly conserved between different species of bacteria and archaea and because of the slow rate of evolution of this region of the

bacterial gene.[1182,1183] In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid and inexpensive alternative to phenotypic methods of bacterial identification.[203]

Jacquier et al.[502] tested 188 NFBs collected from 9 French hospitals and were able to identify 92% to species level and 100% to genus level using 16S rRNA gene sequencing. Cloud et al. compared phenotypic identifications of 96 clinical NFB isolates with identifications obtained by partial 5' 16S rRNA gene sequencing using the MicroSeq 500 system (Life Technologies, Grand Island, NY). Sequencing identified 88 isolates (91.7%) with >99% similarity while 8 isolates (8.3%) could be identified confidently only to the genus level by sequencing. Phenotypic testing was performed by conventional phenotypic and commercial methods in use at each of the participating laboratories and included the Vitek or API 20NE systems (bioMèrieux, Durham, NC) or the MicroScan system (Beckman Coulter, Sacramento, CA). Using phenotypic testing, 50/96 (52.1%) NFB isolates were correctly identified to the species level and 11/96 (11.4%) were correctly identified to the genus level (combined 63.5%).[206] The largest number of discrepancies with phenotypic testing occurred with *P. fluorescens* (7/9) and *S. maltophilia* 11/19).[206] In a separate study conducted by Mignard and Flandrois, 683 clinical NFB isolates were analyzed by 16S rRNA sequencing. Species-level identification was provided for 568 (83.1%) isolates, genus-level identification only for 108 (15.8%) isolates and 7 (1%) isolates could not be identified by sequencing.[733] Bosshard et al. in a prospective study compared 16S rRNA sequencing with two commercially available identification systems (API 20 NE, Vitek-2 Fluorescent card; bioMèrieux, Marcy l'Etoile, France) using 107 NFB (non-*Pseudomonas aeruginosa*) strains recovered from blood cultures and from relevant clinical material where identification was required. Using 16S rRNA sequencing, 92% of the isolates were assigned to species level and 8% to genus level (100% combined). Using API 20 NE, 54% of the isolates were identified to the species level, and 7% to the genus level (61% combined), and 39% of the isolates could not be identified. For Vitek-2, 53% could be identified to the species level, 1% to the genus level (54% combined), and 46% could not be identified.[113]

Resolution of 16S rRNA Gene Sequencing. Although 16S rRNA gene sequencing is highly useful in regards to bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera.[113,503,733] With the NFB this is particularly true for members of the *Burkholderia cepacia* complex, the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex, and some members of the genus *Pseudomonas*, the genus *Achromobacter*, the genus *Bordetella,* and the

genus *Ralstonia*.[113,503]

As for any identification method, limitations for 16S rRNA gene sequencing exist and students and laboratorians should be aware of these pitfalls when using gene sequencing for bacterial identification in the diagnostic laboratory.[503,733]

Selection of a System

Clinical microbiologists must evaluate parameters such as accuracy, cost-effectiveness, and effects on workflow when deciding whether to use a commercially manufactured system for identifying nonfermenters. The commercial systems perform with levels of accuracy equal to or better than conventional methods in identifying *P. aeruginosa*, *Acinetobacter*

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species, and *S. maltophilia;* however, these metabolically active organisms can also be identified easily by using a few simple biochemical tests described earlier in this chapter. Many laboratories have adopted one of the commercial systems as a matter of convenience. However, because of the reported low sensitivity and specificity in the identification of many of the more fastidious and biochemically inactive nonfermenters, supplemental conventional differential media must still be retained. Therefore, the definitive identification of most nonfermenters still requires considerable technical experience and access to a variety of fresh culture media kept under strict quality control. Because relatively few nonfermenters, particularly strains of species other than the three mentioned in the foregoing, are encountered in most medium-sized or small laboratories, the services of a reference laboratory should be seriously considered. Identifying nonfermenters is not difficult if the microbiologist is willing to devote the time and dedication necessary to achieve an acceptable level of accuracy. Packaged systems can be recommended, provided one understands their shortcomings and is willing to set up supplemental tests to identify weakly reactive or fastidious strains.

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CHAPTER 8

Curved Gram-Negative Bacilli and Oxidase-Positive Fermenters

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PART I: CURVED RODS: *CAMPYLOBACTER, WOLINELLA, ARCOBACTER, HELICOBACTER*, AND RELATED BACTERIA

Historical Background

Classification of *Campylobacter* and Related Taxa

Genus *Campylobacter*

Campylobacter jejuni subsp. *jejuni*

Other *Campylobacter* Species

Genus *Arcobacter*

Arcobacter butzleri

Arcobacter cryaerophilus

Arcobacter skirrowii

Genus *Helicobacter*

Helicobacter pylori

Culture and Isolation of *H. pylori*

Identification of *H. pylori*
Other Medically Important *Helicobacter* Species
Other Microaerophilic Gram-Negative Bacilli
Sutterella wadsworthensis
Definitive Identification of Campylobacters and Related Bacteria
Rapid Identification of Campylobacters From Colonies Grown in Culture
Morphologic Identification
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
Commercial Kit Systems
Methods for Direct Detection of Campylobacters From Stool
Direct Immunofluorescence
Enzyme Immunoassay
PCR
PART II: THE FAMILIES *VIBRIONACEAE* AND *AEROMONADACEAE* AND THE GENERA *PLESIOMONAS* AND *CHROMOBACTERIUM*
Phylogeny of the *Vibrionaceae*
Genus *Vibrio*
Taxonomy
Description and Associated Clinical Syndromes of *Vibrio* Species of Human Importance
Methods for Laboratory Isolation of *Vibrios*
Biochemical Characterization and Laboratory Identification of *Vibrio* Species
Listonella, Photobacterium,* and *Shewanella
Aeromonas* and *Plesiomonas
Genus *Aeromonas*
Taxonomy
Clinical Significance
Aeromonas species in Medicinal Leeches
Laboratory Recovery of *Aeromonas* Species From Clinical Specimens
Laboratory Identification of *Aeromonas* Species
Genus *Plesiomonas*
Clinical Significance
Laboratory Isolation and Identification
Genus *Chromobacterium*

PART I: CURVED RODS: *CAMPYLOBACTER, WOLINELLA, ARCOBACTER, HELICOBACTER,* AND RELATED BACTERIA

Historical Background

The microorganism presently classified as *Campylobacter jejuni* was discovered in 1931 by Jones and coworkers[202] as the causative agent of winter dysentery in cattle. Twenty-six years lapsed before King described a group of microaerophilic, motile curved rods isolated from the blood of children with acute dysentery, which she designated "related vibrios" because they were similar in many respects to *Vibrio fetus.*[225] King astutely mentioned that the vibrios isolated from the blood of children might be closely related to the organism described as *V. jejuni* by Jones in 1931 and that the organism might be more important as a cause of childhood diarrheal syndromes of unknown etiology than previously realized.

This was a prophetic statement; nevertheless, another 15 years passed before this association was substantiated in the laboratory. In 1972, Dekeyser and colleagues[96] isolated the "related vibrios" from the feces of patients with acute enteritis using a filtration technique that allowed the small, curved rods to pass through the membrane but retained larger fecal microorganisms. Several other reports followed, linking related vibrios (*V. fetus,* subsp. *jejuni; C. jejuni*) with

gastroenteritis in humans, with a distribution throughout the world.[40,41,211] This relative incidence has since been the experience in most clinical laboratories, although during the past several years, the rates of recovery have declined to some degree.

The history of the discovery of *Helicobacter pylori* (formerly named *Campylobacter pyloridis* and then *C. pylori*) is even more circuitous. Warren and Marshall are credited with the "discovery" of the organism in Perth, Australia, in 1982[444]; however, many previous descriptions of spiral organisms in biopsy specimens of human gastric mucosa have appeared in the literature dating back to the beginning of the 20th century.[132,232] Only after successful cultivation of this bacterium using the unique "*Campylobacter* atmosphere" has serious attention been paid to this organism, which may be the most common cause of human gastrointestinal (GI) infection as well as the most frequent cause of gastritis.[337]

Classification of *Campylobacter* and Related Taxa

Classification of the microaerophilic gram-negative bacilli has changed considerably over the past few decades. Vandamme and associates,[430,433] using a variety of molecular techniques, including DNA–rRNA hybridization, 16S ribosomal RNA (rRNA) sequence analysis, and immunotyping analysis, determined that the *Campylobacter* species and related taxa belong to the same phylogenetic group, which they named rRNA superfamily VI. Five genera, including *Campylobacter, Arcobacter, Helicobacter, Wolinella,* and "*Flexispira*" (which subsequently has been placed in the genus *Helicobacter*), were included in rRNA superfamily VI. Characteristics that differentiate between these related genera are listed in Table 8-1. Solnick and colleagues described two uncultivable human gastric spiral organisms, "*Gastrospirillum hominis*" 1 and 2, which they identified as helicobacters by 16S rRNA analysis and provisionally named "*Helicobacter heilmannii*."[390] Following the successful isolation of "Candidatus *H. heilmannii*" in vitro in 2012, this organism was given full taxonomic recognition as *H. heilmannii*.[384]

Studies of Thompson and coworkers[421] showed that bacterial species included in rRNA superfamily VI could be separated into three distinct rRNA clusters. They reported that only those organisms comprising rRNA group I (*C. fetus, C. coli, C. jejuni, C. lari, C. hyointestinalis, C. concisus, C. mucosalis, C. sputorum*, and *C. upsaliensis*) were the true campylobacters. Paster and Dewhirst[329] found a close relationship between *Wolinella curva, W. recta, Bacteroides gracilis, B. ureolyticus*, and the true campylobacters that made up the rRNA homology group I and suggested that all members of the campylobacter cluster should be placed in the genus *Campylobacter.* Vandamme and colleagues[433] confirmed the findings of Thompson and coworkers[421] and Paster and Dewhirst[329] and proposed an amended description of the genus *Campylobacter* to include all organisms placed in homology group I and the transfer of *W. curva* and *W. recta* to the genus *Campylobacter* as *C. curvus* and *C. rectus,* respectively. Vandamme and coworkers[428] proposed the reclassification of *B. gracilis* as *Campylobacter gracilis.* However, although *B. ureolyticus* was considered a member of the family *Campylobacteraceae*, it remained as a species *incertae sedis* until finally being reclassified as *Campylobacter ureolyticus* in 2010.[429]

The rRNA cluster II contained a homogeneous group of organisms for which Vandamme and associates[433] proposed the genus designation of *Arcobacter.* At the time of this writing, the genus *Arcobacter* includes 18 species isolated from a wide range of farm animals, shellfish, mussels, water sources, and sewage.

rRNA cluster III contained members of three different genera, *Helicobacter, Wolinella,* and "*Flexispira*", and an unnamed species, CLO-3. Vandamme and

associates[433] emended the description of the genus *Helicobacter* and proposed the transfer of *Campylobacter cinaedi* and *C. fennelliae* to the genus *Helicobacter* as *H. cinaedi* and *H. fennelliae,*

TABLE 8-1 Characteristics for Differentiating *Arcobacter, Campylobacter, Wolinella, Helicobacter*, and "*Flexispira*"

Genus	Nitrate Reduction	Growth on 0.5% Glycine	Hydrolysis of Urea	Growth at			Cell Morphology	Flagellar Sheaths
				15°C	30°C	42°C		
Arcobacter	+	NA	V	+	+	–	Curved and spiral rods	Absent
Campylobacter	+	V	–	–	+	V	Curved and spiral rods	Absent
Wolinella	+	–	–	–	–	W	Spiral	Absent
Helicobacter	V	+	V	–	V	V	Curved and spiral rods	Present
"Flexispira"	–	+	+	–	–	+	Straight fusiform rods	Present

+, 90% or more of strains are positive; –, 90% or more of strains are negative; V, 11%–89% of strains are positive; W, weak reaction; NA, results not available.
Data modified from reference 433.

respectively. "*Flexispira*" is now included in the genus *Helicobacter. W. succinogenes* remains as the only species of the genus *Wolinella* and has not been associated with human infections.

Genus Campylobacter

Campylobacter species are microaerophilic (i.e., require decreased O_2) and capnophilic (i.e., require increased CO_2), curved spiral bacteria that are motile by means of a single unsheathed polar flagellum. These organisms are nonfermentative and nonoxidative in their metabolism, deriving energy from the use of amino acids and 4- and 6-carbon Krebs' cycle intermediates. These organisms were once classified with *Vibrio* species, until DNA homology studies showed that they were unrelated to the vibrios. Even among the currently recognized *Campylobacter* species, much genotypic and phenotypic diversity exists. The organisms inhabit a wide variety of ecologic niches and environments. Most species are found in animals (cattle, swine) and cause infertility and abortion.

Campylobacter jejuni subsp. *jejuni*

Clinical Significance. *C. jejuni* subsp. *jejuni* is the most important human pathogen among the campylobacters. It has worldwide distribution, and in industrialized countries, it is recovered from diarrheal stool samples two to seven times

more frequently than *Salmonella* or *Shigella*.[14] It is also ubiquitous in domestic animals—house pets may carry the organism, and the vast majority of chickens, turkeys, and waterfowl are colonized.[150] Raw milk,[75,439] partially cooked poultry,[150] and contaminated water[67,210] are the common sources for human infections.[134] Enteritis with this organism is characterized by crampy abdominal pain, bloody diarrhea, chills, and fever. For most persons, the infection is self-limited and resolves in 3 to 7 days. The organism may continue to be excreted by convalescing patients for 2 weeks to 1 month. The patient may be treated with oral erythromycin if the disease is severe.

Although enteritis and diarrheal syndromes remain the most common manifestations of *Campylobacter* infections, other diseases have emerged during the past few years. Patients with septic arthritis, meningitis, and proctocolitis secondary to *C. jejuni* have been reported.[347] *C. jejuni* has been described to cause bacteremia mostly in elderly or immunocompromised patients,[323] and a patient with prosthetic joint infection after a diarrheal illness has also been described.[438] There have now been several reports that associate *C. jejuni* infection with Guillain–Barré syndrome (GBS), an acute demyelinating disease of the peripheral nerves.[154,233,362,393] Data from both serologic and culture studies show that between 20% and 40% of patients with GBS are infected with *C. jejuni* 1 to 3 weeks prior to the onset of neurologic symptoms.[15] The risk of developing GBS after *C. jejuni* infection, however, is quite small (<1 case of GBS/1,000 *C. jejuni* infections).[14] There is no relation between the severity of GI symptoms and the likelihood of developing GBS after infection with *C. jejuni*; and in fact, even asymptomatic infections may trigger GBS.[15] In the United States and Japan, 30% to 80% of *C. jejuni* isolates from patients with GBS belong to Penner's serotype 0:19.[15,135] *C. jejuni* infection has also been associated with immunoproliferative small intestinal disease (also called alpha chain disease).[249] A review of the epidemiology, pathogenesis, and clinical features of *C. jejuni* infection has been written by Allos.[14]

Presumptive Identification From Stool. It may be possible to make a presumptive diagnosis of *Campylobacter* enteritis by observing characteristic gram-negative, curved, S-shaped or gull-winged, or long spiral forms in Gram-stained preparations of diarrheal stools (Image Plate 8-1A). One could consider examining wet mounts or stained smears of all diarrheal stool specimens for polymorphonuclear leukocytes and the presence of bacterial forms suggestive of *Campylobacter* species. Stool specimens for *Campylobacter* species are not further processed in some laboratories unless polymorphonuclear leukocytes are present. The rationale for this practice is that it is unlikely that *Campylobacter* species will be recovered in clinically significant numbers in stool specimens devoid of leukocytes. The expenditure of time

and use of special culture media for specimens in which there is little chance to recover significant microbes is not considered cost effective.

Methods for Laboratory Isolation. Successful isolation of *C. jejuni* from stool depends on the use of selective media (e.g., Campy-Thio, Campy-BAP), incubation at an elevated temperature (42°C), and the proper incubation atmosphere (5% oxygen, 10% CO_2, 85% nitrogen). A membrane filtration technique that is used with nonselective blood agar plates has been reported to be as effective as the use of selective media for the isolation of *C. jejuni*.[398] This method has the advantage of allowing the isolation of antibiotic-susceptible campylobacters. For the past several decades, the selective culture media and special incubation conditions necessary to recover *Campylobacter* species have been used in most clinical microbiology laboratories.

Various procedures can be used to provide a suitable gaseous atmosphere for cultivating microaerophilic campylobacters. These include evacuation–replacement procedures, disposable gas generators, and the use of the Fortner principle. Two of these procedures, which have been used successfully by various investigators, are outlined in Table 8-2. The use of a CO_2 incubator is not recommended for cultivating campylobacters because only strains that are very aerotolerant grow in the atmosphere provided. Likewise, a candle extinction jar is not recommended because the oxygen level (12% to 17%) is too high for optimal growth of campylobacters.[259,442]

Several selective media have been developed to allow for the isolation of *C. jejuni* from fecal samples. Merino and colleagues[281] evaluated the efficacy of seven selective *Campylobacter* isolation media. The names of these media, their composition, and a summary of the evaluation of each are included in Table 8-3. Butzler selective medium, Blaser medium (Campy-BAP), and Skirrow blood agar have been used in most clinical laboratories. However, Merino and colleagues[281] found that Preston *Campylobacter* blood-free medium with cefoperazone yielded the greatest number of *C. jejuni* isolations. Karmali and colleagues[212] found that a blood-free, charcoal-based selective medium (CSM) consisting of Columbia agar base, activated charcoal, hematin, sodium pyruvate, cefoperazone, vancomycin, and

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TABLE 8-2 Procedures Used by Various Investigators to Create a Microaerophilic Environment Suitable for Cultivating *Campylobacter* Species

Investigators	Procedure
Luechtefeld et	

al.[259] Evacuation-replacement	with a mixture of 10% CO_2 and 90% N_2. Six plates of media were incubated in one jar.
Hébert et al.[164] Evacuation-replacement	Evacuated 75% of air from a modified pressure cooker by twice evacuating the container to −15 in. (−38 cm) Hg and refilling with a mixture of 10% CO_2 and 90% N_2 to atmospheric pressure. Plates occupied no more than one-half the volume of the container.

TABLE 8-3 Formulas for Selective Media for Isolation of *Campylobacter jejuni*

Medium	Base	Additives
Butzler's selective medium	Fluid thioglycollate medium (Difco Laboratories, Detroit, MI)	Agar (3%) Sheep blood (10%) Bacitracin (25,000 IU/L) Novobiocin (5 mg/L) Colistin (10,000 IU/L) Cephalothin (15 mg/L) Actidione (50 mg/L)
Skirrow's blood agar	Blood agar base No. 2 (Oxoid)	Lysed horse blood (7%) Vancomycin (10 mg/L) Polymyxin B (2,500 IU/L) Trimethoprim (5 mg/L)
Blaser's medium (Campy-BAP)	Brucella agar base (Becton Dickinson, Microbiology Systems, Cockeysville, MD)	Sheep blood (10%) Vancomycin (10 mg/L) Trimethoprim (5 mg/L) Polymyxin B (2,500 IU/L) Cephalothin (15 mg/L) Amphotericin B (2 mg/L)
		5% Saponin-lysed horse

Preston *Campylobacter*-selective medium	Nutrient broth No. 2 (Oxoid CM67) 1.2% New Zealand agar	blood Trimethoprim (10 µg/mL) Polymyxin B (5 IU/mL) Rifampin (10 µg/mL) Cycloheximide (100 µg/mL)
Preston *Campylobacter* blood-free medium	Nutrient broth No. 2 (Oxoid CM67) 1.2% New Zealand agar	Bacteriologic charcoal Sodium deoxycholate Ferrous sulfate Sodium pyruvate Casein hydrolysate Cefoperazone (32 mg/L)
Butzler's virion medium	Columbia agar base (Oxoid CM331)	Defibrinate sheep blood Cefoperazone (15 mg/L) Rifampin (10 mg/L) Colistin (10,000 U/L) Amphotericin B (2 mg/L)
Modified Preston medium	Nutrient broth No. 2 (Oxoid)	7% Defibrinated horse blood Cefoperazone (32 mg/L) Amphotericin B (2 mg/L) Campylobacter growth supplement (Oxoid)
Charcoal-based blood-free selective medium	Columbia agar base (GIBCO)	Activated charcoal (Oxoid) Hematin (0.032 g/L) Sodium pyruvate (0.1 g/L) Vancomycin (20 mg/L)

	Cefoperazone (32 mg/L) Cycloheximide (100 mg/L)

Data from references 212 and 281.

cycloheximide is more selective than Skirrow's medium and has a higher isolation rate of *C. jejuni* from mixed cultures. Charcoal, hematin, ferrous sulfate, and sodium pyruvate serve as substitutes for blood in growth media for campylobacters. Casein is added to help grow certain strains of nalidixic acid–resistant thermophilic campylobacters that are environmental organisms.

Endtz and colleagues[108] compared a semisolid blood-free selective motility medium[146] with two blood-free CSM, two blood-based media (Skirrow's medium and Blaser's Campy-BAP), and the membrane filter technique. They found that CSM was the single best medium; however, the highest isolation rates were observed when CSM was used in combination with any other media or the filter technique. Endtz and colleagues also reported that extending the incubation time from 48 to 72 hours led to an increase in the isolation rate regardless of the medium used.[108]

Rectal swabs or swab samples of the stool specimen can be inoculated directly to a small area on the surface of one of the recommended selective agar media. Formed stool specimens may also be processed by emulsifying a small portion (peanut sized) in phosphate-buffered saline or broth before inoculating one or two drops to the surface of the agar with a Pasteur pipette; similarly, one or two drops of liquid stool specimens can be inoculated directly.

An outline of a procedure that will allow isolation of enteric campylobacters from fecal samples is shown in Box 8-1. This technique is consistent with current information from the literature about requirements for cultivation of these bacteria and is suitable for use in most clinical laboratories.

An alternative membrane filter technique, as described by Steele and McDermott,[398] may be used in combination with a Campy-selective medium with equivalent results (Image Plate 8-1B and Box 8-2).

Routine use of enrichment selective "Campy broth" is generally not recommended. Enrichment broths may be beneficial if stool specimens are delayed in transit or left at room temperature too long. Each laboratory director must decide whether an enrichment broth will be beneficial based on local disease patterns and how well the collection and transport of quality specimens can be monitored. Since campylobacters

enrichment broth will be beneficial based on local disease patterns and how well the collection and transport of quality specimens can be monitored. Since campylobacters are microaerophilic, they tend to grow best near

BOX 8-1

Procedure for Isolating *C. jejuni* and Other Enteric *Campylobacter* Species From Fecal Specimens

1. Using a fecal sample or a swab sample in Cary–Blair medium, prepare a turbid suspension of the feces in 10 mL of brain–heart infusion broth. Immediately inoculate one or two plates (two plates are preferable) of a *Campylobacter*-selective medium (best results are obtained with CSM as noted in text); streak to obtain isolated colonies; and hold in a nitrogen-holding jar (see Chapter 16) until the remaining media are inoculated.
2. Lightly centrifuge the specimen (at approximately 1,000 *g*) for 5 minutes.
3. Remove about 5 mL of the supernatant with a syringe and filter through a sterile 0.65-µL Millipore filter, as described by Butzler.[58] Discard the first 3 mL of fluid and use 1 or 2 drops of the remainder to inoculate two plates of chocolate agar without selective agents or a blood agar medium such as the Centers for Disease Control (CDC) anaerobe blood agar that will support the growth of *Campylobacter*. Streak for isolation.
4. Incubate one set of Campy-selective agar and chocolate agar plates at 42°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 and the remaining plates at 35°C–37°C in the same gaseous atmosphere.
5. Inspect the plates after 24, 48, and 72 hours of incubation for colonies characteristic of *Campylobacter* species and identify the isolates with the techniques described in the text. Plates not showing growth after 24 or 48 hours of incubation should be returned for an additional 24–48 hours in the same incubator and gaseous atmospheric conditions, as described above.

BOX 8-2

Steele and McDermott Membrane Filter Technique

1. Mix 1 g stool in 10 mL of sterile saline containing glass beads. Vortex for 30 seconds.
2. Place a 47-mm, 0.45 Gelman cellulose triacetate membrane filter (Gelman No. 63069) centrally onto the surface of a nonselective *Brucella* agar plate

pipette. Take care to ensure that the drops do not extend to the edge of the filter.
4. Remove filter and discard 30 minutes after the suspension is applied.
5. Incubate plate in environment suitable for the cultivation of *Campylobacter* species, as described previously.

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the top of the tube. If a *Campylobacter* broth is used, the following procedure for subculture should be followed:

Use a Falcon brand polyethylene plastic pipette that can be inverted. Place the tip of the pipette 1 inch below the surface of the medium and continuously withdraw sample as you remove the pipette. Invert the pipette to facilitate mixing of the sample, place three drops on a Campy-BAP plate, and streak for isolation. Incubate as you would a primary culture plate.

Identification From Culture. The appearance of colonies on one of the selective *Campylobacter* agars that has been incubated at 42°C in the microaerophilic environment, described previously, is presumptive evidence that the organism is one of the thermophilic *Campylobacter* species (most commonly *C. jejuni*). The morphology of *Campylobacter* species on selective agar varies from flat, gray, irregular-shaped colonies that may be either dry or moist to colonies that are round and convex and glistening with entire edges (Image Plate 8-1C, D). There is a tendency for colonies to form confluent growth along the streak lines on the agar surface. Hemolytic reactions are not observed on blood agar. The identification can be further confirmed by performing rapid catalase and cytochrome oxidase tests (*C. jejuni, C. coli,* and *C. lari* are positive for both these tests.). On occasion, thermophilic bacterial species other than *Campylobacter* species, notably *Pseudomonas aeruginosa,* may break through and grow on the selective media. It is unlikely, however, that *P. aeruginosa* would be confused with *C. jejuni*. The colony morphology of the two organisms is different, and if there were any question, a Gram stain would quickly differentiate *Campylobacter* species from *P. aeruginosa.*

Gram-stained preparations from colonies of *C. jejuni* after 24 to 48 hours incubation on blood agar show characteristic gram-negative, curved, "S"-shaped, gull-winged, or long spiral forms (Image Plate 8-1A). Coccoid forms are more commonly seen in older cultures of *C. jejuni,* particularly after colonies have been exposed to ambient air. Strict adherence to usual Gram stain timing is important because *Campylobacter* species are typically faintly staining. For this reason, one could consider extending the staining time of the safranin counterstain to at least 10 minutes to allow for greater staining intensity.

Once isolated, both subspecies of *C. jejuni* can be easily identified since they are

the only campylobacters that hydrolyze hippurate (Image Plate 8-1E and Table 8-4). In addition, this species is resistant to cephalothin and usually is susceptible to nalidixic acid, although resistant isolates are occasionally encountered (Image Plate 8-1F).

Other *Campylobacter* Species. At the time of this writing, there were 33 species and subspecies of *Campylobacter* described (http://www.bacterio.net/campylobacter.html). Only those species associated with human infections are covered further in this chapter.

Campylobacter coli. *C. coli* is closely related to *C. jejuni* and is also an important foodborne pathogen in humans.[409] It shares several cultural characteristics with *C. jejuni,* including susceptibility to nalidixic acid and resistance to cephalothin. *C. coli* can be differentiated from *C. jejuni* by the hippurate hydrolysis test (*C. jejuni* hydrolyzes hippurate; *C. coli* does not). The report when this organism is recovered in laboratories in which the hippurate test is not performed should read "*C. jejuni/C. coli*." It is estimated that *C. coli* accounts for 5% to 10% of cases of *Campylobacter* enteritis in humans.[299] A patient with a urinary tract infection caused by quinolone-resistant *C. coli* has also been reported.[328]

Campylobacter concisus. *C. concisus* is capable of anaerobic growth and requires hydrogen or formate for growth. The organism is isolated most commonly from human gingival crevices. However, it may also cause GI disease, especially in immunocompromised patients.[1,248,431] *C. concisus* has been suggested as a possible etiologic agent of Crohn's disease.[152,188,243,265,461,462,463] In a study by Zhang et al.,[463] *C. concisus* was more frequently present and higher levels of antibodies to *C. concisus* were detected in children with Crohn's disease than in controls. Recovery of *C. concisus* from stool specimens requires use of the filter method (discussed elsewhere in this chapter).

Campylobacter curvus. *C. curvus* was originally named *Wolinella curva.*[413,433] Cells stain gram negative and are short and slightly curved. Helical or straight cells may also occur. Strains exhibit rapid, darting motility and are asaccharolytic. The organism grows anaerobically and in 5% O_2 atmospheres containing H_2. No growth occurs in air enriched with 10% CO_2. All cultures require formate and fumarate for broth growth. Some strains exhibit a corroding morphology on agar media. Isolates have been recovered exclusively from human sources and include dental root canal, alveolar abscess, liver abscess, bronchial abscess, and blood.[156,413] *C. curvus* and *C. curvus*-like strains have been associated with sporadic episodes of bloody gastroenteritis.[7] Characteristics useful in distinguishing *C. curvus* from similar species are given in Table 8-5.

Campylobacter fetus* subsp. *fetus. *C. fetus* subsp. *fetus* is primarily associated with infective abortion in cattle and sheep and is an infrequent cause of human infections.

Infections usually result in systemic illness and usually affect debilitated persons with chronic hepatic, renal, or neoplastic disease, or individuals with compromised immune function.[68,336] *C. fetus* subsp. *fetus* has been reported to cause proctitis and proctocolitis in homosexual men[99]; premature labor and neonatal sepsis in humans[69]; septic abortion[402]; neonatal meningitis[241]; prosthetic hip joint infection[28,459]; and both native and prosthetic valve endocarditis.[113] *C. fetus* is the most commonly identified *Campylobacter* species to cause bacteremia.[323] In one study, patients with bacteremia due to *C. fetus* were older, were more likely to be male, and were more likely to have both implanted medical devices and no identified cause of immunosuppression compared to patients with bacteremia due to other *Campylobacter* species.[323] *C. fetus* is not believed to cause gastroenteritis, but, because of its susceptibility to cephalothin and its failure to grow at 42°C, it may not be recovered in clinical laboratories where selective media and increased incubation temperatures are used as a screen for *C. jejuni;* therefore, its etiologic role in this infection is not known.[163]

Campylobacter fetus* subsp. *venerealis. *C. fetus* subsp. *venerealis* comprises part of the normal genital tract flora of bulls, but has not been associated with human infection.[385]

Campylobacter gracilis. The name *Bacteroides gracilis* was proposed by Tanner et al.[412] for a group of agar-corroding bacteria that were originally considered to be anaerobic. In 1995, Vandamme and colleagues proposed the transfer of

TABLE 8-4 Differential Characteristics of Campylobacters and Related Taxa of Medical Importance

Organism	Catalase	Nitrate	Hydrogen Sulfide Triple Sugar Iron[b]	Urease	Indoxyl Acetate	Hippurate	Growth							Susceptibility[a]	
							25°C	37°C	42°C	MacConkey	0.1% TMAO	1.5% NaCl	1% Glycine	Nalidixic acid	Cephalothin
RNA Group I															
Campylobacter coli	+	+	–	–	+	–	–	+	+	+	–	–	+	V	R
C. concisus	–	+	+	–	–	–	–	+	C	+	–	+	C	R	R
C. curvus	–	+	+	–	V	V	–	+	V	V	NA	NA	+	C	S
C. fetus subsp. *fetus*	+	+	–	–	–	–	+	+	–	+	–	V	+	V	S
C. fetus subsp. *veneralis*	V	+	–	–	–	–	+	+	–	+	–	V	–	V	S
C. gracilis	–	+	–	–	V	–	–	+	V	V	NA	NA	+	V	S
C. helveticus	–	+	–	–	+	–	–	+	+	–	–	NA	V	S	S
C. hyointestinalis subsp. *hyointestinalis*	+	+	+	–	–	–	V	+	+	+	+	–	+	R	S
C. hyointestinalis subsp. *lawsonii*	+	+	+	–	–	–	–	+	+	V	NA	NA	V	R	S
C. jejuni subsp. *jejuni*	+	+	–	–	+	+	–	+	+	–	–	–	+	V	R
C. jejuni subsp. *doylei*	V	–	–	–	+	+	–	+	W	–	–	–	+	S	S
C. lari	+	+	–	–	–	–	–	+	+	+	+	+	+	R	R
C. mucosalis	–	+	+	–	–	–	C	+	+	+	C	C	C	C	S
C. rectus	–	+	+	–	+	–	–	+	W	–	NA	NA	+	V	S
C. showae	+	+	+	–	V	–	–	+	V	+	NA	NA	V	S	S
C. sputorum biovar *bubulus*	–	+	+	–	–	–	–	+	C	–	+	+	+	R	S
C. sputorum biovar *fecalis*	+	+	+	–	–	–	–	+	+	+	+	+	+	R	S
C. sputorum biovar *sputorum*	–	+	+	–	–	–	–	+	+	+	C	+	+	V	S
C. upsaliensis	– (w)	+	–	–	+	–	–	+	+	–	–	–	C	S	S
RNA Group II															
Arcobacter butzleri	– (w)	+	–	–	+	–	+	+	V	+	NA	V	+	V	R

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A. cryaerophilus	+	+	–	–	+	–	+	+	–	–	–	+	–	V	R
A. nitrofigilis	+	+	+	V	–	–	+	+	–	–	NA	+	–	S	S
A. skirrowii	+	+	–	–	+	–	+	+	V	–	NA	–	V	S	V
RNA Group III															
Helicobacter cinaedi (CLO-1)	+	+	–	–	C	–	–	+	–	–	–	–	+	S	S
H. fennelliae (CLO-2)	+	–	–	–	+	–	–	+	–	–	–	–	+	S	S
CLO-3	+	–	–	–	+	–	–	+	+	NA	NA	NA	+	S	R
H. pullorum	+	+	–	–	–	–	–	+	+	NA	–	NA	NA	S	R
H. pylori	+	–	–	++	–	–	–	+	C	–	–	NA	V	R	S
Helicobacter sp. strain *flexispira*	C	–	–	++	–	–	–	+	+	NA	NA	NA	– (w)	R	R

[a]Susceptibility to antibiotics determined with 30-µg disks.
[b]See Image Plate 8-1G.
+, 90% or more of strains are positive; –, 90% or more of strains are negative; V, 11%–89% of strains are positive; ++, strong positive reaction; W, weak reaction; NA, results not available; C, contradictory reports in literature; R, resistant; S, susceptible; TMAO, trimethylamine oxide; shaded areas indicate key reactions.
Data from references 11, 22, 26, 51, 110, 116, 144, 174, 221, 319, 336, 343, 370, 395, 396, 399, 422, 430, 433, and 435.

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TABLE 8-5 Characteristics Useful for Differentiating *Campylobacter curvus, C. rectus, C. gracilis*, and *C. ureolyticus*

Characteristics	*C. curvus*	*C. rectus*	*C. gracilis*	*C. ureolyticus*
Source	Human clinical	Human clinical	Human clinical	Human clinical
Morphology				
Helical or curved cells dominate	+	−	−	−
Straight cells dominate	−	+	+	+
Cells with tapered ends	+	−	−	−
Motility	+	+	−	−
Urease	−	−	−	+
Growth in 1% glycine	+	+	NA	NA
Indoxyl acetate hydrolysis	+	+	V	NA

+, 90% or more of strains are positive; −, 90% or more of strains are negative; V, 11%–89% of strains are positive; NA, results not available.
Modified from reference 413.

this organism to the genus *Campylobacter* as *C. gracilis.*[428] These bacteria are found in the gingival crevices of humans and have been isolated primarily from sites of deep tissue infection.[200,251,381,414] Johnson and colleagues have reported that 83% of the specimens in which *C. gracilis* was isolated were obtained from patients with serious visceral or head and neck infections.[200] An 80-year-old man with a history of hypertension, hypertensive nephropathy, and chronic obstructive pulmonary disease (COPD) was reported to have developed fatal bacteremia caused by *C. gracilis.*[380] *C. gracilis* is microaerophilic and asaccharolytic and resembles campylobacters in almost all phenotypic characteristics. Individual cells stain gram negative and are small and unbranched, often having both tapered and rounded ends. Growth is stimulated in broth cultures by formate and fumarate. They may be differentiated from other campylobacters by the absence of flagella and oxidase activity.[412] *C. gracilis* isolates appear less susceptible to antimicrobial agents than the closely related species (e.g., *C. ureolyticus*), with only 67% of the isolates reported to be susceptible to penicillin.[200] A selective medium for isolation of *C. gracilis* has been described that contains tryptic soy agar base, formate, fumarate, nitrate and two selective agents, nalidixic acid, and teicoplanin.[252]

Campylobacter helveticus. *C. helveticus* is a thermophilic catalase-negative *Campylobacter* that has been isolated from the feces of domestic cats and dogs.[395] Of note is the fact that almost half of the *Campylobacter* isolates found in cats are *C. helveticus.*[55] Colonies of *C. helveticus* are adherent on blood agar and can be separated from other thermophilic species (*C. jejuni, C. coli,* and *C. lari*) by virtue of a negative catalase reaction. It is indoxyl acetate positive and susceptible to both nalidixic acid and cephalothin.

Campylobacter hyointestinalis. *C. hyointestinalis,* closely related to *C. fetus* subsp. *fetus,* was initially found only in animals, principally as a cause of ileitis in

swine,[137] but more recently has been reported from human clinical specimens. In one report,*C. hyointestinalis* was isolated from stool specimens of four persons, all of whom were experiencing nonbloody, watery diarrhea. The youngest (8 months) and the oldest (79 years) persons were female, and the other two were homosexual men.[107] A 52-year-old woman with chronic myeloid leukemia and nonbloody, watery diarrhea associated with this organism has been reported from France,[287] and an isolate from the rectal culture of a homosexual man with proctitis has been reported in the United States.[115] *C. hyointestinalis* will not be recovered on many *Campylobacter* media formulations because it is susceptible to cephalosporins, such as cephalothin and cefoperazone. Although it will grow at 42°C, growth is more luxuriant at 35°C.[115] The organism is also resistant to nalidixic acid, is hippurate negative, and produces hydrogen sulfide in triple sugar iron agar. The production of hydrogen sulfide in triple sugar iron agar is dependent on the test being incubated in a microaerophilic environment containing hydrogen.[137]

A group of "*C. hyointestinalis*-like" organisms obtained from porcine stomachs have been described. These isolates are sufficiently different from *C. hyointestinalis* to warrant creation of a separate subspecies classification, *C. hyointestinalis* subsp.*lawsonii*.[319] The creation of this new subspecies necessitates that the description of *C. hyointestinalis* be emended to *C. hyointestinalis* subsp. *hyointestinalis*. *C. hyointestinalis* subsp. *lawsonii* can be separated from subsp. *hyointestinalis* by its failure to grow in 1.5% bile. *C. hyointestinalis* subsp. *lawsonii* has been isolated from the intestines and stomachs of pigs; hamster intestines; and the feces of cattle, deer, and humans; but its pathogenicity is not known.[319]

Campylobacter jejuni* subsp. *doylei. A subspecies of *C. jejuni* has been isolated from human clinical specimens including gastric epithelium biopsies[213] and feces from children with diarrhea.[399] The pathogenicity of the organism remains unknown. *C. jejuni* subsp. *doylei* can be distinguished readily from other campylobacters because it does not reduce nitrates and hydrolyzes hippurate.[399] It is susceptible to cephalothin and therefore will not be recovered on media containing cephalosporin-type antibiotics.

Campylobacter lari. Formerly named *C. laridis,* the organism now known as *C. lari* is thermophilic, halotolerant, and nalidixic acid resistant; otherwise, it shares several

features with *C. jejuni* and *C. coli*.[383] Anaerobic growth in the presence of 0.1% trimethylamine oxide (TMAO) and failure to hydrolyze indoxyl acetate help to identify this species (reagents available from Sigma Chemical Co., St. Louis, MO). *C. lari* is

now considered to be part of a group of closely related *Campylobacter* species referred to as the *C. lari* group and is composed of five species (*C. lari, C. insulaenigrae, C. volucris, C. subantarcticus,* and *C. peloridis*), as well as a group of strains termed the *urease-positive thermophilic Campylobacter* (UPTC) and other *C. lari*-like strains.[283] Many laboratories rely on resistance to nalidixic acid to separate *C. lari* from *C. jejuni* and *C. coli;* however, *C. jejuni* resistant to nalidixic acid is being seen with increased frequency. *C. lari* is endemic in sea gulls but is an infrequent human pathogen. However, occasionally it causes enteritis simulating *C. jejuni* infections and, rarely, bacteremia, especially in immunocompromised persons.[32,79,269,300,415]

Campylobacter mucosalis. *C. mucosalis,* formerly classified as *C. sputorum* subsp. *mucosalis,* produces a yellow pigment and is catalase negative. Phenotypically, this species is very similar to *C. sputorum* bv. *sputorum* and *bubulus,* but is able to grow at 25°C. Unlike the majority of campylobacters, this species requires hydrogen and formate as an electron donor for growth, an essential requirement of *C. concisus, C. mucosalis, C. curvus,* and *C. rectus.*[430] Figura and colleagues[118] reported what was thought to be the first isolation of *C. mucosalis* from children with enteritis. However, this finding has been disputed, and the isolates have been shown by molecular probe studies to be *C. concisus*.[244,246,316] These two species are difficult to separate on the basis of biochemical tests alone, and it has been suggested that molecular methods must be used for the precise identification of these two species.[246] On[316] has suggested the use of several media containing various inhibitory agents for separating *C. concisus* and *C. mucosalis.* There are as yet no confirmed reports associating *C. mucosalis* with human infection.

Campylobacter rectus. *C. rectus* was originally named *Wolinella recta.*[412,433] Microscopically, the cells appear small and straight with rounded ends and stain gram negative. Strains exhibit rapid, darting motility and are asaccharolytic. Growth is anaerobic; however, some strains can grow in a 5% O_2 atmosphere but not in air enriched with 10% CO_2. Growth in broth is stimulated by formate and fumarate. Nitrates and nitrites are reduced, and both oxidase and catalase are negative. Additional identifying characteristics are given in Table 8-5. *C. rectus* is found in the gingival crevices of humans[412] and is reported to cause primary endodontic infections.[381] Spiegel and Telford[394] reported isolating this organism along with *Actinomyces viscosus* from an actinomycotic chest wall mass. *C. rectus* has also been reported as a cause of breast abscess,[156] abscess of the palate in a patient with gastroesophageal adenocarcinoma,[263] and extra-oro-intestinal abscesses.[100]

Campylobacter showae. *C. showae* is a recently described species isolated from human gingival crevices.[110] The organism appears as a straight rod with round ends

and contains two to five unsheathed unipolar flagella—a feature that is unique among campylobacters. The organism grows in a microaerophilic atmosphere in the presence of fumarate with formate or H_2, but prefers to grow under anaerobic conditions. Because of the limited number of reliable biochemical traits that can be used to differentiate closely related *Campylobacter* species, serologic or molecular tests or protein profiles may be required to positively identify isolates of this species.[110] An association with human disease has not been shown.

Campylobacter sputorum. *C. sputorum* is capable of anaerobic growth and can be recovered from the oral cavity and gingival crevices of humans. This organism is not recognized as an agent of human disease, although a few clinical isolates have been reported. Three biovars (bv) are described: *C. sputorum* bv. *bubulus, C. sputorum* bv. *sputorum,* and *C. sputorum* bv. *fecalis.*[361]

Campylobacter upsaliensis. *C. upsaliensis* is catalase negative or only weakly positive and thus has been referred to as the CNW strain of *Campylobacter.* However, because weak catalase reactions may also occur for *C. jejuni* subsp. *doylei,* the CNW designation no longer holds. Except for the lack of or weak production of catalase, this organism shares several characteristics with pathogenic campylobacters. It is thermophilic (i.e., grows at 42°C) and is highly susceptible to drugs that are present in selective isolation media, making them unsuitable for the isolation of *C. upsaliensis.*[400] Goossens and coworkers[145] reported the isolation of 99 strains of *C. upsaliensis* by the filter method, with only four strains recovered simultaneously from selective media.

Domestic pets may serve as the reservoir of this species, which was first isolated from healthy dogs, dogs with diarrhea, and later from asymptomatic cats.[129,371] Data from some reports suggest that this organism may be an opportunistic agent of infections in children. Lastovica and associates[245] reported the recovery of *C. upsaliensis* from blood cultures of 16 patients, 10 of whom were 10 months old or younger. Walmsley and Karmali[441] reported the isolation of this organism from the stools of six children. Other reports have associated the isolation of *C. upsaliensis* from the blood of patients with serious underlying disease.[59,81] There has been one report of *C. upsaliensis* isolated from the blood and fetoplacental material of an 18-week pregnant woman who suffered a spontaneous abortion.[151] The patient had no underlying disease, and her only previous pregnancy was uneventful. Numerical analysis of protein profiles revealed that strains isolated from the patient and a healthy household cat were almost identical, implying that the cat might have been the source of the infection.[151] The only report of *C. upsaliensis* from a site other than blood or stool was from a patient with a breast abscess in which *C. upsaliensis* was recovered along with a *Peptostreptococcus* species from purulent exudate obtained through fine-needle aspiration of the infected site.[136] Sandstedt and Ursing[370] have described *C.*

upsaliensis, including its phenotypic characteristics and clinical significance.

Campylobacter ureolyticus. *C. ureolyticus* was formerly known as *Bacteroides ureolyticus* but was reclassified as a species of *Campylobacter* in 2010.[428,429] *C. ureolyticus* differs from other campylobacters in its fatty acid composition and its proteolytic metabolism. It also differs from most campylobacters in its ability to hydrolyze urea (only some *C. lari* and *C. sputorum* strains are also urease positive).[428,429] *C. ureolyticus* strains have been isolated from patients with superficial ulcers, soft tissue infections, nongonococcal nonchlamydial urethritis, and periodontal disease.[104,105,123,124]

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C. ureolyticus has also been reported to be an cause of gastroenteritis.[52] In a study by Johnson and colleagues, *C. ureolyticus* strains were found to be uniformly susceptible to penicillins, cephalosporins, erythromycin, clindamycin, chloramphenicol, metronidazole, and aminoglycosides.[200]

Campylobacter volucris. *C. volucris* is a novel species of *Campylobacter* that was first isolated from a rectal swab specimen from black-headed gulls in 2010 in Sweden.[93] A human with *C. volucris* bacteremia has been reported in an immunocompromised patient with polycythemia vera and alcoholic liver cirrhosis.[234] *C. volucris* is gram negative with a curved-rod morphology. It is oxidase and catalase positive and reduces nitrate. Indoxyl acetate and hippurate are not hydrolyzed. H_2S is not produced in triple sugar iron agar. Strains grow on blood agar plate at 37°C and 42°C under microaerophilic conditions, but do not grow aerobically.[93]

Genus Arcobacter

The genus *Arcobacter* belongs to the family *Campylobacteraceae* and includes the former aerotolerant *Campylobacter* species[433,435]; distinguished by their ability to grow in the presence of atmospheric levels of oxygen.[318] Other characteristics useful in distinguishing aerotolerant "*Campylobacter*" species from other campylobacters include hydrolysis of indoxyl acetate; growth at 15°C, 25°C, and 36°C, but not 42°C; and the inability to hydrolyze hippurate (Table 8-4). Three species were immediately associated with both human and animal diseases, namely *Arcobacter butzleri, A. cryaerophilus,* and *A. skirrowii*.

Arcobacter butzleri. Kiehlbauch and colleagues at the Centers for Disease Control and Prevention (CDC)[221] reported that the strains of aerotolerant campylobacters do not make up a homogeneous group. The majority of human isolates, both from within and

outside the United States, make up a distinct DNA homology group that they named *Campylobacter butzleri*, which changed to *Arcobacter butzleri* following the acceptance of the new genus designation for this group of organisms.[435] Strains of *A. butzleri* can be separated from *A. cryaerophilus* by demonstrating aerotolerance at both 30°C and 36°C (*A. cryaerophilus* is aerotolerant at 30°C, but not at 36°C). In addition, *A. butzleri* grows on MacConkey agar and in glycine and nitrate-containing media (reducing nitrate to nitrite) and in 1.5% and 3.5% NaCl. *A. cryaerophilus* gives the opposite reactions.[221] The majority of isolates of *A. butzleri* from humans have been from stools of patients with diarrheal illness[221,254,434]; however, the organism rarely has been isolated from abdominal contents, peritoneal fluid, and blood.[221,456]

Arcobacter cryaerophilus. *A. cryaerophilus* (formerly *C. cryaerophila*) grows well under aerobic conditions, although it may require microaerophilic conditions for initial isolation. Optimal growth occurs at 30°C, and the organism will not grow at 42°C. Biochemically, this species resembles *C. fetus* subsp. *fetus*; however, *A. cryaerophilus* is indoxyl acetate positive, whereas *C. fetus* subsp. *fetus* is not (Table 8-4). Most strains are susceptible to nalidixic acid and resistant to cephalothin. In a report by Borczyk and colleagues[45] comparing growth of *A. cryaerophilus* on CSM, Skirrow's blood agar, and cefsulodin irgasan novobiocin (CIN) agar, it was noted that the most luxuriant growth was obtained on CIN agar incubated for 24 to 48 hours at 25°C and 36°C. *A. cryaerophilus* has been recovered from human stools from healthy, asymptomatic individuals.[181,369] *A. cryaerophilus* was reported from the stool of a man infected with HIV with intermittent diarrhea; however, this strain was subsequently found to be *A. butzleri*.[416,417]

Arcobacter skirrowii. *A. skirrowii* is the most recently described species of *Arcobacter*. Strains have been isolated mainly from the preputial fluids of bulls; other strains have been isolated from aborted fetuses and diarrheic feces from cows, pigs, and sheep. *A. skirrowii* was isolated from the stool of a 74-year-old man with chronic diarrhea; however, there was no association with farm animals or pets, and it was not clear if the strain was etiologically associated with the patient's diarrhea.[454] Samie et al.[369] found *A. skirrowii* in 1.9% of hospital patients and primary school children in a region in South Africa, but it was not found to be associated with diarrhea.

Genus Helicobacter

The genus *Helicobacter* comprises 35 formally validated species at the time of this writing. Proposed names that are not yet validated by the International Committee on Systematic Bacteriology are enclosed in quotation marks. *Helicobacter* species are strict microaerophiles with a spiral or helical morphology. Many species exhibit strong

urease activity. *Helicobacter* species strain "flexispira" (formerly "*Flexispira rappini*") is the name proposed for an organism that is closely related to *Helicobacter,* but this microorganism is cigar shaped rather than curved and fusiform shaped. Species included in the genera *Helicobacter* possess sheathed flagella. No *Campylobacter* or *Wolinella* species possess sheathed flagella (Table 8-1).

Helicobacter pylori. This species was initially called *Campylobacter pyloridis* and then *Campylobacter pylori*. Molecular analysis provided evidence showing that this organism does not belong to the genus *Campylobacter.*[144] Features that distinguish this organism from campylobacters are its multiple sheathed flagella, its strong hydrolysis of urea, and a unique fatty acid profile (i.e., a high percentage of 14:0 acid, a low percentage of 16:0 acid, and the presence of 3-OH-18:0 acid). 16S rRNA sequencing has shown that this organism is closely related to *W. succinogenes.* However, there are many differences in biochemical features and growth characteristics between *H. pylori* and *W. succinogenes*, which indicates that these species should not be in the same genus. *W. succinogenes* is catalase negative, urease negative, does not possess γ-glutamyltranspeptidase or alkaline phosphatase activity, and does not grow at 30°C or on 0.5% glycine; *H. pylori* has the opposite characteristics.

H. pylori is found only on the mucus-secreting epithelial cells of the stomach. *H. pylori* is an important cause of active chronic antral gastritis[268] and is a major factor in the pathogenesis of peptic ulcer disease.[337] Additionally, there is strong evidence that *H. pylori* is linked to gastric adenocarcinoma and to the development of gastric non-Hodgkin's lymphoma (i.e., mucosa-associated lymphoid tissue [MALT] lymphomas).[327,391] *H. pylori* gastritis is widespread in many countries

in the world and may be one of the most common chronic human infections. The case for *H. pylori* as a causative agent of duodenal ulcer remains controversial. *H. pylori* bacteremia has been reported to occur as a rare complication in patients with undiagnosed gastric infection.[155,307]For excellent reviews, see the work of Blaser,[39] Buck,[51] and Dunn and colleagues.[106]

H. pylori strains are microaerophilic (10% CO_2, 5% O_2, 85% N_2) and will also grow in air with increased (10%) CO_2 content. The optimum temperature for isolation is 35°C to 37°C, although some strains will grow at 42°C. High humidity has also been found to favor growth. Most strains take 3 to 5 days to grow, with occasional isolates requiring 7 days of incubation before growth is evident. They can be cultured on nonselective blood-containing media, producing small, translucent, gray colonies. The characteristic Gram stain (small, curved, slightly plump bacilli) and positive reactions

for catalase, oxidase, and urease provide an identification.

Culture and Isolation of *H. pylori*

Specimens for Recovery of *H. pylori*. For the diagnosis of *H. pylori*-associated gastritis, histologic staining and culturing of biopsy specimens has been considered the "gold standard."[27] Image Plate 8-1H Suitable specimens include gastric and duodenal biopsies. Specimens submitted for culture should be fresh and not delayed in transport for more than 3 hours. Specimens may be kept for up to 5 hours if stored at 4°C. Tissue should be kept moist by the addition of 2 mL or less of sterile isotonic saline.

Isolation Procedure. Grinding of specimens in a ground-glass grinder yields heavier growth than mincing or rubbing the specimen onto an agar surface. Material should be inoculated onto a nonselective blood agar medium, such as Brucella, brain–heart infusion (BHI), or tryptic soy agar plates with 5% sheep or horse blood added. Poor growth is observed on commercially prepared chocolate agar plates; therefore, this medium is not recommended. Because these bacteria are susceptible to cephalothin, *H. pylori* will not grow on any selective medium containing cephalosporins. Many laboratories have had good results using modified Thayer–Martin agar as a selective medium for isolation of *H. pylori* in mixed cultures. Plates are incubated at 37°C in a humid, microaerophilic environment. Growth is usually observed in 3 to 5 days. Unpublished data from experiments conducted at the University of Illinois Hospital Microbiology Laboratory are shown in Table 8-6. Because of the potential hazard of using an anaerobic jar without a catalyst, we recommend the use of the Campy GasPak jar (column 2 in Table 8-6). Growth occurred in the Campy GasPak jar but not in the Poly Bag with Campy gas mixture (column 4 in Table 8-6), presumably because the water added in the former system provides the necessary humidity for growth.

Identification of *H. pylori*. Colonies of *H. pylori* are small, gray, translucent, and weakly β-hemolytic. Gram stain reveals pale-staining, curved, gram-negative bacteria with characteristic gull-wing and "U" shapes. Presumptive identification can be made with positive reactions for oxidase and catalase and an extremely rapid (within minutes) urease reaction. Additional identifying characteristics are listed in Table 8-4.

Biopsy Urease Test (*Campylobacter*-Like Organism Test). A more rapid but somewhat less sensitive and specific technique than the previously mentioned tests is the biopsy urease test (*Campylobacter*-like organism [CLO] test). In this test, a medium containing urea and a pH-sensitive dye is inoculated with the mucosal biopsy specimen. If urease is present in the specimen, urea is split and ammonia causes a rise in pH and subsequent change in the color of the indicator. This test may produce false-negative results if only a small number of organisms are present or false-positive results if other urea-splitting organisms are present in the specimen.[275]

TABLE 8-6 Comparison of Culture Media and Atmospheric Conditions for Growth of *Helicobacter pylori*

Agar Media	Conditions of Incubation[a]				
	Anaerobe Jar (No Catalyst)	Campy GasPak Jar	Anaerobe Jar (Catalyst)	Poly Bag (Campy Gas Mixture)[b]	Atmospheric Air With 5% CO_2
Brucella with 5% sheep blood	Best growth β-hemolytic	Very good growth β-hemolytic	No growth	No growth	No growth
Tryptic soy with 5% sheep blood	Very good growth	Good growth	No growth	No growth	No growth
Brain–heart infusion with 5% horse blood	Very good growth	Good growth	No growth	No growth	No growth
Chocolate (Becton Dickinson Microbiology Systems, Cockeysville, MD)	No growth	No growth	No growth	No growth	No growth
Chocolate (GIBCO)	Small colonies	Small colonies	No growth	No growth	No growth
Chocolate (freshly prepared)	Very small colonies	Very small colonies	No growth	No growth	No growth
Campy-BAP (Blaser's)	No growth	No growth	No growth	No growth	No growth

[a]All cultures were incubated at 37°C and read after 5 days.
[b]5% O_2, 10% CO_2, 85% N_2.
Data from K. Ristow, University of Illinois Hospital, Chicago, IL.

Noninvasive Tests to Diagnose *H. pylori* Infection. *H. pylori* infection may be diagnosed by invasive assays that require endoscopy (culture, stain, polymerase chain reaction [PCR], CLO test), or by noninvasive assays in which endoscopy is not necessary. Included in the latter category are the urease method, antigen detection, and serology.

Urease method. Two urease methods have been described. The first method, called the urea breath test (UBT), requires that the patient ingest [^{13}C]-labeled urea dissolved in water, followed by collection of breath samples that are analyzed for the presence of $^{13}CO_2$ at 60 minutes. A second method utilizes radiolabeled urea containing ^{15}N.[452] After oral ingestion, radiolabeled urea is broken down into ammonia and carbon dioxide by *H. pylori* urease in the stomach. The ammonia is absorbed into the blood and excreted in the urine. The amount of [^{15}N] urea, reflecting the magnitude of *H. pylori* infection, is evaluated by measuring the abundance and excretion rate of ^{15}N in ammonia in the urine. The sensitivity of the $^{15}NH_4$ excretion test is reported to be 96%, with 100% specificity when compared with patients who were *H. pylori* positive by culture and Gram stain.[452] The [^{13}C] UBT is very reliable for the diagnosis of *H. pylori* infection in adults and children older than 6 years, but the specificity varies from 82% to 100% for younger children.[30,187,277,346,363]

Antigen detection. A monoclonal antibody–based enzyme immunoassay (EIA) for direct detection of *H. pylori* antigen in stool specimens is commercially available

(Premier Platinum HpSA, Meridian Bioscience, Cincinnati, OH) and has been found to be highly accurate in all age groups, including children.[20,231,264,288,309]

Serologic methods. Serologic tests for detecting antibody to *H. pylori* have been used mainly for epidemiologic studies, but can also be used to monitor the efficacy of treatment. The principle format is the enzyme-linked immunosorbent assay test for the detection of IgG, although latex agglutination tests are available. IgA and IgM can also be detected, but are less useful diagnostically. One inherent problem is attempting to establish a baseline for positivity since the prevalence of persons with elevated antibody titers is relatively high in certain populations. In an extensive study of Army recruits, Smoak et al.[387] found an overall positivity rate of 26.3%. This rate increased from 24.0% in age group 17 to 18 years to 43% in age group 24 to 26 years. Seropositivity for blacks was 44%, for Hispanics 38% and for whites 14%.

Accuracy of Invasive and Noninvasive Tests to Diagnose *H. pylori* Infection. Cutler and colleagues[91] evaluated the accuracy of several tests for determining *H. pylori* infection, including the UBT, measurement of serum IgG and IgA antibody levels and antral biopsy specimens for CLO test, histology, and Warthin–Starry stain. They found that the Warthin–Starry stain had the best sensitivity and specificity, although the CLO test, UBT, and IgG levels were not statistically different in determining the correct diagnosis. They concluded that the noninvasive UBT and IgG serology tests are as accurate in predicting *H. pylori* status in untreated patients as the invasive tests of CLO and Warthin–Starry. The absence of chronic antral inflammation accurately excludes *H. pylori* infection.[91]

Other Medically Important *Helicobacter* Species. Several species of *Helicobacter* other than *H. pylori* have been isolated from humans and are associated with human disease.[122,278,389,390,391] These include *H. bilis, H. canadensis, H. canis, H. cinaedi, H. felis, H. fennelliae, H. heilmannii* ("*G. hominis*"), *H. hepaticus, H. pullorum*, *H. suis*, *Helicobacter* species strain flexispira ("*Flexispira rappini*"), and the unnamed *Helicobacter* species CLO-3 (Table 8-7).

Helicobacter bilis. *H. bilis* is a fusiform bacterium with 3 to 14 multiple bipolar sheathed flagella and periplasmic fibers wrapped around the cell. It grows microaerophilically at 37°C and 42°C, but not at 25°C. Additional characteristics are provided by Fox et al.[130] *H. bilis* includes some of the former "*Flexispira rappini*" strains previously referred to as *Helicobacter* sp. flexispira 16S rDNA taxa 2, 3, 8, and 9.[157] *H. bilis* is an unusual cause of chronic infections in patients with X-linked agammaglobulinemia (XLA) and is notoriously difficult to diagnose and eradicate.[298,425] It has been reported to cause cellulitis accompanied by bloodstream infection in such patients.[141,425] *H. bilis* has also been recovered in the bile and gallbladder tissue of patients with cholecystitis and gallbladder cancer[126,340]; however,

a causative role has not been shown.[325]

Helicobacter canadensis. *H. canadensis* is named after the country of original isolation. Cells are slender, curved-to-spiral rods with one to three spirals. It is gram negative and motile by means of nonsheathed, single unipolar or bipolar flagella. Cultures grown on solid agar media appear as spreading layers. Growth occurs at 37°C and 42°C. Additional characteristics are found in the report by Fox et al.[125] It has been isolated from humans with diarrhea,[125] and from the blood of a 35-year-old man with pyrexia of unknown origin.[419]

Helicobacter canis. *H. canis* is a gram-negative, helically curved, spiral, slender, rod-shaped bacterium with bipolar flagella and is most frequently detected in the GI tract of dogs and cats.[397] *H. canis* has been reported in cases of human bacteremia[8,16,141,253,344,436] and chronic duodenal ulcerations in a patient with Crohn's disease.[411] Human infection with *H. canis* is most likely zoonotic from regular contact with domestic pets.

Helicobacter cinaedi. *H. cinaedi,* originally designated as CLO-1, and formerly known as "*Campylobacter cinaedi,*" has been isolated from rectal swabs taken from symptomatic as well as asymptomatic homosexual men.[116,422] This organism has also been described as a cause of bacteremia in two homosexual men with concurrent tuberculosis,[331] in patients with AIDS,[82,94,367] and in another who was HIV seropositive but did not have AIDS.[308] Reports, however, suggest that *C. cinaedi* infections are not restricted to homosexual or bisexual men. For example, Vandamme and associates[432] have reported the isolation of *H. cinaedi* from the blood of two women without any record of sexual contact with homosexuals and from the stools of three children, two of whom were girls.

H. cinaedi is known to cause diarrhea as well as bacteremia, cellulitis, and other symptoms after gaining entry into the body through the intestinal mucosa. Although these conditions are primarily observed in HIV-infected patients, their occurrence among both immunocompetent[228,242] and other immunocompromised patients[223,224,286,297] has also been reported. Orlicek and colleagues have reported a neonatal patient with septicemia and meningitis caused by *H. cineadi*.[320]

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TABLE 8-7 *Helicobacter* Species and Related Organisms of Human Origin

Species	Origin	Types of Human Infection
		Cellulitis accompanied by bloodstream infection in patients with

H. bilis	Mice, humans	XLA, bile, and gallbladder tissue of patients with cholecystitis and gallbladder cancer
H. canadensis	Feces of diarrheic humans	Diarrhea and bacteremia
H. canis	Gastric mucosa of dogs and cats	Bacteremia and chronic duodenal ulcerations in patients with Crohn's disease
H. cinaedi	Rectal swabs of homosexual men and intestinal tract of hamsters	Diarrhea, bacteremia, cellulitis, and meningitis in both immunocompetent and immunocompromised patients
H. felis	Gastric mucosa of dogs and cats	Gastric biopsy
H. fennelliae	Rectal swabs of homosexual men	Bacteremia in HIV-infected patients and stools and blood cultures of pediatric patients. Human-to-human transmission has been shown.
H. heilmannii	Cheetahs; gastric mucosa of humans and domestic animals	Chronic gastritis similar to *H. pylori* infection, MALT lymphoma, gastric and duodenal ulcer, and gastric cancer
H. hepaticus	Colons and ceca of mice	Associated with HCC
H. pylori	Gastric mucosa of humans	Gastritis and peptic ulcer disease. Also plays etiologic role in metaplasia and dysplasia of gastric mucosa, gastric adenocarcinoma, and non-Hodgkin's lymphoma of the stomach. Rare cause of bacteremia in patients with undiagnosed gastric infection.

H. pullorum	Intestinal tract and liver of chickens, mice, and humans	Gastroenteritis, chronic hepatobiliary disease, and Crohn's disease
H. suis	Stomach of pigs	Dyspepsia and reflux esophagitis in a pig veterinarian
Helicobacter sp.*flexispira*[a]	Stool of humans, dogs, and from aborted ovine fetuses	Gastroenteritis and bacteremia
CLO-3	Rectal swabs of homosexual men	Diarrhea

[a]Flexispira taxa 1 and 4–6 represent the same species and are named *H. trogontum*,[158] while, Flexispira taxa 2, 3, 8, and 9 represent the same species and are named *H. bilis*.[157] Flexispira taxa 7 and 10 remain unnamed at the time of this writing.
XLA, X-linked agammaglobulinemia; MALT, mucosa-associated lymphoid tissue; HCC, hepatocellular carcinoma.

Since *H. cinaedi* has been identified as a normal intestinal inhabitant of hamsters[138,403] and the mother of the newborn cared for pet hamsters during the first two trimesters of her pregnancy, it is likely that the hamsters served as a reservoir for the transmission of the organism to the mother and that the newborn most likely became colonized with *H. cinaedi* during the birth process.[320] An immunocompetent adult who had daily contact with a kitten for a month developed *H. cinaedi* meningitis.[405] *H. cinaedi* has also been reported to cause infection of an axillobifemoral bypass graft in an immunocompetent 85-year-old man,[404] and was identified in aneurysmal tissues from three patients with abdominal aortic aneurysm.[207] Burman and coworkers[54] have reported the association of skin infections and arthritis due to *H. cinaedi* bacteremia. The most common cutaneous manifestation of *H. cinaedi* bacteremia is superficial cellulitis, which usually takes the

form of painful erythemas or infiltrated erythematous plaques on the extremities.[379] Blood culture isolates are often recovered in automated blood culture instruments only after 5 or more days of incubation. In a report by Araoka et al.,[21] the time required for blood cultures to become positive for *H. cinaedi* was ≤5 days in 69 sets (55%) and >5 days in 57 sets (45%) using the Bactec 9240 and the Bactec FX (Becton, Dickinson, Sparks, MD) blood culture systems. All *H. cinaedi* strains were detected in aerobic bottles only.[21] Since standard laboratory practice is to incubate blood culture bottles for 5 days, the true incidence of *H. cinaedi* bacteremia is likely underreported. In general, organisms are not seen on initial Gram staining of the blood culture material, but can be visualized by darkfield or acridine orange staining. *H. cinaedi* grows only at 37°C, shows intermediate resistance to cephalothin (30-mg disk), and reduces nitrate to nitrite. Additional genotypic and phenotypic characteristics can be found in the report by Kiehlbauch and colleagues.[220] The clinical spectrum of illness associated with *H. cinaedi* infection includes fever, bacteremia, and recurrent cellulitis, with most patients having signs of systemic infection including leukocytosis, and often thrombocytopenia.[222] Kielbauch and colleagues reported that treatment with a penicillin, tetracycline, or an aminoglycoside may be more effective than treatment with cephalosporins, erythromycin, or ciprofloxacin.[222] The only known natural reservoir of *H. cinaedi* found so far is the intestinal tract of hamsters, which may serve as a reservoir of human infections.[138,403] Nosocomial infection

caused by *H. cinaedi* among immunocompromised patients in the same hospital ward has been reported indicating that human-to-human transmission can also occur.[286,356]

Helicobacter felis. *H. felis* is a spiral-shaped, gram-negative rod containing five to seven spirals per cell. Spherical forms are present in older cultures. Cells are motile with a rapid corkscrew-like motion. Growth occurs in microaerophilic atmosphere, but the organism can grow anaerobically. It grows at 37°C and 42°C, but not at 25°C. These organisms are nutritionally fastidious, growing only on media enriched with blood or serum, and are asaccharolytic.[330] These bacteria have been isolated from the gastric mucosa of cats and dogs and also recovered from a gastric biopsy specimen of a 14-year-old girl who presented with persistent epigastric pain of several months duration.[453]

Helicobacter fennelliae. Originally designated CLO-2, and formerly known as "*Campylobacter fennelliae,*"[422] this organism has the distinctive odor of hypochlorite cleaning powders.[116] It is susceptible to cephalothin and does not reduce nitrates to nitrite. As with *H. cinaedi, H. fennelliae* has been isolated from rectal swabs taken

from symptomatic and asymptomatic homosexual men,[116,422] and is reported to cause bacteremia in HIV-infected patients.[218,220,308] Subsequent reports have identified *H. fennelliae* in the blood of a non–HIV-infected heterosexual man with cirrhosis and diabetes,[182] and in stools and blood cultures of pediatric patients in South Africa.[388] Rimbara et al.[355] reported isolation of *H. fennelliae* from the blood of 3 patients from the same hospital ward, which showed the same pulsed-field gel electrophoresis pattern between isolates, suggesting human-to-human transmission.[356] Sharp reported *H. fennelliae* in chronic skin ulcers in a 25-year-old homosexual man with Bruton's XLA.[378]

Helicobacter heilmannii. *H. heilmannii* is a spiral bacterium found in human gastric mucosa that is larger and more tightly coiled than *H. pylori*. The organism is helical, 3.5 to 7.5 μm long and 0.9 μm in diameter with truncated ends flattened at the tips, six to eight tight spirals, and up to 12 sheathed flagella 28 nm in diameter at each pole.[274] McNulty and colleagues proposed the name "*Gastrospirillum hominis*." However, Solnick and coworkers[390] showed that "*Gastrospirillum*" is a member of the *Helicobacter* genus and proposed the name "*H. heilmannii"* after Konrad Heilmann, a German histopathologist who described the first large series of patients infected with the organism.[165] *H. heilmannii* was given full taxonomic status after successful in vitro cultivation in 2012.[384] Heilmann and Borchard reported the prevalence of "*Gastrospirillum*" infection in patients presenting for endoscopy to be less than 1%.[165] *H. heilmannii* appears to be ubiquitous in domestic animals, suggesting that human infection may be acquired as a zoonosis.[103,128,167] Human infection with this bacterium may be accompanied by chronic gastritis similar to that seen with infection by *H. pylori*.[97,120,165,250,274,291,389] Iwanczak et al.[189] recovered *H. heilmannii* from biopsy specimens taken from the gastric mucosa of 22 children with dyspeptic symptoms. In three children, mixed infection with *H. pylori* and *H. heilmannii* was diagnosed.[189] *H. heilmannii* has also been reported to cause gastritis in patients testing negative for *H. pylori*.[272,358] In addition to gastritis, *H. heilmannii* has been associated with low-grade gastric MALT lymphoma,[290,349] gastric and duodenal ulcer,[102,142,199,390,460] and gastric cancer.[147,289,457]

Helicobacter hepaticus. *H. hepaticus* cells are slender, gram-negative, curved-to-spiral rods, which form one to three spiral turns. They are motile by means of sheathed, single, bipolar flagella. Colonies are pinpoint, but cultures often appear as thin spreading layer on agar media.[127] Cells exhibit microaerophilic or anaerobic growth, but there is no growth aerobically. Growth occurs at 37°C, but not at 25°C or 42°C.[127] *H. hepaticus* was originally recovered from the colons and ceca of mice and has been shown to cause chronic active hepatitis and induce hepatocellular carcinoma

(HCC) in mice.[127,443] Yang and colleagues identified *H. hepaticus* infection in patients with primary HCC using serologic and molecular biologic detection, suggesting that *H. hepaticus* infection may be involved in the progression of HCC in humans.[458]

Helicobacter pullorum. *H. pullorum* is a gram-negative, curved, slender, rod-shaped bacterium with a single, unsheathed polar flagellum.[396] It exhibits characteristic darting motility and grows micoaerobically at 37°C and 42°C. No growth occurs under aerobic or anaerobic conditions.[396] It is considered an enterohepatic pathogen that has the ability to colonize the distal intestinal tract and liver of chickens, mice, and humans.[66,396,424] It has been detected in humans with gastroenteritis,[56,396,401] chronic hepatobiliary diseases,[65,126,335,357] and Crohn's disease.[43,236] Sirianni et al.[382] showed that *H. pullorum* has the ability to adhere to and invade human cells and secrete factors that may contribute to the pathogenic potential.

Helicobacter suis. Cells of *H. suis* are gram-negative, tightly coiled spiral rods with up to six turns. In older cultures, coccoid cells predominate. They are motile by means of tufts of 4 to 10 sheathed bipolar flagella. Organisms grow under microaerophilic conditions but not in 5% CO_2, and only weak growth is observed under anaerobic conditions. Growth occurs at 37°C, but not at 25°C or 42°C.[24] *H. suis* is associated with ulceration of the stomach and gastritis in pigs[24] and is reported to infect up to 80% of pigs at slaughter age.[166,326] There has been one report of human infection occurring in a pig veterinarian suffering from general dyspepsia and reflux esophagitis. Biopsy specimens from both the corpus and antrum of the stomach revealed the presence of *H. suis*.[203]

***Helicobacter* sp. Strain *flexispira* ("*Flexispira rappini*").** *Helicobacter* species strain "*flexispira*" (formerly "*Flexispira rappini*") was the proposed name[49] of an organism that is urease positive and possibly genomically closely related to *H. pylori*.[433] However, it is a straight, fusiform-shaped organism rather than a spiral one, and the ultrastructure is highly distinctive: The cell body is cigar shaped, encircled by a complex array of periplasmic fibrils, and has multiple, bipolar tufts of sheathed flagella. It does not possess alkaline phosphatase or grow at 30°C, but does grow at 42°C, and is resistant to metronidazole (5 mg); *H. pylori* has the opposite characteristics.[144] It is separated from the campylobacters by negative reactions for catalase and nitrate and an inability to grow in 1% glycine.[22] *Helicobacter* species strain "flexispira" has been isolated from stool specimens of humans with symptoms of gastroenteritis and from blood cultures, from the stool of dogs,[22,360] and from aborted ovine fetuses.[226,227,360,392,418] Dewhirst et al.[101] examined a collection of *"F. rappini"* strains by using 16S rRNA sequence analysis and found that the strains

fell into 10 taxa, named flexispira taxons 1 to 10, each possibly representing a novel *Helicobacter* species. Two of the taxa corresponded to previously designated *Helicobacter* species, flexispira taxon 6 corresponded to *H. trogontum,*[279] and flexispira taxon 9 corresponded to *H. bilis*.[130] Subsequent phenotypic characterization, analysis of RFLPs of 16S and 23S rDNAs, and SDS-PAGE profiles revealed that flexispira taxa 1, 4, and 5 and the type strain of *H. trogontum* (flexispira taxon 6) represented the same species and are therefore named *H. trogontum*,[158] while flexispira taxa 2, 3, and 8 and the type strain of *H. bilis* (flexispira taxon 9) represent the same species and are therefore named *H. bilis*.[157] Flexispira taxa 7 and 10 remain unnamed at the time of this writing.

CLO-3. An unnamed species originally described by Fennell and coworkers,[116] CLO-3 can be separated from the other CLOs by its ability to grow at 42°C, its resistance to cephalothin, and its inability to reduce nitrate (Table 8-4). One isolate has been reported from a rectal swab obtained from a symptomatic homosexual man.[116]

Other Microaerophilic Gram-Negative Bacilli

Sutterella wadsworthensis. Wexler and colleagues have proposed the name *S. wadsworthensis* for a group of bacteria that were originally identified as *C. gracilis* but differed in genetic and biochemical characteristics from typical *C. gracilis* strains.[447] These organisms are gram-negative straight rods that grow in a microaerophilic atmosphere or under anaerobic conditions. They are differentiated from *C. gracilis* and *Campylobacter* species by being oxidase, urease, and indoxyl acetate negative; resistant to 20% bile disks; and by not reducing tetrazolium tetrachloride under aerobic conditions. They have been isolated mainly from human infections of the GI tract.[447]

Definitive Identification of Campylobacters and Related Bacteria

The colonial morphology and Gram stain characteristics of *C. jejuni*, as described earlier, also pertain to most other *Campylobacter* species. However, definitive species identification depends on the determination of the phenotypic characteristics presented in Table 8-4; when these tests do not provide a species identification, then molecular techniques must be employed.

The differential susceptibility to nalidixic acid and cephalothin may be useful in

differentiating the more commonly encountered *Campylobacter* species according to the scheme in Box 8-3. Because isolates of *C. jejuni* and *C. coli* resistant to nalidixic acid are encountered with increasing frequency, these tests have become less helpful.

Luechtefeld and Wang[260] also found that resistance of *C. jejuni* to triphenyltetrazolium chloride (TTC) is helpful in distinguishing *C. fetus*. The test for hippurate hydrolysis is useful in separating *C. jejuni* from the closely related species *C. coli*. Most strains of *C. jejuni* hydrolyze hippurate to benzoic acid and glycine.[162] The rapid procedure of Hwang and Ederer for hippurate hydrolysis is suitable for testing clinical isolates of *Campylobacter* species. Morris and associates[292] describe a more sensitive method for detecting benzoic acid using gas-liquid chromatography (GLC). This application is an extension of the procedure previously reported by Kodaka and colleagues,[230] who used hippurate formate fumarate medium to detect not only hippurate hydrolysis by GLC, but also the utilization of formate and fumarate.

Mills and Gherna[284] described the use of a rapid test for detecting hydrolysis of indoxyl acetate by *Campylobacter* species. Studies have shown that all strains of *C. jejuni, C. coli, C. curvus, C. helveticus, C. rectus, C. showae, C. upsaliensis, A. butzleri, A. cryaerophilus*, and *H. fennelliae* hydrolyze indoxyl acetate, whereas most other campylobacters are negative.[174,284,317,343] Several additional biochemical and physical characteristics may be helpful in separating the various *Campylobacter* and *Campylobacter*-like species (Table 8-4). An extensive review of identification methods for campylobacters, helicobacters, and related organisms has been published by On.[317]

Rapid Identification of Campylobacters From Colonies Grown in Culture

Morphologic Identification. In most instances, the appearance of small, tan to gray, watery colonies that tend to flatten on *Campylobacter*-selective agar, and that exhibit

BOX 8-3

Definitive Identification of Most Commonly Encountered Campylobacters

	Nalidixic Acid	Cephalothin	TTC	Hippurate Hydrolysis	Indoxyl Acetate Hydrolysis
C. jejuni subsp. *jejuni*	V	R	R	+	+
C. coli	S	R	R	–	+
C. fetus subsp. *fetus*	R	S	S	–	–
C. lari	R	R	S	–	–

TTC, triphenyltetrazolium chloride.

characteristic "S"-shape and gull-wing morphology on Gram's stain and are oxidase and catalase positive, will be sufficient to establish the diagnosis of campylobacteriosis in patients with diarrheal syndromes.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Identification of *Campylobacter* species and related organisms using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to be accurate for several species of *Arcobacter, Campylobacter*, and *Helicobacter*.[12,34,267,270,410,448] In a study by Bessède et al.,[34] there was 100% correlation with MALDI-TOF and molecular identification for six species (*A. butzleri, C. coli, C. fetus, C. lari, C. sputorum*, and *C. upsaliensis*) and 99.4% correlation for *C. jejuni*. In a study reported by Martiny and colleagues, 230/234 (98.3%) *Campylobacter, Arcobacter*, and *Helicobacter* isolates representing 12 species were correctly identified by MALDI-TOF.[270]

Commercial Kit Systems. The API Campy (bioMérieux, Marcy l'Étoile, France) is a miniaturized identification system that includes 11 enzymatic and conventional tests and nine assimilation and inhibition tests.[186,270,352] Martiny et al.[270]reported that the API Campy provided correct identification of *C. jejuni* subsp. *jejuni* for 116/125 (92.8%) isolates tested, but overall correctly identified only 167/234 of 12 species of *Campylobacter, Arcobacter*, and *Helicobacter* isolates tested.

The *Neisseria–Haemophilus* (NH) identification card was developed for use on the Vitek 2 system (bioMérieux, Marcy l'Étoile, France). The NH card is based on colorimetric technology utilizing dehydrated media containing chromogenic substrates that are used to identify 27 taxa of gram-negative fastidious bacteria, including *C. jejuni, C. coli*, and *C. fetus*.[270,354,427] In a study reported by Martiny et al.,[270] 101/125 (80.8%) *C. jejuni* subsp. *jejuni*, 47/65 (72.3%) *C. coli*, and 0/7 *C. fetus* isolates were correctly identified to the genus and species level with the Vitek 2 NH

card.

Methods for Direct Detection of Campylobacters From Stool

Non–culture-based methods that allow for the direct detection of *Campylobacter* antigens or DNA in stool specimens have been developed. These include direct immunofluorescence, EIAs, and PCR assays.

Direct Immunofluorescence. Hodge and coworkers[175] advocate the use of direct immunofluorescence techniques in the rapid screening of stool specimens from patients with an acute diarrheal syndrome. This approach has the potential for removing the guesswork involved in interpreting Gram-stained smears. However, these reagents are not commercially available.

Enzyme Immunoassay. There are currently three *Campylobacter* EIA methods commercially available: the Premier CAMPY EIA (Meridian Bioscience, Cincinnati, OH), the ProSpecTCampylobacter EIA (Remel, Lenexa, KS), and the ImmunoCard STAT! CAMPY test (Meridian Bioscience, Cincinnati, OH). In a study conducted by Granato et al.[149] on 485 stool specimens (127 culture-positive and 358 culture-negative) tested by the Premier CAMPY and ProSpecT Campylobacter microwell plate EIAs, both assays had identical sensitivities of 99.3% and comparable specificities (99.3% vs. 98%), with positive and negative predictive values of 95% and 99.7% for each EIA, respectively, after arbitration of discrepancies with culture results by PCR. In a subset of 300 (127 culture-positive and 173 culture-negative) stool specimens tested using the ImmunoCard STAT! CAMPY assay, the sensitivity, specificity, positive, and negative predictive values were 98.5%, 98.2%, 97.8%, and 98.8%, respectively, after arbitration of discordant results with PCR.[149] Several studies have shown that the ProSpecT Campylobacter EIA and ImmunoCard STAT! CAMPY assay can detect *C. upsaliensis* in culture-negative specimens because of the cross-reactivity between *C. upsaliensis* antigen with *C. jejuni/C. coli* antigens.[89,95,172] Bessède et al. evaluated two culture-based methods (culture after filtration and culture on selective medium without filtration) with two different PCR methods and three different immunoenzymatic methods. The main result of their study was the lack of sensitivity of culture methods for detecting *Campylobacter* species, the filtration methods detected only 15 cases (65%) and selective media 13 cases (54.4%).[33] In contrast to these findings, researchers from CDC conducted a study of diagnostic methods used by laboratories within eight states for detection and isolation of *Campylobacter* from stool including four *Campylobacter*-selective media and four EIA assays. They reported that a total of 3.1% (87/2772) of specimens tested were positive by culture of which 13.8% (12/87) were negative in all four EIA tests utilized

by the participants. As compared to culture, the sensitivity/specificity/positive predictive values of four EIA tests were as follows: Premier CAMPY, 81.6/97.6/52.9%, ProSpecT Campylobacter 82.7/97.9/56.7%, ImmunoCard STAT! CAMPY, 72.4/96.3/38.6%, XpecT, 73.6/99.3/76.2%.[121] Given the low incidence of *Campylobacter* disease and the performance data generated in their study, these authors concluded that rapid EIA tests do not appear reliable as standalone tests for direct detection of *Campylobacter* in stool.[121]

PCR. There are currently five commercially available diagnostic systems for the detection of enteric pathogens using multiplex PCR that include targets for one or more *Campylobacter* species. These include Prodesse ProGastro SSCS assay (Hologic Inc., San Diego, CA),[50] Luminex xTag GI pathogen panel (GPP) assay (Luminex Corp., Austin, TX), [83,219,306,332] FilmArray GI panel (BioFire Diagnostics LLC, Salt Lake City, UT),[57,219] Verigene Enteric Pathogens (EP) test (Nanosphere, Northbrook, IL),[312] and BD Max enteric bacterial panel (EBP) (BD Diagnostics, Baltimore, MD).[19,36,161] The Prodesse ProGastro SSCS assay and the BD Max EBP assay detect the same pathogens: *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. (*C. jejuni* and *C. coli*), and Shiga-like toxin genes. In a study of the BD Max EBP performed on 3,401 specimens by Harrington et al.,[161] the positive percent agreement and negative percent agreement for *Campylobacter* was 97.5% and 99.0%, respectively. Biswas et al.[36] reported 100% sensitivity and 100% specificity for *Campylobacter* with the BD Max. The Prodesse ProGastro SSCS panel was evaluated by Buchan et al.,[50] who reported

96.4% sensitivity and 93.5% specificity for *C. coli/C. jejuni*. The Verigene EP test includes seven bacterial and two viral targets, including *Campylobacter* group. In an assessment of 611 prospective and 839 contrived specimens, conducted on the Verigene EP, Novak et al.[312] reported 97.1% sensitivity for *Campylobacter* species. The FilmArray panel contains 23 bacterial, viral, and parasitic targets, including *C. jejuni, C. coli*, and *C. upsaliensis*. In a multicenter study of 1,556 samples, the sensitivity and specificity of the FilmArray GI panel for *Campylobacter* species were 97.1% and 98.4%, respectively.[57] Khare et al.[219] reported 100% sensitivity and specificity with *Campylobacter* species and the FilmArray GI panel, although only three positive *Campylobacter* samples were included in the study. The Luminex xTag GPP assay can detect 15 targets (9 bacteria, 3 viruses, 3 parasites) and includes *C. jejuni, C. coli*, and *C. lari*. Several studies on the xTag GPP assay have been

published with reported sensitivities ranging from 90% to 100%.[83,219,306,332] All of the molecular panels report sensitivities and specificities greater than conventional culture and can be performed directly on stool specimens, with results available in 1 to 5 hours depending on the method. The choice of a particular system will depend on the throughput required for the test volume of the laboratory, the turnaround time of the assay, the technical time and expertise required to perform the assay, and the choice of menu desired depending on whether a focused or broad approach to pathogen detection is desired.

PART II: THE FAMILIES *VIBRIONACEAE* AND *AEROMONADACEAE* AND THE GENERA *PLESIOMONAS* AND *CHROMOBACTERIUM*

Phylogeny of the *Vibrionaceae*

The name *Vibrionaceae* was originally proposed by Veron in 1965 with the intent of grouping a number of nonenteric, fermentative, gram-negative rods that were oxidase positive and motile by means of polar flagella. This grouping was intended as a convenience for the purpose of differentiating these organisms from the *Enterobacteriaceae* and did not necessarily imply a taxonomic relationship among the included species. The *Vibrionaceae* once included four genera: *Vibrio*, *Aeromonas*,*Photobacterium*, and *Plesiomonas*. However, in the past few decades, a variety of methods for nucleic acid analysis have revolutionized microbial taxonomy and have resulted in the restructuring of this family along phylogenetic lines and the establishment of two new genera, *Listonella* and *Shewanella,* and a new family, *Aeromonadaceae.*[88,261,364] The genus *Plesiomonas* is now placed in the family *Enterobacteriaceae*.

Genus Vibrio

Vibrio species have both historical and contemporary interest. *Vibrio cholerae* is the etiologic agent of Asiatic cholera in humans, a potentially severe diarrheal disease that has been the scourge of humanity for centuries. The organism was first described and named by Pacini in 1854; 32 years later Koch isolated the organism, which he called "Kommabacillus" because of the characteristic curved or comma-shaped appearance of the individual bacterial cells.

There have been seven pandemics of cholera in recorded history; the last three pandemics are known to be due to *V. cholerae* serogroup O1. The most recent

pandemic, caused by *V. cholerae* O1, biotype El Tor, began in 1961 in Indonesia, rapidly spread to Asia, Europe, Africa, and the South Pacific, and reached South and then Central America in 1991. In addition, a number of minor epidemics of diarrheal syndromes and extraintestinal infections caused by non-O1 serogroups of *V. cholerae* and by several newly described halophilic species have been reported in the United States in several Gulf Coast states since the early 1970s.[183,209] Most infections have occurred following ingestion of contaminated and poorly cooked seafood. Wound infections have also been reported following trauma while swimming or working in infected waters or on exposure to marine animals.[183]

The point of this discussion is that clinical microbiologists cannot discount *Vibrio* species as a possible isolate from diarrheal stool specimens and must remain informed on how to recover and identify the various species. Those working in inland laboratories will have fewer potential encounters than those working in the Gulf Coast hospitals; nevertheless, with open world travel and shipping of seafoods to inland markets, everyone must remain alert.

Taxonomy. The strains of *V. cholerae* that have been recovered from classic patients with pandemic cholera agglutinate in what has been designated O1 antiserum. Strains not agglutinating in this antiserum are called either non-O1 *V. cholerae* (if this species is determined biochemically) or a variety of other *Vibrio* species names, such as *V. parahemolyticus, V. mimicus,* and so on. Since the non-O1 species usually do not cause diarrheal syndromes as severe or potentially life-threatening as O1 species, or more commonly may be associated with extraintestinal infections, early differentiation between the two groups can be of considerable clinical importance. Although 35 or more distinct *Vibrio* species have been identified, all but 11 are environmental organisms, called "marine vibrio species," and have not been associated with human infections.[197]

Description and Associated Clinical Syndromes of *Vibrio* Species of Human Importance. The species that are recovered from humans and potentially cause disease can be divided into two groups, namely *V. cholerae* and the noncholera vibrios.

Vibrio cholerae. *V. cholerae* is the etiologic agent of epidemic and pandemic cholera in humans. Within the species of *V. cholerae*, there is much dissimilarity among the strains in both their pathogenic and epidemic potential (Table 8-8). The strains can be divided according to differences in their cell wall composition (somatic O antigen), which forms the basis of the serotyping scheme that classifies the organisms into 139 different serogroups. All share a common flagellar (H) antigen. It was determined in the mid-1930s that all of the pandemic strains were agglutinated with a single antiserum that has been designated O1. The O1 type *V. cholerae* strains

can be further separated into one of three serogroups: Inaba,

TABLE 8-8 Characteristics of *Vibrio cholerae*

Classification Method	Epidemic-Associated	Not Epidemic-Associated
Serogroups	O1	Non-O1 (serogroups O2-O138)[a]
Biotypes	Classical, El Tor	Biotypes not applicable to non-O1 strains
Serotypes	Inaba, Ogawa, Hikojima	These three serotypes not applicable to non-O1 strains
Toxin	Produce cholera toxin	Usually do not produce cholera toxin; sometimes produce other toxins[a]

[a]A serogroup designated O139 Bengal emerged in Calcutta, Bangladesh, and parts of India in 1992 that produced cholera toxin in quantities similar to that produced by *V. cholerae* O1, and has spread in epidemic proportions across the Indian subcontinent.

Ogawa, and Hikojima. These serogroups are important for epidemiologic studies. For example, the current pandemic of cholera that began in 1961 is caused by the Ogawa serogroup, whereas a focus of cholera that is endemic in the Gulf Coast region of the United States is associated with a *V. cholerae* O1 Inaba serotype.[215] In January 1991, epidemic cholera appeared simultaneously in several coastal cities of Peru and has since been reported in Ecuador, Chile, Colombia, Brazil, and the United States. This outbreak is apparently also due to a strain other than the pandemic strain and has been identified as O1 serotype Inaba, biotype El Tor.[70]

Epidemic strains of serovar O1 may be further divided into the classic and El Tor bv. El Tor is an actively hemolytic biotype of *V. cholerae* that was isolated at the El Tor Quarantine Station in Egypt. The El Tor strain has been found to be hardier and better capable of surviving in the environment; furthermore, chronic carriers of the El Tor strain have been reported in the literature.[214] The El Tor vibrio is now recognized as a biotype of *V. cholerae* and is responsible for most current epidemic outbreaks of classic cholera. The present pandemic of cholera that began in 1961 is caused by the El Tor bv, as are the Gulf Coast and South American outbreaks. The classic bv has

almost disappeared except for rare isolations in India. Studies from Bangladesh indicate that the classic biotype has reemerged.[197]

Cholera: World history. There have been seven pandemics of cholera in recorded history; the last three pandemics are known to be due to the *V. cholerae* serogroup O1. The seventh pandemic of cholera, caused by the El Tor vibrio, originated in Celebes, Indonesia, in 1961 and subsequently spread worldwide, reaching the South American continent in 1991. The emergence and rapid spread of cholera caused by a new serotype designated O139 Bengal in October 1992 in nine countries (India, Bangladesh, Pakistan, Thailand, Nepal, Malaysia, Burma, Saudi Arabia, and China) suggests the possibility for the beginning of the eighth pandemic.[10,114,266,406] For a synopsis of the cholera pandemics of the 19th and 20th centuries, the reader is referred to the review published by Lacey.[235]

Toxigenic* V. cholerae *0139 Bengal. In October 1992, an epidemic of cholera-like illness began in Madras, India, and spread to Calcutta and Bangladesh and many other places in India and in southeast Asia.[293] The strain could not be identified as any of the 138 known types of *V. cholerae* and thus represented a new serogroup, O139 (synonym Bengal to indicate its first isolation from the coastal areas of the Bay of Bengal).[10] The strain caused large epidemics of cholera in Bangladesh, India, and neighboring countries and, for a time, largely replaced *V. cholerae* O1 strains in affected areas. From 1996 to 2002, most cases of cholera in Bangladesh were caused by *V. cholerae* O1 biotype El Tor, but in March 2002, *V. cholerae* O139 reemerged as the predominant pathogen.[114] There are three important points to consider with regard to this new serotype: (1) the symptoms associated with *V. cholerae* O139 infection suggest that it is indistinguishable from cholera caused by *V. cholerae* 01 and should be treated with the same rapid fluid replacement, (2) the rapid spread of *V. cholerae* O139 suggests that preexisting immunity to *V. cholerae* O1 offers little or no protective benefit and travelers to affected areas should not assume that cholera vaccination is protective against the *V. cholerae* O139 strain, and (3) laboratory identification methods for *V. cholerae* O1 depend on detection of the O1 antigen on the surface of the bacterium, and therefore do not identify this new strain.[72] The phenotypic, serologic, and toxigenic traits of *V. cholerae* O139 Bengal have been reported by Nair and coworkers.[301]

Cholera: Western Hemisphere. In January 1991, epidemic cholera, which had not been reported in South America in the 20th century, appeared simultaneously in several coastal cities of Peru and rapidly spread throughout South and Central America.[153,324]

Cholera: United States. In the United States, a small environmental focus of potentially epidemic *V. cholerae* exists along the Gulf Coast.[216] Cholera caused by this strain has in most cases been linked to undercooked crab or raw oysters harvested

from the Gulf Coast. In 1973, the first patient with cholera in the United States since 1911 was reported from Texas.[446] This was followed in 1978 by the report of 11 patients in Louisiana and in 1981 with two additional outbreaks in Texas involving 18 patients.[37] Crabs harvested from nearby estuaries were found to be the vehicle of infection in the Louisiana cases, whereas the largest of the two Texas outbreaks was traced to contamination of cooked rice following accidental rinsing with water from the environment containing the outbreak strain.[214] It is now known that 44 toxigenic *V. cholerae* O1 infections were acquired in the United States between 1973 and 1987.[197] All resulted from exposures in Louisiana and Texas near the Gulf Coast. In 1991, 26 patients with cholera were reported in the United States; 18 were linked to the South American outbreak.[71] In 1992, 103 patients with cholera were reported in the United States; 75 were associated with an outbreak on board an Aerolineas Argentinas flight between Argentina and Los Angeles in February 1992. In 1993 and 1994,

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22 and 47 cholera cases were reported in the United States, respectively. Of these, 65 (94%) were associated with foreign travel. Three of these were culture-confirmed cases of *V. cholerae* 0139 in travelers to Asia.[73] From 1995 to 2000, 14 patients with cholera were reported; 6 were associated with the endemic environmental focus and 8 with imported seafood.[294] Through 2015, cholera continues to be rare in the U.S. and is most often acquired during travel to countries where *V. cholerae* is circulating.

Cholera: Non O1. *V. cholerae* non-O1 serotypes have been associated with isolated cases of diarrheal disease, although the majority of non-O1 strains do not produce cholera toxin, they appear to produce an enterotoxin different from cholera toxin. Strains have also been isolated from wounds and systemic infections. Safrin and coworkers,[368] in a review of patients with non-O1 *V. cholerae* bacteremia, reported that the case-fatality rate for 13 cases in which the outcome was known was 61.5%. The majority of known infections have occurred in immunocompromised patients, particularly those with hematologic malignancy or cirrhosis. Pitrak and Gindorf[342] reported a patient with bacteremic cellulitis caused by non-O1 *V. cholerae* that was acquired in a freshwater inland lake in northern Illinois.

Pathophysiology of* Vibrio cholerae *-induced gastroenteritis. *V. cholerae* is the prototype of diarrheal syndromes in which disease is caused not by tissue invasion of microorganisms but through the production of toxins that interrupt normal intraintestinal exchanges of water and electrolytes. Toxigenic strains produce a toxin that binds to a receptor on the epithelial cell membrane and activates adenylate cyclase, causing increased levels of cyclic adenosine monophosphate (cAMP) and hypersecretion of salt and water, resulting in the characteristic “rice water” diarrhea of

cholera. Box 8-4 provides a brief account of the step-by-step sequence for *V. cholerae*-induced gastroenteritis. Figure 8-1 is a schematic illustration of the mode of action of the cholera toxin. For a comprehensive review of cholera including pathogenesis and virulence factors, the reader is referred to publications by Kaper and colleagues and Sack and colleagues.[208,366]

Treatment and prevention of V. cholerae infections. Most *V. cholerae* are rapidly killed by tetracycline; however, fluid secretion may persist for several hours after treatment from the effect of toxin already bound to the mucosal cells. Correction of fluid and electrolyte losses is essential with as much as 1 L or more of fluid per hour required. Antibiotic therapy (e.g., trimethoprim-sulfamethoxazole or tetracycline) will

BOX 8-4

Events Leading to *V. cholerae*-Induced Gastroenteritis

1. Organisms ingested in contaminated water must first pass the highly acidic secretions in the stomach. An estimated 10^{10} organisms per milliliter are required to survive gastric passage in healthy persons; only about 100 organisms per milliliter are required in hypochlorhydric persons, either because of previous gastrectomy or from ingestion of antacids in treatment of gastric ulcer disease.
2. To cause disease, *V. cholerae* bacterial cells must adhere to the gastric and intestinal mucosal epithelial cells. These bacteria are motile and secrete mucin, two properties that aid in the penetration of the protective mucin layer that coats the surface of the gastroenteric mucosa. Bacterial attachment is a complex mechanism requiring the recognition by the bacterial cells of a surface marker on the epithelial cells to which they can bind.
3. *V. cholerae* produces an enterotoxin molecule composed of two subunits: an A (active) subunit and a B (binding) subunit. The A subunit is composed of two peptides: A_1 with toxin activity and A_2, which facilitates penetration of the A subunit into the cell. The B subunit binds the toxin molecule to cholera toxin–specific G_{M1} ganglioside receptors on the intestinal epithelial cell membrane. There are five B subunits per toxin molecule, arranged in a ring around a central core that contains the enzyme A1. Initial binding occurs rapidly, followed by a slow conformational change in the toxin molecule, leading to internalization of the A_1 enzyme into the host cell; thus, there is a short lag phase (15–60 minutes) between the time cholera-infected water is ingested and the onset of symptoms. Through a series of steps, A_1 catalyzes the ADP-ribosylation of the G_s (stimulatory) regulatory protein, locking it in the active state. The G_s protein acts to return adenylate cyclase from its inactive to active form, which in turn causes an intracellular rise in cAMP (Figure 8-1).[282]

4. cAMP prevents the reabsorption of sodium ions across the brush-border membrane of the intestinal epithelial cell and the excretion of sodium bicarbonate and potassium into the bowel lumen. The intestinal chyle has high concentrations of sodium and chloride (isotonic), bicarbonate (twice that of plasma), and potassium (three to five times that of plasma). Water, therefore, is passively passed from the epithelial cells into the intestinal lumen in response to high osmotic pressure gradients, following the old adage, "where goes the sodium, there goes the water."
5. Thus, there is diffuse fluid secretion from the gut epithelial cells and the accumulation of large quantities of water in the intestinal lumen. The rate of fluid production increases between 3 and 10 hours after exposure. Fluid loss persists for up to 5 days in patients who receive no antibiotics, after which the bacterial cells are washed from the bowel by an unknown host mechanism. The result is varying degrees of dehydration and electrolyte imbalance, which can lead to metabolic acidosis, hypokalemia, shock, and death in extreme cases.

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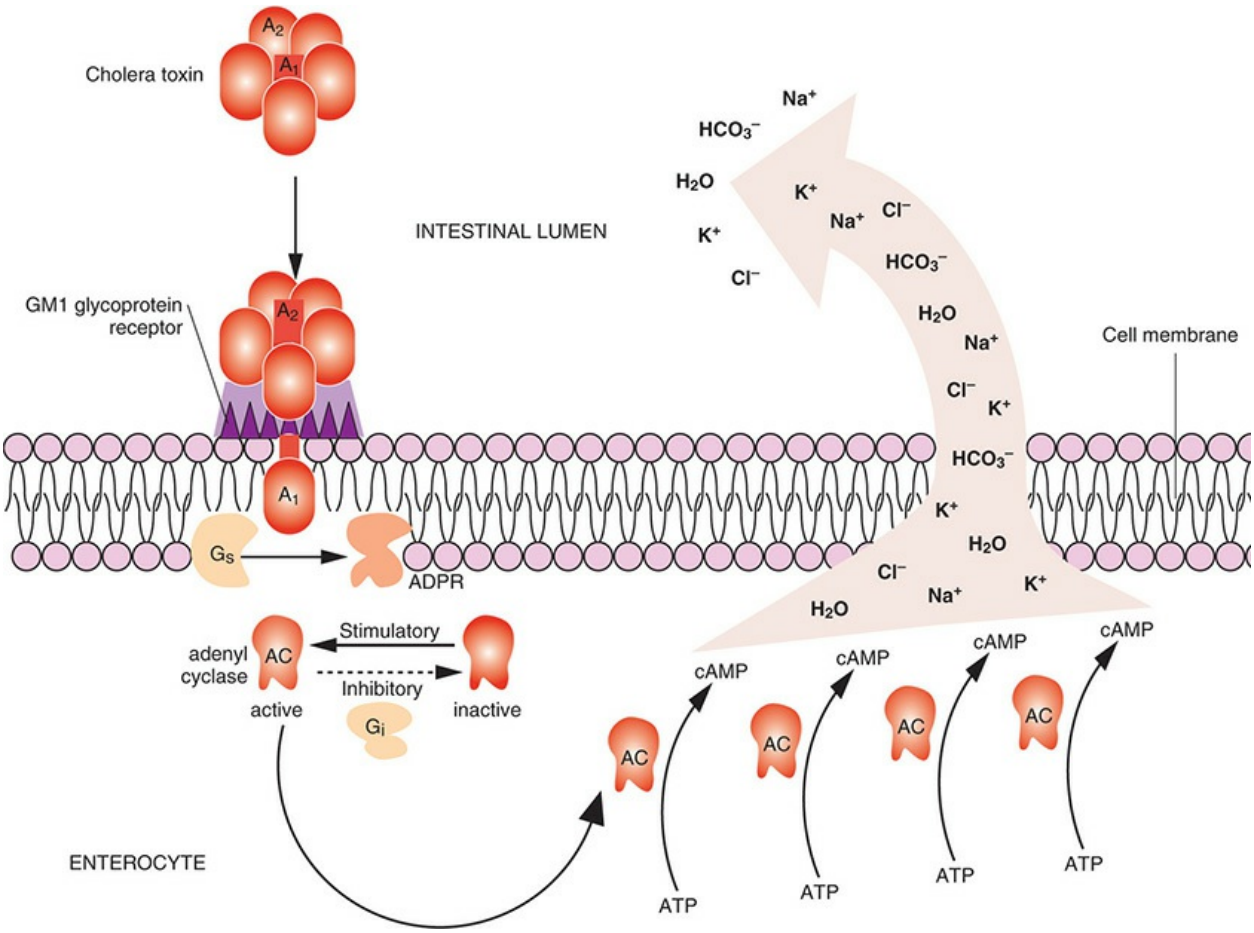

■ **FIGURE 8-1** The action of cholera toxin (see Box 8-4 for details).

help shorten the duration of the diarrhea and reduce the period of carriage; however, antibiotics should be considered ancillary to vigorous rehydration. Additionally, antimicrobial-resistant strains of *V. cholerae* have emerged during the past few decades.[294,366] The risk for cholera and traveler's diarrhea can be reduced by following the general rule "boil it, cook it, peel it, or forget it." In particular, travelers should not consume (1) unboiled or untreated water or ice made from such water; (2) food and beverages from street vendors; (3) raw or partially cooked fish and shellfish, including ceviche; and (4) uncooked vegetables. Cold seafood salads are particularly hazardous. Considerable work has been done regarding development of oral cholera vaccines.[366] Two oral vaccines are licensed in several countries outside the United States. One consists of killed *V. cholerae* plus the cholera B subunit; the other is an avirulent strain of *V. cholerae*. Currently no cholera vaccine is commercially available in the United States.

Noncholera Vibrios. Most *Vibrio* infections in the United States have been caused

by nonepidemic species other than *V. cholerae.* The natural habitat and geographic distribution, the culture media required for optimal recovery, the key biochemical reactions, and the clinical syndromes associated with the noncholera species of human importance are listed in Table 8-9. Although these *Vibrio* species are "noncholera," they should not be dismissed, as many strains can cause severe diarrheal disease in addition to extraintestinal infections that can result in fatal septicemia. In most intestinal infections, however, the symptoms are less severe and of shorter duration than that experienced in classic epidemic cholera.

Certain species of noncholera vibrios can produce enterotoxins similar to those described for *V. cholerae.* In addition, some species cause invasive disease and more closely simulate *Shigella* dysentery; other species, *V. vulnificus* in particular, may invade the intestinal lymphatics and result in septicemia. Extraintestinal *Vibrio* infections are most commonly cutaneous wounds or otitis externa, where breaks in the skin have become contaminated while swimming or boating in marine environments or after handling contaminated raw seafood.[276,377] The following is a brief account of the microbiology and clinical syndromes associated with each of the noncholera *Vibrio* species reported to cause human infection.

Vibrio alginolyticus. *V. alginolyticus* was originally classified as biotype 2 of *V. parahaemolyticus.* Most clinical

TABLE 8-9 Characteristics of Clinically Important *Vibrio* Species Other Than *V. cholerae*

Species	Natural Habitat, Geographic Distribution; Modes of Human Infection	Media for Optimal Growth in Laboratory Cultures	Key Biochemical Reactions		Clinical Syndromes
V. alginolyticus	Habitat: marine environment Infection: exposure of traumatized skin to seawater or infected animals	NaCl supplement needed for growth on nonselective media. Grows on blood agar and enteric media. Yellow colonies on TCBS agar.	Lysine Arginine Voges–Proskauer 8%, NaCl 10%, NaCl	+ – + + V	Associated with soft tissue infections; seems to have also an etiologic role in wound and ear infections
V. damsela CDC Group EF-5 (transferred to genus *Photobacterium*)	Habitat: marine environment Infection: exposure of broken skin or traumatic wounds to infected marine animals or contaminated seawater	Requires 1% NaCl in nonselective culture media. Good growth on blood agar. Green colonies on TCBS agar. Optimum growth 25°C.	Arginine Voges–Proskauer Ferment: Glucose Mannitol Galactose Trehalose	+ + + – + +	Associated with human wound infections
V. fluvialis	Habitat: worldwide—endemic in Bangladesh. In the United States—Gulf Coast, New York, and Pacific Northwest estuaries. Infection: ingestion or contact with contaminated water	Na^+ supplement of culture media less critical than other halophilic *Vibrio* species. Yellow colonies on TCBS agar.	Arginine 6% NaCl Glucose (gas) Hydrolysis of esculin Aminovalerate Glutarate	+ + – – + –	Cholera-like gastroenteritis and diarrheal syndrome—watery diarrhea, vomiting, dehydration; probably enterotoxin induced
V. furnissii	Habitat: endemic in the marine waters and estuaries in Asia Infection: ingestion or contact with contaminated water	Na^+ supplement for optimal growth in nonselective culture media. Yellow colonies on TCBS agar.	Glucose (gas) Hydrolysis of esculin Aminovalerate Glutarate	+ – – +	Isolated from patients with diarrhea and gastroenteritis, particularly tourists returning from Asia
V. hollisae (transferred to genus *Grimontia*)	Habitat: marine environment in Gulf Coast and Chesapeake Bay states Infection: consumption of raw seafood	1%–2% NaCl supplement needed for growth. Poor growth on TCBS or MacConkey agars. Screen for oxidase-positive colonies on blood agar.	Indole Lysine Arginine Ornithine Motility (after 7 days) Urea	+ – – – – –	Isolated from patients with diarrhea and gastroenteritis. Invasion of bloodstream reported in persons with liver abnormalities.
V. metschnikovii	Habitat: worldwide in fresh and brackish marine waters, rivers, sewage; also in shrimp, crabs, and lobsters Infection: cause of fowl cholera; exposure or ingestion of contaminated water or animals	Grows well on most laboratory isolation media. Sodium supplement not as critical as other halophilic vibrios. Yellow colonies on TCBS agar.	Oxidase Nitrate Voges–Proskauer	– – +	Associated with rare and isolated human infections: septicemia, urinary tract infections, wounds, peritonitis
V. mimicus	Habitat: coastal waters, and oysters and shrimp Infection: ingestion of undercooked seafood, particularly oysters	Grows on enteric isolation media. Green colonies on TCBS.	Sucrose Mannitol Ornithine Lipase Voges–Proskauer Polymyxin	– + + V – S	Diarrheal syndrome related to production of heat-labile and heat-stable toxins; also swimmer's ear infections
V. parahaemolyticus	Habitat: worldwide distribution in fresh- and seawaters. Endemic in Japan. Infection: ingestion of contaminated seafood—raw fish and shellfish	Grows slow on nonselective media. Screen for oxidase-positive colonies on blood agar. Green colonies on TCBS agar.	Lysine Arginine Voges–Proskauer Lactose Salicin Urease Indole	+ – – – – V +	Acute gastroenteritis—nausea, vomiting, abdominal cramps, fever, chills. Positive Kanagawa test (i.e., clear hemolysis on specially prepared media). Extraintestinal: wounds and septicemia.
V. vulnificus (lactose-positive *Vibrio*)	Habitat: coastal water and estuaries Infection: ingestion of raw oysters; exposure of traumatic wounds to infected marine animals or contaminated water	1% NaCl needed for growth. Growth good on blood agar. Green (85%) or yellow (15%) colonies on TCBS agar.	Lactose Lysine Arginine Salicin	+ + – +	Life-threatening septicemia; 50% fatality rate. High association with preexistent liver disease. Wounds are painful, with skin and muscle necrosis.

S, susceptible, 90% or greater; +, 90% or more of strains are positive; −, 90% or more of strains are negative; V, 11%–89% of strains are positive.

isolates are recovered from superficial wounds[109,271,338,374] or the external ear.[159,197] Conjunctivitis,[256] acute gastroenteritis,[350] bacteremia,[44,109,194] and necrotizing fasciitis[143] caused by *V. alginolyticus* have also been reported.

Vibrio cincinnatiensis. Human infection caused by this *Vibrio* species is rare. It has been recovered from blood and cerebrospinal fluid of a 70-year-old patient with bacteremia and meningitis.[46] The patient was treated with ampicillin (day 1) and moxalactam for 9 days, followed by an uneventful recovery.

Vibrio damsela (Photobacterium damsela). *P. damsela* was formerly called CDC Group EF-5, *V. damsela*, and *Listonella damsela*. It has been reported as the cause of human wound infections, primarily after exposure to saltwater.[86,177,295] Most strains are resistant to penicillin, but susceptible to gentamicin, chloramphenicol, and tetracycline. MacDonell and Colwell[261] proposed transfer of *V. damsela* to the genus *Listonella.* Subsequently, Smith and colleagues proposed that *L. damsela* should be reassigned to the genus *Photobacterium*.[386]

Vibrio fluvialis. Formerly designated as CDC Group EF-6, *V. fluvialis* has been isolated from humans with diarrhea. This species was isolated from fecal cultures of more than 500 patients with diarrhea at the Cholera Research Laboratory in Bangladesh during a 9-month period from 1976 to 1977.[184] In the United States, the organism has been isolated from a wound of a patient in Hawaii, from water and sediment in the New York Bay, from shellfish in Louisiana, from water and shellfish in Pacific Northwest estuaries, from an 81-year-old man from Laredo, Texas, with diarrheal illness,[408] from the stool of a 1-month-old infant,[169] and from the stool of a 43-year-old man with a history of AIDS.[176]

Vibrio furnissii. Formerly designated *V. fluvialis* biogroup 2, *V. furnissii* has been isolated from patients with acute gastroenteritis in at least two outbreaks of food poisoning[48] and from the stool of a 1-month-old infant.[169]

Vibrio harveyi. *V. harveyi* (formerly *V. carchariae)*[334] is a pathogen of sharks, but has been reported in a human wound infection in an 11-year-old girl who was attacked by a shark while wading in knee-deep water off the South Carolina coast.[333] *V. harveyi* can be differentiated from biochemically similar species (*V. alginolyticus, V. parahaemolyticus*, and *V. vulnificus*) by negative gelatin hydrolysis at 22°C, negative motility at 36°C, and negative ornithine decarboxylase reaction. The other species have the opposite reactions.

Vibrio hollisae (Grimontia hollisae). Formerly designated as CDC Group EF-13, and reclassified as *Grimontia hollisae* in 2003,[420] this species has most commonly been isolated from stool specimens of persons with diarrhea and abdominal pain and a

history of eating raw seafood.[4,168,295] Rare patients with systemic infection caused by *G. hollisae* have been described and most often involve bacterial sepsis in persons with underlying immune deficiencies.[173,258,348] Evidence suggests that *G. hollisae* may share with *V. vulnificus* a predilection for bloodstream invasion in persons with liver abnormalities.[348]

Vibrio metschnikovii. *V. metschnikovii,* formerly CDC Enteric Group 16, is often isolated from the environment but rarely isolated from human clinical specimens. The first documented human infection was from the blood of a patient with cholecystitis at Cook County Hospital in Chicago.[198] More recently, Hansen and colleagues reported patients with *V. metschnikovii* septicemia: one fatal case involving a patient with liver cirrhosis, renal insufficiency, and diabetes; and another in an 82-year-old woman with respiratory problems and an infected leg lesion, who was treated successfully.[160] Human isolates referred to the CDC for identification include two isolates from urine and four isolates from unknown sources.[112]

Vibrio mimicus. Formerly classified as sucrose-negative *V. cholerae, V. mimicus* has been isolated from shellfish and water as well as from human diarrheal stools and ear infections.[92,377]

Vibrio parahaemolyticus. *V. parahaemolyticus* causes gastroenteritis in humans following ingestion of contaminated seafood,[294] the mechanism of which has not been elucidated. Since 1996, pandemic spread of *V. parahaemolyticus* infections due to a single clone has been reported in several Asian countries.[426] Symptoms include watery and sometimes bloody diarrhea, abdominal cramps, nausea, vomiting, headache, low-grade fever, and chills. The illness is usually mild to moderate and self-limiting, with a duration of 2 to 3 days. Extraintestinal infections by *V. parahaemolyticus* have also been described, mostly from wounds in contact with seawater, although primary septicemia rarely has been reported.[294] A urea-positive biotype has appeared and has been the cause of several recent outbreaks, often associated with ingestion of contaminated raw oysters.[5,238,310,313] Janda and associates[197] reveal that 70% of the *V. parahaemolyticus* cultures submitted for identification to the Microbial Diseases Laboratory in the Department of Health Laboratory at Berkeley, California, were urease positive. Similar findings have been reported in the Pacific Northwest.[217]

More than 95% of *V. parahaemolyticus* strains that have been isolated from patients with diarrhea are Kanagawa positive; that is, they hemolyze human erythrocytes on Wagatsuma agar.[206] The hemolysin is both cytotoxic and cardiotoxic. Only about 1% of strains isolated from marine environments are Kanagawa positive.[197] The implication is that Kanagawa hemolysin activity is associated with the pathogenesis of *V. parahaemolyticus* gastroenteritis. However, the association of Kanagawa hemolysin with pathogenesis has never been proven, and in fact Honda and coworkers[180] report

that 11 of 12 *V. parahaemolyticus* isolates recovered from patients in a 1985 gastroenteritis outbreak in the Maldives were Kanagawa negative. Rehydration is usually the only treatment required.

Vibrio vulnificus. Formerly CDC Group EF-3, *V. vulnificus* was first termed *lactose-positive Vibrio* by Hollis and coworkers in 1976.[177] *V. vulnificus* is a particularly virulent species associated with wound infections after exposure to contaminated seawater and primary septicemias and death following consumption of contaminated seafood, most commonly raw oysters.[74,78,294] A high fatality rate (40% to 60%) is associated with septic infections.[197] The organisms reach the bloodstream by invading the intestinal mucosa. Patients with hepatic disease are particularly susceptible to septicemia.[38,111,407]

Medical conditions that predispose to *V. vulnificus* bacteremia include liver dysfunction and syndromes leading to increased iron deposition: chronic cirrhosis, hepatitis, thalassemia major, hemochromatosis, and a history of heavy alcohol consumption.[197] The chief symptoms associated with sepsis are fever, chills, and vomiting, which begin about

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38 hours after ingesting raw oysters. Diarrhea is often not a component of the syndrome.

Methods for Laboratory Isolation of *Vibrios*. There are four possible laboratory approaches to the isolation of *Vibrio* species from clinical specimens:

1. Use normal procedures, and make no specific effort to search for *Vibrio* species.
2. Use normal procedures and plating media, and look for oxidase-positive colonies.
3. Incorporate thiosulfate citrate bile sucrose (TCBS) agar as an extra plate for stool cultures and also for other likely specimens such as those from wounds, blood, eye, and ear.
4. Use other special procedures to enhance the isolation of *V. cholerae*, *V. parahaemolyticus*, and other *Vibrio* species.

For laboratories in the American Midwest, where positive cultures for *Vibrio* species may be few, the first or second approaches may be most appropriate. In laboratories near oceans, particularly those located in endemic areas, the third or fourth approach may be indicated. However, disadvantages to the routine use of TCBS agar include the increased cost for a relatively low return, and some *Vibrio* species or strains may

not grow well on TCBS agar. It is helpful for laboratory personnel to be informed when clinical cases of cholera or extraintestinal *Vibrio* infections are suspected. In these cases, the use of a selective medium or alkaline broth enrichment, to be discussed in a later section, may still be in order. In such instances, a *Vibrio* culture may be offered. However, when this is considered, education of the medical staff regarding when this test should be ordered is important to assure appropriate utilization.

Specimen Collection, Processing, and Media Selection. Laboratory personnel should be notified if the physician suspects a cholera syndrome or extraintestinal infections with *Vibrio* species. Specimens should be collected as early in the disease as possible. In the acute diarrheal stages of disease, specimens may be collected from the rectum with a soft rubber catheter or a rectal swab or from a small portion of the passed liquid stool. Culturing of vomitus may also be productive of organisms, particularly in the early stages of disease.

Specimens should be transported in closed containers to preserve moisture and transferred to culture media as soon as possible. *Vibrio* species are generally quite sensitive to drying, exposure to sunlight, and the development of an acid pH. They are also easily inhibited by the normal intestinal flora or contaminating organisms. If cultures cannot be set up immediately, *Vibrio* species will remain viable in Cary–Blair semisolid transport medium for an extended time. The use of buffered glycerol saline transport medium should be avoided. If a transport medium is not available, a 2 × 1/2-inch strip of thick blotting paper can be soaked in the fecal specimen, placed in a sealed plastic bag, and then mailed to the nearest reference laboratory.[215] Specimens suspicious for harboring *Vibrio* species should be inoculated to 5% sheep blood and MacConkey agar. Whether to also inoculate a plate of TCBS agar and/or a tube of alkaline peptone water (APW) enrichment must be determined by each laboratory supervisor depending on the prevalence of *Vibrio*-related diseases in any given locale. If TCBS agar is not used, hemolytic colonies that appear on sheep blood agar after overnight incubation should be tested for cytochrome oxidase activity. Either representative colony can be individually touched and spot tested for the oxidase reaction using Kovac's reagent or one or two drops of Kovac's reagent can be dropped in an area on the surface of the plate where suspicious colonies are present. The rapid development of a blue color is indicative of a positive test. Oxidase-positive colonies can be transferred to TCBS agar for further species identification using biochemical and other characteristics.

APW enrichment broths should be subcultured to TCBS agar for further evaluation of colonies that grow after an additional 24 to 48 hours of incubation. APW, which contains 1% peptone and 1% NaCl at pH 8.6, is a simple-to-use enrichment broth that can be recommended in situations where low concentrations of organisms in the specimen are anticipated (e.g., in convalescent stages of disease). The high pH of the

medium serves to suppress the growth of many commensal intestinal bacteria while allowing uninhibited multiplication of *V. cholerae.* Subcultures to TCBS or gelatin agar should be made within 12 to 18 hours, since other organisms can begin to overgrow in the broth after prolonged incubation. APW is also an excellent transport medium if specimens cannot be immediately delivered to the laboratory for processing. It is recommended that about 1 mL of liquid or 1 g of formed stool be placed into 10 mL of APW in a screw-capped tube; alternatively, rectal swabs can be placed into a tube containing 1 to 2 mL of APW.[215]

Presumptive Identification of *Vibrio* Species Based on Colonial and Microscopic Morphology. Vibrios grow readily on most isolation media; growth of all species is enhanced by adding 1% NaCl to the medium. Colonies are typically smooth, convex, creamy in consistency, gray-white, and have entire margins. Rough colonies are occasionally encountered that adhere to the agar. Certain marine vibrios are able to swarm on the surface of agar media, associated with the formation of long cells with lateral flagella. This phenomenon is not seen with most human isolates.

Microscopically, straight or curved gram-negative bacilli are observed (Image Plate 8-2D). The curved character of the cells may be best seen in early stationary phase in broth cultures; in log phase, straight and rounded coccoid forms are intermixed. Although a presumptive diagnosis of cholera can be made by observing large numbers of curved bacilli in direct gram-stained stool specimens, recovery of the organism in culture is needed to make a definitive identification.

The differential reactions on TCBS agar are helpful in making a presumptive identification of *V. cholerae, V. alginolyticus, V. parahaemolyticus*, and *V. vulnificus.* After 18 to 24 hours of incubation on TCBS agar, *V. cholerae* grows as smooth, yellow colonies, 2 to 4 mm in diameter with an opaque center and transparent periphery (Image Plate 8-2A). The colonies of *V. alginolyticus,* which also ferment sucrose, will also produce yellow colonies on TCBS agar; *V. parahaemolyticus* and *V. vulnificus,* which do not utilize sucrose, produce blue-green colonies (Image Plate 8-2B). On gelatin agar, *V. cholerae* grows as transparent colonies surrounded by an opaque halo indicating liquefaction of gelatin (Image Plate 8-2C). O'Brien and Colwell have described a

modified taurocholate–tellurite gelatin agar for the differentiation of *V. cholerae* (β-galactose positive) versus *V. parahaemolyticus* (β-galactose negative) based on the hydrolysis of 4-methylumbelliferyl-β-D-galactose, in addition to determination of gelatin hydrolysis and tellurite reduction.[314]

Biochemical Characterization and Laboratory Identification of *Vibrio* Species.

Members of the genus *Vibrio* are facultative anaerobes capable of both respiratory and fermentative metabolism. However, because they grow and react in carbohydrate test media designed for fermentative metabolism, they are classified with the fermenters. The natural habitat for *Vibrio* species is aquatic, in both fresh and salt water. The growth and biochemical reactivity of most species are enhanced in differential test media supplemented with 1% to 2% sodium chloride.

Most *Vibrio* species produce cytochrome oxidase, a characteristic that separates them from the *Enterobacteriaceae*. Therefore, *Vibrio* species are included in the group of oxidase-positive fermenters—*Aeromonas* species, *Plesiomonas* species, and *Chromobacterium* species—from which they must be differentiated (Table 8-10). Since *V. cholerae* ferments glucose, an acid-deep/alkaline-slant reaction is seen on Kligler's iron agar. Since sucrose is also fermented, an acid-deep/acid-slant reaction is seen on triple sugar iron agar. *V. cholerae* produces both lysine and ornithine decarboxylases.

Those laboratory workers who use lysine iron agar to screen stool isolates will note that *V. cholerae*, *A. hydrophila*, and *Plesiomonas shigelloides* all produce a purple slant/purple deep reaction because of the decarboxylation of lysine. Arginine can be used to separate *V. cholerae* (negative) from both *Aeromonas* and *Plesiomonas* (positive). Most strains of *A. hydrophila* hydrolyze esculin, differentiating it from the other organisms included in Table 8-10. Differences in the utilization of lactose, sucrose, mannitol, and inositol also serve to differentiate these genera.

V. cholerae, including the El Tor biotype, can be distinguished from other *Vibrio* species by the ability to produce a positive string test (Image Plate 8-2E). To perform this test, bacterial colonies are mixed with a few drops of 0.5% sodium deoxycholate on a glass slide. An inoculating loop is immersed into the mixture and pulled away from the drop. *V. cholerae* produces a long string that becomes more tenacious after 60 seconds or more (other vibrios may give an initial string reaction that diminishes or disappears 45 to 60 seconds later). A positive slide agglutination with

TABLE 8-10 Oxidase-Positive, Fermentative, Gram-Negative Bacilli: Differential Characteristics of *Aeromonas hydrophila, Plesiomonas shigelloides, Chromobacterium violaceum*, and *Vibrio cholerae*

Characteristics	*A. hydrophila*	*P. shigelloides*	*C. violaceum*	*V. cholerae*
Kligler's iron agar (slant/deep/ hydrogen sulfide)	K/A/–	K – A/A/–	K/A/–	K/A/–
Catalase	+	+	+	+
Esculin	+	–	–	–
Motility	+	+	+	+
ONPG	+	+	–	+
Indole	+	+	–	+
Voges–Proskauer	(+)	–	–	(–)
Lysine decarboxylase	+	+	–	+
Ornithine decarboxylase	–	+	–	+
Carbohydrates:				
Lactose	(–)	(+)	–	–
Sucrose	+	–	(–)	+
Mannitol	+	–	–	+
Inositol	–	+	–	–
Growth in 1% peptone with:				
0% NaCl	+	+	+	+
7% NaCl	–	–	–	–
11% NaCl	–	–	–	–

+, 90% or more of strains are positive; (+), 51%–89% of strains are positive; (–), 51%–89% of strains are negative; –, 90% or more of strains are negative; V, variable; K/A, alkaline slant/acid deep; K – A/A, alkaline to acid slant/acid deep; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

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TABLE 8-11 Differentiation Between *Vibrio cholerae* Biotypes

Test	Classic	El Tor
String test	+	+
β-Hemolytic on sheep blood agar	–	+
CAMP test	–	+
Voges–Proskauer test	–	+
Chicken red blood cell agglutination	–	+
Susceptibility to 50 U polymyxin B	S	R
Phage IV susceptibility	S	R

+, positive test; −, negative test; S, susceptible; R, resistant.

polyvalent O antiserum is also helpful in differentiating *V. cholerae* from other closely related strains (Image Plate 8-2F). The El Tor biotype can be distinguished from classic strains of *V. cholerae* by several characteristics (Table 8-11). El Tor strains are actively β-hemolytic on blood agar (Image Plate 8-2G) and are capable of agglutinating chicken erythrocytes (Image Plate 8-2H). The chicken erythrocytes test is performed by mixing a loopful of washed chicken erythrocytes (2.5% suspension in saline) with bacterial cells from a pure culture to be tested. Visible clumping of the erythrocytes indicates the El Tor biotype, in contrast to classic O1 strains of *V. cholerae* that do not have this property. Classic strains of *V. cholerae* are susceptible to 50 IU of polymyxin B in the disk diffusion test; El Tor strains are resistant. El Tor strains are also Voges–Proskauer positive, whereas classic strains of *V. cholerae* are Voges–Proskauer negative. Lesmana and coworkers[255] described using a modified CAMP test to differentiate the classical biotype (CAMP negative) from the El Tor biotype (CAMP strong positive). *V. cholerae* O139 strains also demonstrate a strong positive CAMP reaction, whereas non-O1 and non-O139 isolates give a weak positive CAMP reaction. The test is performed by inoculating a β-lysin-producing *Staphylococcus aureus* strain (ATCC 25178) onto a 5% sheep blood agar plate by making a single straight line streak and then inoculating the *Vibrio* species to be tested in a line perpendicular to and a few millimeters from the *S. aureus* streak. Plates are incubated in a candle jar at 37°C for 18 to 20 hours and observed for zones of synergistic hemolysis (Chart 8-1). For laboratories capable of performing phage IV susceptibility tests, El Tor strains are resistant to this phage.

As an initial first step in the identification of *Vibrio* species, Kelly and colleagues[215] have devised a dichotomous scheme for separating the *Vibrio* species into six groups based on their reactions with seven tests: requirement for 1% NaCl for growth in nutrient broth, oxidase production, reduction of nitrate to nitrite, *myo*-inositol fermentation, and production of arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase. Their scheme is reproduced in Table 8-12. Grouping the species this way will provide simple presumptive identification of most clinical isolates. Additional key reactions and clinical correlation can be obtained by consulting Table 8-9. Overman and associates[322] recommended the use of the API 20E strip for performing the biochemical tests necessary to make identifications of the more commonly encountered members of the *Vibrionaceae*. However, a more recent evaluation of commercially available identification systems showed that the six methods evaluated correctly identified only 63% to 81% of the *Vibrio* tested to the species level.[315] For *V. cholera* in particular, accuracy ranged from 50% to 97%.

Thus, the authors concluded that extreme care should be taken in interpreting results of commercial systems for identification of *Vibrio* species.

Nonculture Methods. An immunochromatographic dipstick test for rapid detection of *V. cholerae* O1 and O139 from stool specimens has been developed. In an evaluation of the test in Madagascar and Bangladesh, where cholera is endemic, sensitivity ranged from 94% to 100% and specificity from 84% to 100%.[305] Bhuiyan and colleagues modified the procedure by performing a 4-hour enrichment in APW prior to the dipstick assay.[35] With this added step, the sensitivity and specificity, respectively, were 96% and 92% for *V. cholerae* O1 and 93% and 98% for *V. cholerae* O139.

MALDI-TOF MS. Rychert et al.[365] evaluated the Vitek MS v2 MALDI-TOF MS platform with Knowledgebase 2.0 (bioMérieux, Marcy l'Étoile, France). The Vitek MS identified all 42 isolates of *V. cholera* O1 and O139 and 7 of 9 non-O1/O139 isolates. Cheng et al.[77] evaluated the Bruker Biotyper MALDI-TOF MS (Bruker Daltonics Inc. Billerica, MA) for ability to identify genetically confirmed *Vibrio* species that were recovered from patients with bloodstream infections. The Bruker MS identified all isolates of *V. vulnificus* (n = 20), *V. parahaemolyticus* (n = 2), and *V. fluvialis* (n = 1), but none of the isolates of nonserogroup O1, non-O139 *V. cholera* (n = 33) to the species level due to the fact that *V. cholera* was not included among the 53 *Vibrio* species listed in the Bruker Biotyper MALDI-TOF MS library (DB 5627).

Listonella, Photobacterium, and *Shewanella*

MacDonell and Colwell[261] proposed restructuring of the *Vibrionaceae* to include the establishment of two new genera, *Listonella* and *Shewanella,* within the family. Through rather complex 5S rRNA ribonucleotide sequencing studies and cluster analysis, these authors concluded that several *Vibrio* species, notably *V. anguillarum, V. pelagius*, and *V. damsela*, should be transferred to a proposed new genus *Listonella* as *L. anguillara, L. pelagia*, and *L. damsela*. They also conclude from these studies that *Alteromonas* (*Pseudomonas*) *putrefaciens* and *Alteromonas hanedai* should comprise the new genus *Shewanella* along with a proposed new species, *Shewanella benthica.* Subsequent to the work of MacDonnell and Colwell, Smith and colleagues proposed that *L. damsela* be reassigned to the genus *Photobacterium* as *P. damsela* based on phenotypic data.[386] Members of the genera *Listonella, Photobacterium*, and *Shewanella* are associated with marine environment and are pathogenic for fish.

TABLE 8-12 Key Differential Tests to Divide the 12 Clinically Significant *Grimontia, Photobacterium*, and *Vibrio* species Into 6 Groups

	Reactions of the Species in:											
	Group 1		Group 2	Group 3	Group 4	Group 5			Group 6			
Test	*V. cholera*	*V. mimicus*	*V. metschnikovii*	*V. cincinnatiensis*	*G. hollisae*	*P. damsela*	*V. fluvialis*	*V. furnissii*	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*	*V. carchariae*
Growth in Nutrient Broth With												
No NaCl	+	+	–	–	–	–	–	–	–	–	–	–
1% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	–	+	+	+	+	+	+	+	+	+
Nitrate →nitrite	+	+	–	+	+	+	+	+	+	+	+	+
myo-Inositol fermentation	–	–	V	+	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	V	–	–	+	+	+	–	–	–	–
Lysine decarboxylase	+	+	V	V	–	V	–	–	+	+	+	+
Ornithine decarboxylase	+	+	–	–	–	–	–	–	V	+	V	–

All data are for reactions within 2 days at 35°C–37°C. +, most strains (generally 90%–100%) are positive; –, most strains are negative (generally 0%–10% positive); V, between 11% and 89% are positive. Key test results are shaded.
Data from reference 215.

Members of the genus *Shewanella* (notably *S. putrefaciens*) are straight or curved, gram-negative bacilli, motile by means of a single polar flagellum. Characteristic colonies are dome shaped, circular, slightly viscous or mucoid, and usually red-brown or salmon-pink. They possess cytochrome oxidase activity and produce abundant hydrogen sulfide in Kligler's iron agar. Nitrates are reduced to nitrites, and gelatinase, ornithine decarboxylase, and DNase tests are positive. *S. putrefaciens* has been recovered from human clinical specimens and is discussed in detail in Chapter 7 of this text.

Aeromonas and *Plesiomonas*

Several years ago, *Aeromonas* species were included along with *Vibrio* species and *P. shigelloides* in the *Vibrionaceae*.[29] However, based on molecular genetic evidence, *Aeromonas* species have been placed in a separate family, *Aeromonadaceae*.[88,261] It has also been proposed that *Plesiomonas* be placed in the *Enterobacteriaceae* as a member of the genus *Proteus*. Phenotypic differences between *Vibrio, Aeromonas*, and *Plesiomonas* species are listed in Table 8-10.[139]

Genus Aeromonas

As the species name hydrophila ("water loving") indicates, the natural habitat of

Aeromonas species is freshwater or seawater, where they commonly cause infectious diseases in cold-blooded aquatic animals. These bacteria also reside in sink traps and drainpipes and can be recovered from tap water faucets and distilled water supplies, which are potential sources of organisms involved in nosocomial infections.

Taxonomy. The genus *Aeromonas* has undergone considerable taxonomic and nomenclature revisions during the past several years. Only five species were recognized in 1988.[196] By 2000, the number of valid published genomospecies had risen to 14.[204] At the time of this writing, the genus *Aeromonas* includes 32 recognized species and 12 subspecies (http://www.bacterio.net/aeromonas.html). The genus *Aeromonas* is divided into two groups: psychrophilic and mesophilic. *A. salmonicida*, a fish pathogen, is the only species in the psychrophilic group. It is nonmotile and does not grow at 37°C. The mesophilic group of motile species are potential human pathogens. A group of *Aeromonas*-like bacteria that were mannitol, sucrose, and indole negative and known by the vernacular name CDC Enteric Group 501 were found in 1988 to be a new species of *Aeromonas* and were named *A. schubertii*[170] One of the original eight strains of CDC Enteric Group 501 was found to be only 61% related to the type strain of *A. schubertii* and was retained within the vernacular name *Aeromonas* group 501, also named *Aeromonas* sp. HG13. This strain deviated from the *A. schubertii* phenotype by being indole positive and lysine decarboxylase negative[170] and was subsequently assigned to a new species, *A. diversa*.[285]

Clinical Significance. Four categories of human infections have been described: gastroenteritis, cellulitis and wound infections, septicemia, and miscellaneous. However, as our knowledge of *Aeromonas* has grown, the miscellaneous category of diseases they cause has expanded.[191]

Gastroenteritis. Janda and others[190,191,193] have reviewed the infectious disease spectrum of *Aeromonas* species and concluded that there is strong evidence supporting *Aeromonas* species as a causative agent of diarrhea, although the data are not absolute. On one hand, they cite several reports indicating that there is little significant difference between symptomatic and asymptomatic persons who harbor *Aeromonas* species in their stools.[119,178,423] In contrast, other reports link *Aeromonas* species with gastroenteritis.[53,148] Watson and associates[445] proposed that the virulence factors of *Aeromonas* species that cause intestinal infections are similar to those of other enteric pathogens, that is, adherence of bacterial cells to intestinal mucosa, toxin production, and mucosal invasion. About 20% of their patients with intestinal infections from *Aeromonas* species had symptoms of dysentery similar to those caused by *Shigella* species and by invasive strains of *C. jejuni*. Most of the invasive strains included in their study, as determined by invasiveness in cell culture, were *A. sobria*; a minority

were *A. hydrophila.* The majority of *A. sobria* and *A. hydrophila* produce a cholera-like extractable toxin (Asao toxin) that causes watery diarrhea.[80,311] The previous notion that *A. caviae* does not produce an enterotoxin, is not invasive, and is not considered to be a human pathogen[140] may be questioned in light of a few reports in which this species was found to be a cause of gastroenteritis.[17,133,303,304]

Cellulitis and Wound Infections. Wounds, ranging in severity from mild, primarily cutaneous infections (e.g., cellulitis) to severe infections involving muscle, joints, and/or bone, are the second most common type of infection caused by *Aeromonas* species, after gastroenteritis. Clinical settings associated with *Aeromonas* wound infections include lacerations/abrasions associated with water sports, puncture wounds or other penetrating injury, crush injuries (e.g., motor vehicle accident), and invasive medical procedures (e.g., intra-abdominal surgery, catheterization).[191] In a fraction of cases, no preceding trauma or precipitating event is recognized.

Septicemia. Four categories of persons who develop *Aeromonas* septicemia have been proposed, based on portal of entry, underlying disease state, immune status, and exposure to freshwater. The two most common of these groups are immunocompromised adults and infants less than 2 years of age who have complicating underlying medical conditions (e.g., malignancy, hepatobiliary disease, diabetes) and *Aeromonas* in their GI tract. *Aeromonas* septicemia in these groups of patients can be rapidly fatal, especially in those with hepatic cirrhosis or underlying malignancy.[247] A third group are persons who develop secondary *Aeromonas* sepsis from trauma-related myonecrosis. The fourth, and smallest, group includes adults with no underlying defects, who are exposed to freshwater sources. *Aeromonas* bacteremia in burn patients has also been described.[25] *Aeromonas* septicemia is caused in most instances by one of three species: *A. hydrophila, A. veronii*, or *A. caviae.*[191,192,229] *A. schubertii* has also been responsible for infections in a few patients with bacteremia.

Miscellaneous *Aeromonas* Infections. Uncommon presentations of *Aeromonas* infections include urinary tract

infections, hepatobiliary disease, meningitis, ear infections, endocarditis, hemolytic uremic syndrome, peritonitis, respiratory tract disease, and ocular infections.[23,42,64,85,296] Posttraumatic brain abscess caused by *A. hydrophila* was reported in a patient following an assault during which the patient sustained a right frontal scalp laceration.[262] Only a few cases of *Aeromonas* meningitis have been documented in adults. In one patient, meningitis occurred after a craniotomy following head trauma.[345] In other patients with *Aeromonas* meningitis, the infection has occurred in association with medicinal leech therapy,[321] and following exposure to

compost, where inhalation of the organism was the proposed mechanism of entry.[131] Two pediatric patients with *Aeromonas* meningitis have also been reported.[376,455]

Infections Caused by Less Commonly Isolated *Aeromonas* species. A number of less commonly isolated *Aeromonas* species have been described to cause human infections.

- ***A. caviae*** has been reported to cause keratitis associated with contamination of a contact lens case.[339]
- ***A. dhakensis*** (formerly *A. hydrophila* subsp. *dhakensis* and *A. aquariorum*)[31] has been reported in severe soft tissue and bloodstream infections,[117,229,449,450,451]
- ***A. enteropelogenes*** (formerly *A. trota*)[185] has been isolated from human feces and human appendix,[61] and reported to cause gastroenteritis in a child[351] and wound infection accompanied by septic shock in a patient with liver cirrhosis.[237] It has a unique biochemical profile, including negative reactions for esculin hydrolysis, arabinose fermentation, and the Voges–Proskauer test; positive reactions for cellobiose fermentation, lysine decarboxylation, and citrate utilization; and susceptibility to ampicillin.[61,87] This finding may invalidate the use of ampicillin-containing selective media (discussed in the next section) for screening stool specimens for *Aeromonas* species.
- ***A. jandaei*** (formerly genospecies DNA Group 9; *A. sobria*). This organism is sucrose, esculin, and cellobiose negative. It has been isolated from blood, wounds, and stool.[62]
- ***A. schubertii*** (formerly CDC Enteric Group 501)[170] has been incriminated as the cause of wound infections.[63]
- ***A. veronii*** (formerly CDC Enteric Group 77).[171] This organism is ornithine decarboxylase positive and has been reported to cause bacteremia, wound infections, and diarrhea.[6,171,205]

***Aeromonas* Species in Medicinal Leeches.** The medicinal leech *Hirudo medicinalis* has enjoyed a revival as a treatment for venous congestion following microvascular or plastic surgery. Unfortunately, *Aeromonas* species are present in the leech gut, where they aid in the breakdown of ingested red blood cells. As a result, an increasing number of *Aeromonas* infections have been associated with leech application.[201,321,373] Although some of the reported patients had relatively trivial episodes of wound drainage, other patients had significant episodes of cellulitis,

abscess, tissue loss, sepsis, and meningitis.[9,257,280,321] It has been recommended that leech applications be restricted to tissue with arterial perfusion to minimize contamination of necrotic tissue with *Aeromonas*. Furthermore, the prophylactic administration of antibiotics has been proposed when leeches are applied.[257]

Laboratory Recovery of *Aeromonas* Species From Clinical Specimens. Differential or selective agars should be utilized when *Aeromonas* is suspected as the etiologic agent of gastroenteritis or when fecal specimens are submitted for workup on patients whose peak of diarrheal symptoms has subsided (Box 8-5). Most strains grow on selective enteric media as lactose fermenters and, therefore, may be overlooked as unimportant or commensal enteric organisms.

Laboratory Identification of *Aeromonas* Species. *Aeromonas* species are cytochrome oxidase positive and can be quickly excluded from the *Enterobacteriaceae* by performing an oxidase test. A drop or two of tetramethyl-*p*-phenylenediamine-dihydrochloride (oxidase reagent) can be placed on surface colonies and observed for the evolution of a black discoloration characteristic of the colonies of *Aeromonas* species. Mesophilic *Aeromonas* species are motile with polar rather than peritrichous flagella similar to *Pseudomonas* species; however, *Aeromonas* species can be differentiated from the latter because they utilize glucose fermentatively rather than oxidatively, and most *Aeromonas* species are indole positive (*Pseudomonas* species are negative). The phenotypic characteristics of *Aeromonas* species,[60] reviewed by Altwegg and colleagues[18] and more recently by

BOX 8-5

Selective Agars Used to Culture *Aeromonas*

1. *Blood agar with or without ampicillin:* Blood agar can be made selective by incorporation of 10μg/mL ampicillin. Janda and associates[195] recommend the use of a selective sheep blood agar containing ampicillin (SB-A agar) to improve the recovery of *Aeromonas* species from stool specimens.
2. *Alkaline peptone water (APW, pH 8.6):* Initially developed for isolation of *Vibrio* species, APW can be used to recover aeromonads present in low numbers (10 CFU/mL) in stools. After overnight enrichment, APW is subcultured to the agar medium of choice.
3. *CIN agar:* Originally developed for the isolation of *Yersinia enterocolitica*, CIN agar is also suitable for recovery of *Aeromonas* from feces.
4. *Enteric agars:* Deoxycholate, MacConkey, and Xylose Lysine Deoxycholate gave the highest overall plating efficiencies of eight routine enteric agars tested

for recovery of *Aeromonas* species from feces.[98]

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Abbott and colleagues,[2] are summarized in Table 8-13. Janda and Duffey[196] point out that one pitfall in the identification of *Aeromonas* species is that some of the miniaturized, semiautomated test kit systems cannot efficiently distinguish between *Aeromonas* species and *V. fluvialis.* The latter is only rarely encountered in clinical laboratories, however, and can be differentiated from *Aeromonas* species by demonstrating the ability of *V. fluvialis* to grow in 6.5% salt solutions, to produce yellow colonies on TCBS agar (sucrose positive), and to be sensitive to the vibriostatic agent 0/129.[196]

Lamy and colleagues compared the accuracy of six commercial systems for *Aeromonas* identification including API-20E, API-32GN, ID-GN card with the Vitek2 system (bioMérieux, Marcy l'Étoile, France), the identification portion of the NFC47 panel (MicroScan Walk/Away system, Beckman Coulter, Sacramento, CA), ID69 (Phoenix system; BD Diagnostic Systems, Sparks, MD), and GN2 microplates (Omnilog system; Biolog, Hayward, CA). Correct identification at the genus and species level for the six systems was 77.1%, 91.9%, 82.7%, 80.5%, 73.5%, and 67.8%, respectively.[240] Confusion with *Vibrio* affected 6.9% of results obtained with NFC47 and 16.1% of results obtained with API-20E. Overall accuracy of identification was hampered by outdated databases and taxonomy, weak algorithms, and impractical additional tests. For *Aeromonas* identification, additional tests were not required with the Phoenix and GN2 microplate systems, but were always necessary with the API-20E and NFC47 MicroScan W/A systems. Additional tests were also required with the API-32GN and ID-GN card Vitek2 systems, but only when the probability of identification was below the acceptable minimum defined by the corresponding system.[240]

Namdari and Bottone[302] also describe the suicide phenomenon for the rapid differentiation of *Aeromonas* species. This phenomenon is expressed when unknown strains are grown in broth media containing 0.5% glucose. The supplied glucose suppresses the tricarboxylic acid cycle, resulting in accumulation of acetic acid and cell death. *A. hydrophila* is nonsuicidal, aerogenic, and esculin positive; *A. sobria* is suicide variable, aerogenic, and esculin negative; and *A. caviae* is suicidal, anaerogenic, and esculin positive. Further studies on the biochemical characteristics and serologic properties of the genus *Aeromonas* have been published by Janda and colleagues and Abbott and colleagues.[2,193]

MALDI-TOF MS has been shown to be effective in species identification of *aeromonads*. Lamy and colleagues evaluated the accuracy of MALDI-TOF MS for identifying *aeromonads* with an extraction procedure. Genus-level accuracy was 100%. Compared to *rpoB* gene sequencing, species-level accuracy was 90.6% (29/32) for type and reference strains and 91.4% for a collection of 139 clinical and environmental isolates.[239] Chen et al.[76] reported that MALDI-TOF MS could accurately identify *A. dhakensis, A. hydrophila, A. veronii*, and *A. caviae* with accuracy rates of 96.7%, 90.0%, 96.7%, and 100%, respectively.

Genus Plesiomonas

The term *plesiomonas* is derived from the Greek word meaning "neighbor," indicating a close association with *Aeromonas.* However, as mentioned before, *Aeromonas* species have been reclassified within their own family, whereas *Plesiomonas* is believed to be more closely related to *Proteus* than *Aeromonas,*[261,364] and is now included in the family *Enterobacteriaceae*. *P. shigelloides* is the only species in the genus.

Clinical Significance. *P. shigelloides* is ubiquitous in surface waters and in soil and commonly infects various cold-blooded animals (frogs, snakes, turtles, lizards). Humans become infected primarily by ingesting contaminated or unwashed food. Although less frequently recovered from human feces than *Aeromonas* species, *Plesiomonas*-induced gastroenteritis has been reported in children[273] and in adults.[178] In the latter study, 28 of 31 patients with gastroenteritis had no other organisms to account for the acute symptoms. In Thailand, a carrier rate as high as 5.5% has been reported.[341]

Plesiomonas-related gastroenteritis in humans usually manifests as a mild watery diarrhea in which the stools are free of blood and mucin. Severe colitis or a cholera-like illness may be seen in patients who are immunosuppressed or who have GI malignancies.[359] The infection is more prevalent in the subtropical and tropical regions of the world and during the warm summer months. Pathogenicity is probably related to the production of an enteropathogenic enterotoxin; Sanyal and colleagues demonstrated that 13 clinical strains they studied produced significant fluid accumulation in the ileal loop test.[372] *P. shigelloides*-associated diarrhea has occurred in epidemics but also in isolated cases. It has been described after uncooked shellfish consumption and as a cause of travelers' diarrhea.[179] A few isolated cases of extraintestinal infections including septicemia, neonatal meningitis, cellulitis, septic arthritis, and acute cholecystitis have also been reported.[47,84,353] There are also reports of an overwhelming postsplenectomy infection with *P. shigelloides* in a patient cured of Hodgkin's disease[90] and a *P. shigelloides*-associated persistent dysentery and

pseudomembranous colitis in a 42-year-old Bangladeshi woman.[437] The clinical disease spectrum and pathogenic factors associated with *Plesiomonas* infections have been reviewed by Brenden and colleagues.[47]

Laboratory Isolation and Identification. *P. shigelloides* is a straight-to-rounded, short, motile gram-negative bacillus with polar, generally lophotrichous flagella (*Vibrio* species and *Aeromonas* species are monotrichous). The organism grows well on sheep blood agar and on most enteric media. Isolates are nonhemolytic on sheep blood agar, and in 24 hours at 30°C to 35°C (growth is optimum at 30°C), colonies average 1.5 mm in diameter; are gray, shiny, smooth, and opaque; and may be slightly raised in the center. *P. shigelloides* is readily isolated on enteric agars such as MacConkey, deoxycholate, Hektoen, and xylose lysine deoxycholate. However, ampicillin-containing selective media that are frequently used for the isolation of *Aeromonas* species are not suitable for the isolation of *P. shigelloides*.[440]

Glucose is fermented; therefore, the deep of Kligler's iron agar or triple sugar iron agar tubes will appear yellow. *P. shigelloides* will appear as a nonlactose fermenter on MacConkey agar and may be confused with *Shigella* species. The cytochrome oxidase reaction is positive, and indole is produced. *P. shigelloides* decarboxylates arginine, lysine, and ornithine. It does not produce DNase or extracellular proteases, and it ferments inositol but not mannitol. These are

TABLE 8-13 Differentiation of *Plesiomonas shigelloides* and Clinically Important *Aeromonas* species

							Decarboxylase					Fermentation			
Organism	Hemolysis Sheep Blood	Oxidase	Motility	DNase	Indole	Voges–Proskauer	Lysine	Ornithine	Arginine	Esculin	Gas From Glucose	L-Arabinose	Sucrose	Mannitol	Inositol
A. hydrophila complex	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–
A. caviae complex															
A. caviae	–	+	+	+	+	–	–	–	+	+	–	+	+	+	–
A. media	NA	+	–	+	V	–	–	–	+	+	–	+	–	NA	NA
A. eucrenophila	NA	+	+	+	NA	–	–	–	+	+	+	V	V	+	–
A. sobria complex															
A. sobria	+	+	+	+	+	+	+	–	+	–	+	V	+	+	–
A. veronii biotype *sobria*	+	+	+	+	+	+	+	–	+	–	V	–	+	+	–
A. veronii biotype *veronii*	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–
A. jandaei	+	+	+	NA	+	+	+	–	+	–	+	–	–	+	–
A. schubertii	V	+	+	+	–	V	+	–	+	–	–	–	–	–	–
A. enteropelogenes	+	+	+	NA	+	–	+	–	+	–	+	–	–	+	–
P. shigelloides	–	+	+	–	+	–	+	+	+	–	–	–	–	–	+

+, 90% or more of strains are positive; –, 90% or more of strains are negative; V, 11%–89% of strains are positive; NA, results not available. Shaded areas indicate key reactions.
Data from references 3, 13, 18, 47, 61, 170, 171, 196, and 375.

key characteristics by which it is separated from *Aeromonas* species. Additional key identifying characteristics are listed in Table 8-13.

P. shigelloides can be resistant to penicillin, ampicillin, carbenicillin, and other β-lactamase susceptible penicillins. Most strains are susceptible to the aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole and the quinolones, ciprofloxacin, and norfloxacin.[47,353]

Genus *Chromobacterium*

Brief mention of the genus *Chromobacterium* is made here because some strains are oxidase-positive fermenters and can be confused with *Aeromonas* species and *Vibrio* species. *Chromobacterium violaceum* is the species most commonly encountered in clinical laboratories, although it is seldom associated with human disease. *C. violaceum* grows well on blood agar, and most strains produce abundant violet pigment that makes recognition easy. Select biochemical characteristics are shown in Table 8-10. In addition, the ability of the organism to utilize citrate, reduce nitrates, and strongly hydrolyze casein is also helpful in making a final definitive identification.

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CHAPTER 9

Miscellaneous Fastidious Gram-Negative Bacilli

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Introduction to the Family *Pasteurellaceae*

The family *Pasteurellaceae* was established as a distinct taxonomic family in 1981 and includes a diverse group of gram-negative coccobacilli that are primarily isolated from animals.[1038] Initially, the various genera classified in the family were delineated by phenotypic and metabolic characteristics, and several additional "species" were subsequently assigned to the family based on DNA–DNA hybridization studies.[926]

With the application of more advanced molecular taxonomic methods, the phylogenetic structure of the family began to undergo significant revision beginning in the early 1990s.[339,354] At that time, the family *Pasteurellaceae* comprised three genera—*Haemophilus*, *Pasteurella*, and *Actinobacillus*—that included both human and animal isolates. Early 16S rRNA sequencing studies of these three genera demonstrated that organisms in the *Pasteurellaceae* were phylogenetically diverse and intermixed.[339,354] During the last decade, existing genera and species in the family have undergone reexamination by molecular methods, resulting in reclassification of some species and rejection of others as invalid members of the current genera. Several new genera have been created to accommodate both misclassified species and newly described bacterial isolates. Consequently, the family *Pasteurellaceae* now includes 15 genera, the majority of which are composed of bacteria found in various animals (Box 9-1). These genera are classified in the proposed order "Pasteurellales" in the γ-division of the *Proteobacteria*.

The family *Pasteurellaceae* now includes the following genera: *Haemophilus, Pasteurella, Actinobacillus, Aggregatibacter, Mannheimia, Bibersteinia, Lonepinella, Phocoenobacter, Histophilus, Avibacterium, Gallibacterium, Nicoletella, Volucribacte, Chelonobacter,* and *Basfia*. *Haemophilus* species are part of the normal microbiota of the upper respiratory tract of humans except for *H. ducreyi*, the causative agent of the sexually transmitted infection chancroid. *Pasteurella* species are primarily animal isolates that are occasionally recovered from human infections. With the exception of *Actinobacillus ureae* and *Actinobacillus hominis*, members of the genus *Actinobacillus* are also primarily recovered from animals. The genus *Aggregatibacter* includes the former *Haemophilus* species *H. aphrophilus (Aggregatibacter aphrophilus)*, *H. paraphrophilus (Aggregatibacter aphrophilus), H. segnis (Aggregatibacter segnis),* and *Actinobacillus actinomycetemcomitans (Aggregatibacter actinomycetemcomitans).*[956] The genus *Mannheimia* was created in 1999 to accommodate the trehalose-negative "*[Pasteurella] haemolytica*" complex and *Pasteurella granulomatis*.[46] The "*[Pasteurella] haemolytica*" complex included biovars A and T. Organisms belonging to biovar A were subsequently reassigned to the genus *Mannheimia* and distributed among the six species of *Mannheimia: M. haemolytica, M. granulomatis, M. glucosida, M. ruminalis, M. varigena,* and *M. succiniciproducens*.[46,206,772] Organisms assigned to the T biovar were reclassified [*Pasteurella*] *trehalosi*, despite evidence that this species is not closely related to other organisms in the genus *Pasteurella*. In 2009, [*Pasteurella*] *trehalosi* was placed in the new genus *Bibersteinia* as *Bibersteinia trehalosi*.[137]

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BOX 9-1

Members of the Family *Pasteurellaceae*

Genus	Species
Haemophilus	*H. influenzae, H. parainfluenzae, H. haemolyticus, H. parahaemolyticus, H. paraphrophaemolyticus, H. pittmaniae, H. ducreyi*
Pasteurella	*P. multocida* subsp.*multocida, P. multocida* subsp. *gallicida, P. multocida* subsp.*septica, P. dagmatis, P. canis, P. stomatis*
	Species incertae sedis: [*P.*] *aerogenes,* [*P.*] *pneumotropica,* [*P.*] *bettyae,* [*P.*] *caballi,* [*P.*] *mairii,* [*P.*] *testudinis,* [*P.*] *skyensis,* [*P.*] *langaaensis,* [*P.*] *lymphangitidis*
Actinobacillus	*A. ureae* (formerly *Pasteurella ureae*), *A. hominis, A. lignieresii, A. pleuropneumoniae, A. equuli* subsp. *equuli* subsp. *haemolyticus, A. suis, A. arthriditis* (Bisgaard taxon 11),*Actinobacillus* genomospecies 1,*Actinobacillus* genomospecies 2 (sorbitol-negative *A. arthriditis*), Bisgaard taxa 8 and 26
	Species incertae sedis: [*A.*] *muris,* [*A.*] *seminis,* [*A.*] *succinogenes,* [*A.*] *rossii,* [*A.*] *porcinus,* [*A.*] *capsulatus,* [*A.*] *indolicus,* [*A.*] *delphinicola,* [*A.*] *scotiae,* [*A.*] *minor,* [*A.*] *porcitonsillarum*
Aggregatibacter	*A. aphrophilus* (formerly *Haemophilus aphrophilus* and *Haemophilus paraphrophilus*), *A. segnis* (formerly *Haemophilus segnis*), *A. actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*)
Mannheimia	*M. haemolytica* (formerly *Pasteurella haemolytica*), *M. granulomatis* (formerly *Pasteurella granulomatis*), *M. glucosida, M. ruminalis, M. varigena,* and *"M. succiniciproducens"*
Bibersteinia	*B. trehalosi* (formerly [*Pasteurella*] *trehalosi*)
Lonepinella	*L. koalarum*
Phocoenobacter	*P. uteri*
Histophilus	*H. somnus* (formerly *"Haemophilus somnus," "Histophilus ovis,"* and *"Haemophiilus agni"*)
	A. gallinarum (formerly [*Pasteurella*] *gallinarum*), *A. paragallinarum* (formerly *"Haemophilus paragallinarum"*), *A. avium* (formerly

	[*Pasteurella*] *avium*), *A. volantium* (formerly [*Pasteurella*] *volantium*), *A. endocarditidis*
Gallibacterium	*G. anatis* biovar *haemolytica, G. anatis* biovar *anatis* (formerly [*Pasteurella*] *anatis*), *G. melopsittaci, G. trehalosifermentans, G. salpingitidis, Gallibacterium* genomospecies 1, 2, and 3, unnamed taxon (group 5)
Nicoletella	*N. semolina*
Volucribacter	*V. psittacicida, V. amazonae*
Chelonobacter	*C. oris*
Basfia	*B. succiniciproducens*

Newer genera in the family *Pasteurellaceae* include the genus *Lonepinella*, *Phocoenobacter, Histophilus*, *Avibacterium*, *Gallibacterium*, *Nicoletella*, *Volucribacter, Chelonobacter, and Basfia*. The genera *Lonepinella* and *Phocoenobacter* each include single species named *Lonepinella koalarum* and *Phocoenobacter uteri*, respectively.[437,983] *L. koalarum* is an environmental isolate and has also been recovered from koala bears. *P. uteri* was isolated from the uterus of a dead harbor porpoise found stranded on the Scottish coastline. The genus *Histophilus* also comprises isolates from animals and currently includes the single species *Histophilus somni*.[45] This newly described species includes three isolates that were formerly incertae sedis (i.e., of uncertain placement): "*Haemophilus somnus,*" "*Haemophilus agni,*" and "*Histophilus ovis.*" *Histophilus somnus* causes infectious thromboembolic meningoencephalitis in cattle, while *Histophilus ovis* and *Histophilus agni* cause mastitis, septicemia, synovitis, epididymitis, and vaginitis in sheep. The new genus *Avibacterium* accommodates V-factor-dependent and V-factor-independent avian species formerly classified in the genera *Haemophilus* and *Pasteurella*. The four species in the genus *Avibacterium* form a monophyletic group, showing greater than 96.8% DNA sequence homology with one another, and include *A. gallinarum* (formerly *Pasteurella gallinarum*), *A. paragallinarum* (formerly *Haemophilus paragallinarum*), *A. avium* (formerly *Pasteurella avium*), *A. volantium* (formerly *Pasteurella volantium*), and the new species *A. endocarditidis*.[132,138]

The genus *Gallibacterium* includes former members of the "[*Pasteurella haemolytica*]-"*Actinobacillus salpingitidis*"-*Pasteurella anatis* complex. The genus includes *G. anatis* biovar *haemolytica*, *G. anatis* biovar *anatis*, *G. melopsittaci*, *G. trehalosifermentans*, *G. salpingitidis*, *Gallibacterium* genospecies 1, 2, and 3, and an unnamed taxon (group 5).[134,269] These organisms have been isolated from avian

unnamed taxon (group 5).[134,269] These organisms have been isolated from avian species, including chickens, turkeys, pigeons, parrots, and parakeets. The genus *Nicoletella* was described in 2004 and includes the single species *Nicoletella semolina*, which has been isolated from the airways of horses with respiratory disease.[739] The new genus *Volucribacter* includes members of the formerly unnamed Bisgaard taxon 33 and includes two species, *V. psittacicida* and *V. amazonae*.[267] These species have been isolated

from parrots, budgerigars, parakeets, and chickens with respiratory disease and septicemia. In 2009, genotypic analysis of [*P.*] *testudinis* and other isolates from diseased tortoises resulted in the description of the new genus *Chelonobacter*, which was demonstrated to be a member of the *Pasteurellaceae*.[515] Genus *Chelonobacter* contains a single species, *C. oris*, that is distinct genotypically and phenotypically from [*P.*] *testudinis*, which remains unclassified at this time. The genus *Basfia* is represented by the single species, *B. succiniciproducens*, which was isolated from the bovine rumen.[740] Characteristics for differentiation of the various genera within the family *Pasteurellaceae* are presented in Table 9-1.

Haemophilus Species

Taxonomy

In the 2005 edition of *Bergey's Manual of Systematic Bacteriology*, which reflects bacterial phylogeny based largely on 16S rRNA gene sequence comparisons, 15 *Haemophilus* species are included in the family *Pasteurellaceae*.[702] These species are dispersed among 10 of 21 rRNA "clusters."[975] *Haemophilus* sensu stricto is placed in rRNA cluster 16 and includes *H. influenzae*, *H. aegyptius*, and *H. haemolyticus*. These X-factor (hemin)-dependent species comprise a closely related group. The "Aphrophilus" cluster (rRNA cluster 13) includes *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, and *Actinobacillus actinomycetemcomitans*. *H. parainfluenzae* and *H. ducreyi* comprise rRNA clusters 2 and 19, respectively. The "Parahaemolyticus cluster" includes *H. parahaemolyticus* and *H. paraphrohaemolyticus*. By 16S rRNA sequencing, *H. parainfluenzae* and members of the "Parahaemolyticus cluster" appear to occupy an intermediate taxonomic position between *Haemophilus* sensu stricto and other genera included in the *Pasteurellaceae*. DNA–DNA hybridization studies, however, clearly indicate the correct placement of *H. parainfluenzae* and the "Parahaemolyticus cluster" within the genus *Haemophilus*. In order to assess the

taxonomists have started to examine the phylogeny of *Haemophilus* species sensu stricto using alternate gene sequences along with 16S rRNA sequencing. These examinations have included partial sequencing of several "housekeeping" genes, including *sodA* and *sodC* (superoxide dismutase genes), *infB* (translation initiation factor 2 gene), *fucK* (fuculokinase gene), *hap* (penetration protein gene), *pgi* (glucose-6-phosphate isomerase gene), *adk* (adenylate kinase gene), and *recA* (recombination protein gene).[207,952,957]

Based on these data, the genus *Haemophilus* can be divided into four clusters, which are shown in Box 9-2. Members of cluster 2 have since been reassigned to the new genus *Aggregatibacter*.[956] *H. ducreyi* differs in several respects from other members of the genus and was originally assigned to genus *Haemophilus* because of its requirement for X factor (hemin). *H. ducreyi*, however, is unrelated to *H. influenzae* by DNA–DNA hybridization studies and is only distantly related to *H. influenzae* and the other haemophili, but 16S rRNA sequencing has confirmed its membership in the *Pasteurellaceae*.[975] *H. ducreyi* will likely be reclassified in the near future. Although traditionally defined by requirements for factors found in blood (i.e., X factor [hemin] and V factor [nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP)]), these requirements are not exclusive features of *Haemophilus* species. V-factor-dependent avian isolates (e.g., *Avibacterium* [*Pasteurella*] *avium*, *Avibacterium* [*Pasteurella*] *volantium*) are more closely related to the type species of the genus *Pasteurella* (*P. multocida*) than to that of the genus *Haemophilus* (*H. influenzae*).[138] Some animal haemophili (e.g., "*Haemophilus agni*," "*Haemophilus somnus*") have never been validated as *Haemophilus* species; these organisms are now included in the genus *Histophilus*.[45] Sequencing of the *infB* (elongation factor 2) gene from *Haemophilus* species has indicated that all species that require X and V factors are closely related and that *H. parainfluenzae* constitutes a heterogeneous group within the genus. Using 16S rRNA sequencing as well as the various "housekeeping" genes, taxonomists are attempting to determine the relationship of typeable (i.e., encapsulated) *H. influenzae* to nontypeable strains, the phylogenetic relatedness of genospecies of haemophili to *Haemophilus* species sensu stricto, the relationship of *H. influenzae* cryptic genospecies biotype IV and *Haemophilus* sensu stricto, the status of haemophili in the "Parahaemolyticus" and "Parainfluenzae" groups, and the relationship of *H. influenzae* and *H. haemolyticus* to haemophili that are nonhemolytic but phenotypically resemble *H. haemolyticus*.[569,877,920,953,957] During the course of these taxonomic investigations, a new *Haemophilus* species, *H. pittmaniae*, was described.[954] This organism, along with other *Haemophilus* species, is part of the microbiota of the upper respiratory tract of humans.

Haemophilus influenzae

Haemophilus influenzae

Haemophilus species are part of the normal bacterial microbiota of the oropharynx and nasopharynx in more than 85% of adults. Most oropharyngeal isolates are nonencapsulated *H. influenzae* and *H. parainfluenzae*, although encapsulated *H. influenzae* (capsular serotypes a, b, c, d, e, and f) may be found as part of the normal microbiota of the upper respiratory tract of both children and adults. Prior to the availability of *H. influenzae* type b conjugate vaccines, 2% to 6% of children carried capsular serotype b strains and as many as 60% of children in day care centers were colonized in the oropharynx. Recovery of *H. influenzae* from sputum specimens may reflect the presence of these organisms as normal microbiota. However, in chronic respiratory ailments (e.g., bronchitis, chronic obstructive pulmonary disease [COPD]), both encapsulated (typeable) and nonencapsulated (nontypeable) *H. influenzae* may cause severe infections.[919,1171] Infections caused by *H. influenzae* and members of the genus other than *H. ducreyi* are described in Box 9-3.

Among the haemophili, *H. influenzae* serotype b is considered the most pathogenic of the six capsular serotypes (i.e., types a, b, c, d, e, and f). The type b polysaccharide is the only capsular type that contains two pentose monosaccharides rather than hexose sugars as subunit carbohydrates. The native type b capsule comprises a linear teichoic acid containing ribose, ribitol (a 5-carbon sugar alcohol), and phosphate linked by phosphodiester bonds, and is called PRP (polyribosylribitol phosphate) (Fig. 9-1). Although virulence of *H. influenzae* type b is multifactorial, the PRP capsule is

TABLE 9-1 Phenotypic Characteristics for Differentiation of Genera in the Family *Pasteurellaceae*

Characteristic	*Haemophilus*	*Actinobacillus*	*Aggregatibacter*	*Pasteurella*	*Mannheimia*	*Lonepinella*	*Phocoenobacter*	*Avibacterium*	*Gallibacterium*	*Nicoletella*	*Volucribacter*	*Histophilus*	*Bibersteinia*	*Chelonobacter*	*Basfia*
HEM SBA	V	V	–	–	V	–	–	–	β	–	–	V	β^{w}	β	–
CAT	V	V	V	V*	+	–	–	V	+	+	V	–	V	+	–
OX	V	V	V	V*	+	–	+	+	+	+	V	+	+	+	+
ALA	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Requires X factor	V	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Requires V factor	+	V	V	V	–	–	–	V	NA	–	–	–	+	–	–
IND	V	–	–	V	V	–	–	–	–	–	–	+	–	V	–
URE	V	+	–	V	–	–	–	–	–	+	–	–	–	–	–
ACET	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
MOT	–	–	–	–				–	–	–	–	–	–	–	–
ODC	V	V	–	V	V	–	–	V	–	–	–	–	–	–	–
NO_3 RED	+[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	–	V	V	V-	V	NA	+	V	+	V	V	NA	–	+	+
Acid produced from:															
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MAL	V	V	+	V	V	NA	–	V	V	V	V	–	+	+	–
FRU	V	V	+	V	NA	NA	NA	+	+	+	+	–	+	+	+
SUC	V	V	V	V	NA	NA	–	+	+	+	+	–	+	+	+
LAC	–	V	V	V	V	NA	–	V	NA	–	V	–	–	–	+
XYL	V	V	V	V	V	NA	–	V	+	–	V	–	–	+	–
ARAB	–	V	–	–	V	V	NA	V	V	–	V	–	–	+	–
M-INO	–	V	NA	–	V	–	–	V	V	–	V	–	NA	NA	–
MNTL	–	V	V	V	+	–	–	V	+	–	–	–	+	+	+
MANN	V	V	V	+	–	NA	–	+	+	–	NA	V	+	+	+
MEL	–	V	V	–	–	V	–	–	–	–	V	–	–	–	V
SBTL	–	V	–	V	V	NA	–	V	V	–	–	–	+	+	–
TREH	–	V	V	V	–	NA	–	V	V	–	–	–	+	+	+

+, positive reaction; –, negative reaction; V, variable reaction; NA, data not available; HEM SBA, hemolysis on sheep blood agar; CAT, catalase; ALA, aminolevulinic acid porphyrin test; OX, oxidase; IND, indole; URE, urea; ACET, acetoin (VP); MOT, motility; ODC, ornithine decarboxylase; NO_3 RED, nitrate reduced to nitrite; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; GLU, glucose; MAL, maltose; FRU, fructose; SUC, sucrose; LAC, lactose; XYL, xylose; ARAB, arabinose; M-INO, myoinositol; MNTL, mannitol; MANN, mannose; MEL, melibiose; SBTL, sorbitol; TREH, trehalose; β^{w}, weak $^{beta-}$hemolysis; V*, Variable reaction, most species positive.
[a]Refer Image Plate 9-1H.

BOX 9-2

Phylogeny of Genus *Haemophilus* Sensu Stricto Isolated From Humans

Cluster 1	Cluster 2	Cluster 3	Cluster 4
Haemophilus influenza	*Aggregatibacter (Haemophilus) aphrophilus*	*Haemophilus parainfluenzae*	*Haemophilus parahaemolyticus*
Haemophilus aegyptius	*Haemophilus paraphrophilus*	*Haemophilus pittmaniae*	*Haemophilus paraphrohaemolyticus*
Haemophilus haemolyticus	*Haemophilus segnis*		
	Actinobacillus actinomycetemcomitans		

BOX 9-3

Infections Caused by *Haemophilus* Species

Infection	Comments
Meningitis	In the prevaccine era, *H. influenzae* was the most common cause of bacterial meningitis in children between 1 month and 2 years of age, with the peak incidence between 6 and 12 months of age. Between the ages of 2 and 6 years, *H. influenzae* and *Neisseria meningitidis* occurred with equal frequency, and *H. influenzae* meningitis was uncommon in children older than 6 years of age. More than 90% of isolates obtained from these patients belonged to capsular serotype b. Meningitis caused by this agent now occurs most frequently among infants who are either not immunized or who are too young to have completed the primary immunization series.[215] Prior nasopharyngeal colonization in a susceptible host leads to invasion of the bloodstream and subsequent seeding of the meninges.[706] Symptoms of an upper respiratory tract infection of viral etiology and otitis media often precede the development of meningitis.[1014] Onset of signs and symptoms may be abrupt or insidious, with the latter being the most common pattern. Children present with fever, malaise, and occasionally vomiting; nuchal rigidity is often absent. Some children may develop peripheral and/or cranial nerve palsies and seizures. A high index of suspicion, aggressive diagnostic approaches (i.e., lumbar puncture for collection of CSF, collection of blood cultures, early administration of appropriate antimicrobial therapy), and close communication with the laboratory are essential for proper diagnosis. Although relatively unusual, complications of *H. influenzae* type b meningitis include brain and brainstem abscesses, subdural effusion, pericarditis, septic arthritis, and localized infections at other body sites due to hematogenous dissemination.[231,417,1222] *H. influenzae* meningitis in adults is uncommon and usually complicates underlying conditions, such as CSF leakage following head trauma or neurosurgical procedures. Meningitis may also result from direct extension from a contiguous focus of infection in the sinuses or the middle ear.[188,1143] Debilitating underlying diseases, such as diabetes, chronic alcoholism, pneumonia, HIV infection, and other immunodeficiency states, also predispose adults to *H. influenzae* meningitis. Approximately half of the adult patients with *H. influenzae* meningitis have disease caused by nontypeable *H. influenzae* strains, while the other half are caused by type b and other encapsulated strains.[196,381] Rare instances of *H. influenzae* type b meningitis have been reported in children who have completed the primary immunization series and patients with meningitis due to nontypeable *H. influenzae* have been reported.[607,629] *H. influenzae* is spread via respiratory secretions, and an increased risk of secondary, invasive disease exists for unvaccinated or incompletely vaccinated household contacts 4 years of age and younger. Rifampin, the drug of choice for chemoprophylaxis, eradicates oropharyngeal carriage and interrupts transmission. According to the American Academy of Pediatrics (AAP), prophylaxis should be administered to all household members (including adults) when the household contains a contact or contacts younger than 48 months whose immunization status is incomplete.[32] The AAP defines a contact as a child who is a member of the immediate household or who has spent 4 or more hours each day with the index case for at least 5 of the 7 days preceding the onset of disease. The dosage of rifampin for children is 20 mg/kg once daily for 4 days and for adults is 600 mg twice daily for 2 days. Rifampin must be given to close contacts within 7 days after disease onset in the index case in order to be effective. All members of a household with an immunized child less than 12 months of age (i.e., those children who have not yet received the 12–15 month "booster") should receive rifampin. Chemoprophylaxis is not necessary when all household contacts younger than 48 months of age have completed their immunization series. Children attending day care facilities who are contacts of index cases should receive rifampin prophylaxis if they are unvaccinated or incompletely vaccinated and are exposed to the index case for 25 or more hours in the week prior to disease onset in the index case.

	H. influenzae meningitis has been described as a complication of CSF shunt infections.[1364] These infections have occurred primarily in infants and children and have involved ventriculoperitoneal, ventriculoatrial, and lumboperitoneal shunts. While shunt infections caused by *Staphylococcus epidermidis* or *Staphylococcus aureus* usually develop from intraoperative/perioperative contamination of the shunt and ventricular CSF, *H. influenzae* shunt infections result from seeding of the shunt and CSF during bacteremia. Patients presenting with meningeal symptoms often have other infections associated with *H. influenzae* (e.g., otitis media). Bacterial isolates have either been capsular type b or nontypeable. While shunt infections caused by gram-positive bacteria require removal/replacement of the foreign body for cure, systemic antimicrobial therapy without shunt removal is often successful in eliminating type b strains, although antimicrobial monotherapy has been less successful in treatment of shunt infections caused by nontypeable strains.[1364]
Supraglottitis	*H. influenzae* type b has historically been associated with supraglottitis (epiglottitis), a cellulitis of supraglottic tissues that results in obstructive laryngeal edema.[919] Epiglottitis seldom occurs in infants, and is typically seen in children 2–7 years of age and in adult males 20–30 years old. The presentation is acute, with sore throat, dysphagia, fever, and swelling of the epiglottis above the larynx at the base of the tongue.[521,1246] In children, this abrupt onset helps to clinically differentiate epiglottitis from croup caused by viral agents (e.g., respiratory syncytial virus (RSV), parainfluenza viruses) and from pertussis. As the disease progresses, swallowing becomes increasingly difficult, resulting in drooling, inspiratory stridor, and incipient respiratory distress.[521] Nasotracheal intubation and vigorous administration of antimicrobial and supportive therapy may be life-saving in cases of airway obstruction. While posterior pharyngeal cultures are not helpful because of the presence of other nasopharyngeal microbiota, *H. influenzae* type b may be recovered from blood cultures. Epiglottitis also occurs in adults; fever, sore throat, dysphagia, and respiratory distress are the presenting signs and symptoms.[1363] The introduction of effective vaccine strategies has also affected the incidence of epiglottitis. The incidence of pediatric epiglottitis prior to widespread immunization was 3.47–6.0 per 100,000 patients per year and currently is estimated to be 0.3–0.7 per 100,000 patients per year in the postvaccine era.[1173] Disease incidence in adults has remained at 1–4 cases per 100,000 patients per year. Despite effective vaccines, *H. influenzae* type b epiglottitis has been reported in children who have received the entire vaccination series.[1311]
Otitis media	Otitis media refers to inflammation of the middle ear and is clinically categorized as uncomplicated, persistent, recurrent, or chronic.[776] Otitis media occurs most frequently in children aged 6 months to 5 years, with the highest incidence among children less than 3 years of age. The high incidence in young children reflects the underdeveloped protective mechanisms of the eustachian tube. Acute otitis media often occurs as a complication of viral infections (e.g., RSV, parainfluenza viruses). Symptoms include ear pain, hearing loss, and sometimes discharge from the external otic canal, while signs include fever, irritability, headache, and occasionally nausea and vomiting. Otoscopy reveals a red, opacified bulging tympanic membrane that is relatively immobile. Definitive etiologic diagnosis requires needle aspiration of middle ear fluid (tympanocentesis), which is not performed routinely. If drainage is present, however, it should be collected for culture. Culture of CSF and blood may be necessary and helpful if systemic signs and symptoms are present. Common bacterial causes of acute otitis media include *Streptococcus pneumoniae*, *H. influenzae*, and *Moraxella catarrhalis*, with one-quarter to one-third of infections due to *H. influenzae*. The causative agent cannot be differentiated on clinical grounds, although fever, otorrhea, and tympanic membrane inflammation are less commonly seen with *H. influenzae* compared with *S. pneumoniae*.[777,921,1096] Over 90% of *H. influenzae* strains recovered from appropriate specimens are nontypeable, with the remaining 10% being type b. Up to one-fourth of children with *H. influenzae* type b ear infection also have bacteremia and/or meningitis.[141] Complications include recurrent otitis media, persistence of middle ear effusions necessitating insertion of drainage tubes, hearing impairment, mastoiditis, meningitis, chronic otitis media, brain abscess, and sepsis.[786] Amoxicillin clavulanate combined with amoxicillin (PO), cefuroxime axetil (PO), or ceftriaxone (IM) has been recommended as the antimicrobial agents of choice for treatment of recurrent and persistent otitis media.
Sinusitis	Acute sinusitis is characterized by persistent cold symptoms, purulent nasal/postnasal discharge, cough, fever, headache, and often facial pain.[183,878] The acute presentation blends into chronic sinusitis, which is characterized by symptomatic persistence beyond 3 months, with cough and purulent nasal drainage being the principal symptoms.[1149] A sensation of facial fullness or the presence of edema or periorbital discoloration may indicate extension of infection from the sinuses into the soft tissues of the orbit. Diagnosis of sinusitis is made most often by a clinical assessment, with imaging techniques (i.e., radiographs, computed tomographs, magnetic resonance imaging) being used for visualizing ethmoid and sphenoid sinuses and for determining orbital involvement. In studies where specimens were obtained surgically or by needle aspiration of the maxillary sinus spaces, *H. influenzae* and *S. pneumoniae* are the organisms most frequently isolated.[1149] Careful clinical and bacteriologic studies have demonstrated *H. influenzae* as the etiologic agent in 20%–25% of adults and in 36%–40% of children.[183,1205] Most *H. influenzae* isolates from these infections are nontypeable. Serious intracranial infection (e.g., epidural abscess) may arise by direct extension of infection from the sphenoid and ethmoid sinuses.
Bronchitis and COPD	Chronic bronchitis is an ill-defined clinical entity involving bronchial inflammation without significant involvement of the pulmonary parenchyma, and is characterized by a persistent, nonproductive cough, wheezing, and shortness of breath.[1354] This condition is common, particularly among men over age 40 years who smoke. Environmental factors (e.g., air pollution) and viral infections further irritate and inflame the respiratory mucosa, rendering them increasingly susceptible to bacterial colonization

	and eventually leading to the production of purulent sputum. In patients with stable chronic bronchitis, the lower airways are often colonized with multiple strains of nontypeable *H. influenzae*; during acute exacerbations, the numbers of these organisms increase.[86] Chronic cough with sputum production for at least 3 consecutive months for more than 2 years in succession is termed *chronic obstructive pulmonary disease*. COPD classification is currently undergoing characterization using spirometry, which involves measurements of airflow limitations that are progressive and associated with abnormal responses of the lung to noxious gases or particulates. Patients with severe exacerbations of chronic bronchitis and COPD present with dyspnea, hypoxia, and usually a low-grade fever. During exacerbations, sputum production is increased and the consistency changes from mucoid to mucopurulent to purulent. Gram's stains of properly collected sputum specimens generally show many PMNs associated with a particular bacterial morphotype (e.g., the pale-staining coccobacilli of *H. influenzae*).[918] The most common agents recovered from purulent sputum are nontypeable strains of *H. influenzae*, followed by *S. pneumoniae* and *M. catarrhalis*.[1007,1170] In patients with *H. influenzae*-associated disease, nontypeable strains can be isolated from the lower airways between exacerbations of symptoms.[782] During exacerbations, empiric therapy with oral antimicrobial agents are prescribed, along with bronchodilators and other supportive measures. In patients with airway obstruction who are producing purulent sputum, antimicrobial therapy shortens the duration of symptoms. Drugs with demonstrated efficacy in this setting have included extended-spectrum cephalosporins, tetracyclines, quinolones, and newer macrolides. Chronic bronchitis, acute exacerbations of purulent bronchitis, COPD, and pneumonia are a continuum of pathologic pulmonary processes in which nontypeable *H. influenzae* strains are a primary player. Several host factors (e.g., immune status, genetic predisposition, other pulmonary compromises) contribute to the pathogenesis of these ill-defined conditions. Treatment options include second- and third-generation cephalosporins, amoxicillin clavulanate, azithromycin, and the fluoroquinolones.[632]
Pneumonia	*H. influenzae* pneumonia can be a manifestation of systemic *H. influenzae* infection, or may develop as a complication of COPD. *H. influenzae* pneumonia in patients with systemic disease (i.e., meningitis, epiglottitis, bacteremia, otitis media) is lobar, segmental, and purulent; characteristics are similar to pneumococcal pneumonia. In these infections, encapsulated type b organisms are the usual etiologic agents. Nontypeable *H. influenzae* are the important causes of pneumonia with and without bacteremia in elderly patients with underlying respiratory conditions (e.g., chronic bronchitis, COPD, bronchiectasis), or with other underlying diseases (e.g., immunodeficiency, diabetes, alcoholism, neoplasms).[655,1143] Bacteremic pneumonia due to *H. influenzae* capsular types other than type b has also been reported in patients with underlying conditions that impair local or systemic host defense mechanisms. Definitive diagnosis of *H. influenzae* pneumonia through the assessment of respiratory specimens is challenging since the organism is present in the upper respiratory tract of healthy individuals, as well as those with pneumonia. Expectorated sputum specimens may provide inadequate or misleading results; bronchial washings, bronchoscopy, or BAL specimens may be necessary for definitive cultural diagnosis.
Bacteremia and infectious complications of bacteremia	Bacteremia is a frequent, early manifestation of acute *H. influenzae* type b meningitis, but some infants may present with bacteremia without meningitis. Acute presentations are seen mostly in children with underlying disease (e.g., sickle cell disease) and include fever, lethargy, and a high peripheral neutrophil count. Bacteremia may seed soft tissues, joints, and bone, resulting in cellulitis, septic arthritis, and osteomyelitis, respectively.[919] Cellulitis often appears in children as violaceous or reddish blue swellings on the cheeks and periorbital areas. This manifestation of *H. influenzae* type b sepsis has virtually disappeared since the introduction of type b conjugate vaccines.[30,1089] Septic arthritis is characterized by pain, swelling, and decreased mobility of the infected joint. Hematogenous osteomyelitis presents as swelling, bone pain, and constitutional symptoms.[1407] Howard and colleagues reviewed 851 cases of septic arthritis and osteomyelitis from 1977 through 1997 in Ontario, and found that hematogenous *H. influenzae* type b septic arthritis and osteomyelitis accounted for 30% and 3%, respectively, of these infections prior to 1992, but only one patient with type b septic arthritis and no patients with osteomyelitis were seen from 1992 to 1997.[610] Widespread vaccination has resulted in the virtual disappearance of type b organisms as etiologic agents of these complications. *H. influenzae* bacteremia may also occur in neonates and the elderly. In these cases, meningitis may or may not be present. Most cases of neonatal infection result from maternal–fetal or maternal–perinatal transmission (see below), and the majority of isolates from bacteremia in neonates and older children are nontypeable.[978] While *H. influenzae* type b sepsis has been reported in elderly adults, the majority of isolates from these individuals are also nontypeable with the respiratory tract as the usual source. Nontypeable *H. influenzae* bacteremia occurs in older patients with underlying diseases (e.g., alcoholism, SLE, rheumatoid arthritis, diabetes, malignancies) and is associated with a several complications, including cellulitis, septic arthritis, osteomyelitis, empyema, intra-abdominal infections, pyelonephritis, and endocarditis.[1143]
Endocarditis	*H. parainfluenzae* and *A. aphrophilus* (formerly *H. aphrophilus/paraphrophilus*) are the frequently isolated species from patients with endocarditis and intravascular infections, with typeable and nontypeable *H. influenzae* being relatively uncommon.[327,482,613,666] The incidence of endocarditis is highest in young to middle-aged adults. Patients usually have a subacute clinical course with low-grade fever, malaise, chills, and respiratory tract symptoms. Preexisting or underlying valvular disease is found in only about half of the patients, with the presence of a heart murmur being the only suggestive finding. Echocardiography studies have demonstrated that *A. aphrophilus* endocarditis is associated with a high incidence of valvular vegetations and consequent septic complications resulting from embolization. Risk factors for endocarditis with *Haemophilus/Aggregatibacter*

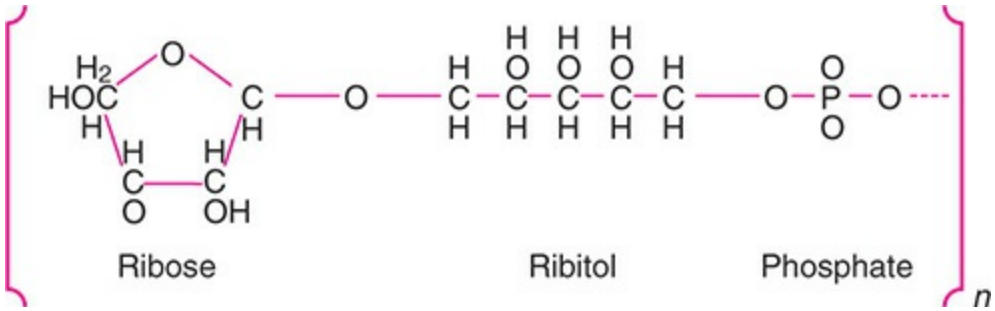
	species include the presence of dental or oral lesions, antecedent dental manipulations, oral surgery, diseased or damaged cardiac valves, and valvular prostheses. *H. parainfluenzae* has also been implicated in cases of endocarditis in persons with no underlying predisposing factors. Intravenous drug users may present with right-sided endocarditis, tricuspid valve vegetations, and clinical and radiographic evidence of septic embolization.[256,275,590,960] *H. parainfluenzae* has also been associated with sustained bacteremia resulting from infection of vascular implants, such as pacemaker wires.[996]
Urogenital, maternal, perinatal, and neonatal infections	*Haemophilus* species, particularly nontypeable *H. influenzae* and *H. parainfluenzae*, are uncommon causes of urethritis, female genital tract infections, vulvovaginitis, obstetrical and gynecological infections, postpartum bacteremia, septic abortion, tubo-ovarian abscess, intrauterine fetal death, and neonatal sepsis with/without meningitis.[78,110,202,313,566,609,675,734,1188,1292] In a study conducted in Houston between 1976 and 1981, *H. influenzae* was recovered from blood cultures of 16 women with postpartum bacteremia and 36 neonates with bacteremia or meningitis.[1316] Another 50 *H. influenzae* were cultured from genital sites (vagina, endometrium, cervix, Bartholin's glands, fallopian tubes, male urethra, and prostatic fluid) and fetal tissues (amniotic fluid, placental tissues). Of these isolates, 94% were serologically nontypeable. Quentin and colleagues recovered *Haemophilus* species from genital cultures of 83 women over a 90-month period.[1051] Forty-two women had significant bacterial genital tract infections, including endometritis, salpingitis, and Bartholin's gland abscesses. Genital tract infection was linked to the presence of intrauterine devices in 62% of the patients with endometritis and in 4 of 6 patients with salpingitis, suggesting that the genital haemophili may behave as opportunistic agents in genital sites. Prenatal and perinatal acquisition of *Haemophilus* species from the maternal genital tract has been associated with premature rupture of membranes, amnionitis, septic abortion, and neonatal sepsis.[246,849,1331] Biotyping of genital isolates has shown that nontypeable biotype IV *H. influenzae* and biotype I and II *H. parainfluenzae* are inhabitants of the genital tract and may be associated with genital tract infections, postpartum maternal, and neonatal systemic infections.[1051] Studies of serologically nontypeable *H. influenzae* strains recovered from these infections have established the existence of a novel, cryptic genospecies of *H. influenzae*.[1049,1050] This novel genospecies types as biotype IV (i.e., indole-negative; urease and ornithine decarboxylase-positive) but is distinct by other morphologic, phenotypic, and genetic criteria (i.e., peritrichous fimbriae, a distinctive outer membrane protein profile, variant outer membrane proteins, unique multilocus enzyme electrophoresis patterns).[922,1050] Molecular studies indicate that these isolates are clonal and genetically homogeneous, share less than 70% of overall genomic similarity with *H. influenzae* sensu stricto, and are most closely related to strains of *H. haemolyticus*.[1050] This cryptic genospecies cannot be phenotypically differentiated from other *H. influenzae* biotype IV strains.
Ocular infections	*H. influenzae* ocular infections vary widely in severity and include conjunctivitis, scleritis, subconjunctival abscess, and endophthalmitis. *H. influenzae* causes a contagious, acute conjunctivitis colloquially called "pinkeye." Localized outbreaks of acute conjunctivitis occur among persons who share towels, handkerchiefs, or other objects that come in direct contact with the skin of the face or eyes. The diffuse pink color of the sclera and the presence of a serous or purulent discharge are virtually diagnostic of *Haemophilus* conjunctivitis. Hemorrhagic conjunctivitis may also occur as part of invasive *H. influenzae* type b disease.[1012] *H. influenzae* endophthalmitis is uncommon but may occur as an iatrogenic complication of ocular surgery.[22,27,1264,1402] Sykes and colleagues reported on three cases of *H. influenzae* scleritis.[1233] These elderly patients had severely decreased visual acuity, pain, and nodular scleral abscesses from which *H. influenzae* was recovered in culture. Brooks and colleagues reported a spontaneous subconjunctival abscess due to *H. influenzae* in a 27-year-old immunocompetent woman with no underlying or preexisting ocular pathology.[184] *H. influenzae* may also be isolated from superinfected herpetic corneal ulcers and from corneal specimens of patients with atopic keratoconjunctivitis.[1191] Historically, isolates from conjunctival infections were designated as a separate species called "*H. aegyptius*." Phenotypically, these isolates are similar to *H. influenzae* biotype III. The distinctive clinical presentation, biochemical characteristics, and serologic studies have been used to support the status of "*H. aegyptius*" as a separate species within the genus. However, genomic nucleotide studies have demonstrated significant sequence homology between "*H. aegyptius*" and *H. influenzae*; so this organism is now called *H. influenzae* biogroup *aegyptius*. A unique, highly virulent clone of *H. influenzae* biogroup *aegyptius* emerged in the early 1980s as a causative agent of a clinical syndrome called Brazilian purpuric fever (BPF; see below).
Brazilian purpuric fever	During the 1980s, a subgroup of strains that were phenotypically indistinguishable from *H. influenzae* biotype III ("*H. aegyptius*") were recognized in association with outbreaks of a severe illness called BPF.[160–162] BPF was first reported in 1984 in Promissão, São Paulo State, Brazil, when 10 children died from an acute illness characterized by a high fever, abdominal pain with vomiting, a petechial/purpuric rash, hypotensive shock, and vascular collapse. A subsequent outbreak among 10 children in the nearby town of Serrana resulted in four additional deaths.[162] Blood cultures from these children grew "*H. aegyptius*." During epidemiologic investigations, cases were found to have had recent or concurrent purulent conjunctivitis. Since the initial outbreaks, sporadic cases have been reported in other areas of Brazil and in central and western Australia.[874,1351] Outbreak-associated "*H. aegyptius*" strains possess several characteristics not found in "*H. aegyptius*" isolated from cases of purulent conjunctivitis without BPF.[562] These strains are nontypeable, possess a unique 24-kDa plasmid, a distinctive 25-kDa outer membrane fimbrial protein, and are resistant to the bactericidal effects of normal human serum. BPF strains possess a

conserved cell surface epitope of the 48-kDa P1 outer membrane protein that is not present in non–BFP-associated strains. Outbreaks of BPF were caused by a unique clone of "*H. aegyptius*" that has been designated "*H. influenzae* biogroup *aegyptius*." In vivo studies using a rat model and studies with a human microvascular endothelial cell line also have shown that BPF-associated *H. influenzae* biogroup *aegyptius* are more cytotoxic than non–BPF-associated *H. influenzae* strains.[1118,1344] Based on multilocus enzyme electrophoresis methods, biogroup *aegyptius* isolates form three distinct lineages within the species *H. influenzae*. The BPF isolates are not closely related genetically to non-BPF biogroup *aegyptius* strains but do show a close genetic relationship with encapsulated *H. influenzae* capsular serotype c.[925]

Miscellaneous *H. influenzae* infections

H. influenzae type b may cause other uncommon infections; in many instances, the recovery of *Haemophilus* species was unexpected. *H. influenzae* has been isolated from abdominal infections (i.e., peritonitis, pancreatic and pelvic abscesses, appendicitis) and hepatobiliary tract infections (i.e., cholecystitis, pyogenic liver abscess).[31,522,852,964,971,1047,1349]

While sporadic infections due to encapsulated non-type b *H. influenzae* occurred in the United States prior to widespread vaccination with type b vaccines, serious infections caused by both non-type b capsular serotypes and nontypeable strains of *H. influenzae* are being increasingly recognized in the vaccine era. In 2001, five cases of invasive disease caused by *H. influenzae* serotype a were seen over a 10-month period in Utah.[4] These children (median age 12 years) presented with meningitis, bacteremia, and, in one instance, purpura fulminans. Examination of 48 invasive non-type b *H. influenzae* by restriction endonuclease typing showed that type a strains segregated into two major clonal groups, while type e and type f strains represented single clones, respectively. In another Utah study, Bender and colleagues reviewed all cases of invasive *H. influenzae* disease in children less than 5 years old from 1998 through 2008 and found that *H. influenzae* serotype a was the most common, representing the etiologic agent in 28% of all infections.[109] Increased incidence of encapsulated non-type b *H. influenzae* may reflect either the emergence of hypervirulent clones or limited genetic diversity among non-type b strains.[976] Serious infections (i.e., meningitis, pneumonia) due to *H. influenzae* serotypes other than type b have been reported.[421,678,703,869]



■ **FIGURE 9-1** Structure of the repeating unit of the *Haemophilus influenzae* type b capsular polysaccharide PRP. This molecule consists of the 5-carbon monosaccharide ribose, linked by an ester bond to ribitol, and a 5-carbon sugar alcohol, which, in turn, is linked to a phosphate group.

of singular importance. Animal studies with isogenic strains transformed with DNA coding for capsule production confirmed that type b strains were more virulent than the other capsular types. The PRP capsule allows the organism to resist phagocytosis and intracellular killing by neutrophils. Anti–type b antibodies promote complement-dependent phagocytosis and killing (opsonization) of these organisms in vitro and in vivo. In addition to the type b capsule, *H. influenzae* possesses several other virulence factors, most of which are related to adherence, colonization, and invasion. Some of these factors are found in both typeable and nontypeable strains, while others are

found only in nontypeable *H. influenzae* isolates.

***Haemophilus Influenzae* Type b Vaccines and Immunity.** Prior to the introduction of effective vaccines against *H. influenzae* type b, this organism was the most common cause of bacterial meningitis in children younger than 5 years, causing approximately 16,000 cases of invasive disease annually. The majority of systemic type b infections occurred in children aged 2 years and younger. Inadequate levels of protective, anti-PRP bactericidal antibodies at this age play a major role in the development of disease. During the immediate newborn period, immunity to *H. influenzae* type b is acquired by transplacental antibodies, but this is lost within the first few months of life. These antibodies reappear following exposure to type b organisms or to other microbial antigens that engender cross-reactive antibodies. Most individuals who develop systemic *H. influenzae* type b disease have low or undetectable levels of anti-PRP capsular antibodies. The antigenic capabilities of native, purified PRP were exploited in the first *H. influenzae* type b vaccine preparations. Purified PRP elicited antibodies in older children, but failed to elicit a response in children less than 2 years of age, the group most at risk for serious illness. PRP also did not predictably elicit a "booster" response on subsequent antigenic challenge. This failure was due to the T-cell-independent nature of the primary immune response to polysaccharide antigens. Clinical trials were subsequently undertaken with four PRP/protein conjugate vaccines. The vaccines consisted of PRP conjugated covalently to tetanus toxoid (PRP-T), to diphtheria toxoid (PTP-D), to an outer membrane protein complex from Neisseria meningitidis (PRP-OMPC), and to CRM197, a "non-toxic" diphtheria toxin isolated from a mutant strain of Corynebacterium diphtheriae (PRP-HbOC). Using this approach, the immunogenicity of the PRP material would likely be enhanced, since conjugation of carbohydrate moieties to proteins as haptens elicits a T-cell dependent immune response with generation of memory cells.

Two conjugate vaccines are currently licensed and available in the United States: PRP-T (ActHIB *Haemophilus* b Tetanus Conjugate Vaccine, Sanofi Pasteur, Swiftwater, PA) and PRP-OMPC (PedvaxHIB *Haemophilus* b Conjugate Vaccine [Meningococcal Outer Membrane Protein Conjugate],

Merck and Company, Inc., West Point, PA). PRP-T is also available as a vaccine containing diphtheria, tetanus, and acellular pertussis vaccine combined with ActHIB (TriHIBit DTaP/Hib, Connaught Laboratories, Swiftwater, PA), and PRP-OMPC is also available in combination with the hepatitis B vaccine (Recombivax). Infants should receive a dose of PRP-T conjugate vaccine at 2, 4, and 6 months of age or two doses of PRP-OMPC administered at 2 and 4 months of age. A booster dose is

recommended at 12 to 15 months of age. The food and drug administration (FDA) also states that TriHIBit (DTaP/Hib) should not be used for dosing at ages 2, 4, or 6 months, but can be used for the final dose in children aged 12 months or older. The PRP-OMPC/hepatitis B combination vaccine can be used for immunizations at 2, 4, 12, and 15 months of age.

In the United States, widespread use of these conjugate vaccines has almost completely eradicated invasive *H. influenzae* type b disease in children aged 5 years and younger. In 1987, the incidence of invasive pediatric *H. influenzae* type b was 41 cases per 100,000 per year, and this incidence decreased to about 0.11 cases per 100,000 per year by 2007 in the same pediatric population.[1225] Large decreases in disease incidence among children less than 18 months of age were noted long before the conjugate vaccines were in widespread use, and it is now recognized that conjugate vaccines not only prevent disease by inducing active immunity, but also decrease oropharyngeal carriage of and, consequently, exposure to *H. influenzae* type b. In premature infants or those with underlying disease (e.g., HIV infection), the vaccines may not be as effective as in term infants with normal immune systems, since antibody responses are less robust, which results in lower levels of anti-PRP antibodies.[833] The reduced antibody responses to the vaccines in HIV-infected infants is likely due to the T-cell-depleted state of the patients and the T-cell-dependent nature of the immune response to the conjugate vaccines. *H. influenzae* type b conjugate vaccines are immunogenic in HIV-seropositive adults, but the ability to develop protective levels of antibody is related to baseline CD4 T-cell counts and IgG levels. Occasionally, severe infections with nontypable *H. influenzae* may occur, as evidenced by the report of a previously healthy 7-year-old boy who developed an intracranial abscess.[1134]

Other Haemophilus *Species*

H. parainfluenzae, *H. haemolyticus*, *H. parahaemolyticus*, and *H. paraphrophaemolyticus* and *H pittmaniae* are part of the normal microbiota of the respiratory tract and are rarely associated with infections. In recent years, however, several reports documenting *H. parainfluenzae* as an important agent of native and prosthetic valve endocarditis have appeared in the literature.[256,275,590,960] *H. parainfluenzae* infections of pacemaker lead wires and an implantable cardioverter defibrillator have also been described.[314,996] *H. parainfluenzae* has been isolated from patients with bronchitis, sinusitis, otitis media, brain abscess, COPD/pneumonia, cellulitis, abscesses, neonatal sepsis, and native/prosthetic joint infections.[159,200,201,243,481,1032,1170,1278] *H. parainfluenzae* has also been isolated as an unusual cause of urinary tract infection.[556] *H. parainfluenzae* may occasionally be isolated from the genital tract and may cause urethritis in men, chorioamnionitis in

women, and early-onset sepsis in the neonate.[408,1008,1079] Rare *H. parainfluenzae* hepatobiliary tract infections, including liver abscess, retroperitoneal abscess, and peritonitis, have also been reported.[29,449,454,863,1008] In these latter infections, it was postulated that *H. parainfluenzae* gained access to the hepatobiliary tree from the intestinal tract rather than via a hematogenous route.

Haemophilus ducreyi

H. ducreyi is the causative agent of chancroid, a highly contagious sexually transmitted infection characterized by painful genital and perianal ulcers and tender inguinal lymphadenopathy (Image Plate 9-2B). Chancroid is a major cause of genital ulcer disease in Latin America, Africa, East and Southeast Asia, and India. In these countries, chancroid accounts for 20% to 60% of genital ulcer disease.[900] Outbreaks of chancroid have been reported in a number of coastal cities in the United States and Canada. In these outbreaks, female sex workers have been the usual reservoir, with the exchange of sex for money or drugs being a major behavioral risk factor.[148] In these outbreaks, male-to-female case ratios of 3:1 to 25:1 have been observed. In undeveloped countries, infection with *H. ducreyi* is associated with transmission of human immunodeficiency viruses (HIV-1 and HIV-2). Studies in Africa have provided evidence that chancroid is a risk factor for sexual transmission of HIV-1. Chancroid facilitates the transmission of HIV due to increased viral shedding into the genital tract from ulcer exudates and bleeding of ulcers during and after intercourse.[426] The lesions of chancroid also disrupt mucosal integrity, thereby providing a portal of entry for HIV. In addition, the cellular immune response to *H. ducreyi* involves infiltration of the lesion tissues with large numbers of CD4-positive T-helper cells in the lesions themselves, thereby increasing the risk of transmission to sexual contacts. Antigens of *H. ducreyi* also stimulate the CD4 T lymphocytes locally, resulting in upregulation of HIV replication and increased titers of infectious virus and virally infected cells in the genital tract.[1295]

Although several potential virulence factors have been identified in *H. ducreyi*, their roles in pathogenesis are poorly understood at the present time. All strains appear to have fine, tangled, surface fimbriae, unique outer membrane proteins, lipooligosaccharide, and a cytotoxin/hemolysin, which contributes to epithelial cell invasion and the formation of ulcerative lesions.[1324] The role of these molecules in disease pathogenesis is not clear. *H. ducreyi* also has a specific growth requirement for iron, and, although it lacks siderophores, the organism has a receptor on its surface that binds hemoglobin and is essential for iron uptake. *In vitro*, *H. ducreyi* is able to attach, invade, and cause cytopathic effects in cultured human foreskin cells, and several animal models for chancroid have been developed to study virulence and pathogenicity, including rabbit, piglet, primate, and human volunteer models. The latter

studies have helped to identify genes and gene clusters that are essential for organism virulence.[99]

Genital lesions caused by this organism are called "soft chancres" because, unlike the primary chancre of syphilis, the borders of the lesion are ragged and pliable rather than sharply demarcated and indurated. Trauma or localized abrasion of the skin or mucosa is required for entry of the organism, and it is estimated that about 10,000 organisms constitute

an infective dose. After an incubation period of 4 to 7 days, the lesions begin as tender erythematous papules that become pustular, eroded, and ulcerated within the next 48 to 72 hours. In males, chancroid lesions are usually tender, painful, and are covered with a yellow-gray exudate overlying a beefy, bleeding base. In males, lesions are usually present on the external genitalia (prepuce, frenulum, coronal sulcus, glans penis, and penile shaft); in uncircumcised men, these lesions are often found beneath the foreskin. HIV-infected males may have increased numbers of genital ulcers that heal more slowly and that become superinfected more readily.[708] In females, multiple lesions may be present on the fourchette, labia, vulva, the vaginal wall, the clitoris, and in the perianal area. Lesions may coalesce to form large ulcerative areas that may become superinfected. Extragenital chancroid is uncommon, but lesions have been described on the glabrous skin of the thighs, the breasts, the oral mucosa, and the conjunctivae. In about half of the infected patients, painful unilateral inguinal lymphadenopathy develops. These enlarged lymph nodes may actually suppurate through the overlying skin to form large inguinal abscesses, draining fistulas, and sinus tracts. Untreated genital ulcers may persist for several months, but the organism does not disseminate via the blood or lymphatics.

Laboratory Diagnosis of Haemophilus *Infections*

Direct Examination of Clinical Specimens

Gram's Stain. A rapid presumptive diagnosis of *H. influenzae* meningitis can be made by direct examination of cerebrospinal fluid (CSF) using the Gram's stain. If sufficient (i.e., more than 1 to 2 mL) CSF is received, the specimen is centrifuged to obtain a pellet of material for examination and culture. Cytocentrifugation of CSF specimens enhances detection of small numbers of organisms and considerably increases the sensitivity of the Gram's stain in comparison with conventionally centrifuged or uncentrifuged specimens.[236] On Gram's stain preparations, *Haemophilus* organisms appear as small, pale-staining, gram-negative coccobacilli (Image Plate 9-1A). Occasionally, slender filamentous cells may be observed.

Although *H. influenzae* may be the likely pathogen on the basis of the Gram's stain appearance and the clinical presentation of the patient, the organisms cannot be identified on the basis of the Gram-stained smear. Furthermore, a negative Gram's stain (i.e., a Gram's stain with no organisms seen) does not rule out the possibility of *Haemophilus* infection because very few organisms may be present in the specimen. Gram's stain smears of other specimen types may also be useful for presumptive diagnosis.

Detection of Type b Capsular Antigen. For rapid diagnosis of *H. influenzae* type b infections, techniques are available for the detection of the type b PRP capsular antigen in CSF, serum, and urine. Commercially available methods include latex agglutination (LA) (e.g., Directigen Meningitis Combo Test, Becton-Dickinson and Company, Sparks, MD) and staphylococcal protein A coagglutination (COA) (e.g., Phadebact CSF test, Bactus AB, Huddinge, Sweden). These kits contain reagents for detecting *H. influenzae* group b PRP antigen along with reagents for *S. pneumoniae*, *N. meningitidis*, and group B streptococcal antigen detection in body fluids. Despite the performance of these tests in clinical trials that led to their approval for use, many laboratories have discontinued offering these tests routinely. Aside from the high cost of the latex/COA reagents themselves, their diagnostic and prognostic utility has not been borne out after years of experience. Perkins et al. reviewed all latex tests performed over a 10-month period at two hospitals and found 57 positive results.[1021] Review of these cases revealed that 31 results were false-positives, 22 were true positives, and 4 were indeterminate. False-positive tests were noted most often with urine specimens. Patients with false-positive test results received unnecessary treatment, resulting in prolonged hospitalization and additional complications. Furthermore, among the 22 patients with true-positive LA results, there was no instance where the antimicrobial therapy or clinical management of the patient was altered on the basis of the LA result. These tests may be most useful in cases of suspected meningitis where the initial Gram's stain is negative and/or where CSF cultures are negative after 48 hours. The recommended method for optimal direct detection of *H. influenzae* in CSF depends on careful inspection of a Gram-stained smear prepared from a cytocentrifuged specimen.

Recovery of *Haemophilus* Species in Culture. Optimal recovery of *Haemophilus* species from clinical specimens depends on proper collection and transport of specimens, and the use of appropriate culture media and incubation environments. Because of their fastidious nature, specimens containing them should not be exposed to drying or to temperature extremes. Crucial specimens such as CSF should be hand-carried to the clinical laboratory as soon after collection as possible. Conventional sheep blood agar is not suitable for recovery of *Haemophilus* species that require V factor for growth because of the presence of V-factor-inactivating enzymes in native sheep blood. Rabbit or horse blood does not contain these enzymes, and agar media

containing either of these blood products will support the growth of most *Haemophilus* species. Isolation of *Haemophilus* requires incubation at 35°C to 37°C in a moist environment with increased CO_2 (3% to 5%). This atmosphere is provided by incubation in a CO_2 incubator or in candle extinction jars. Primary isolation of *Haemophilus* species from clinical specimens is accomplished by using chocolate agar, *Haemophilus* isolation agar, or the *Staphylococcus* streak technique (Image Plate 9-1B).

Chocolate Agar. Chocolate agar is prepared by adding sheep blood to an enriched agar base when the temperature of the medium is high enough to lyse the red cells without inactivating the NAD in the blood lysate (i.e., about 80°C). Most clinical laboratories purchase chocolate agar and other media from commercial vendors. Commercially prepared "chocolate agar" contains a mixture of hemin and chemically defined growth factors that are added to GC (gonococcal) agar base medium. GC agar base contains proteose peptone, cornstarch, phosphate buffers, sodium chloride, and agar. The chemically defined supplement contains NAD, vitamins ($B1_2$, thiamine hydrochloride), minerals (iron, magnesium), amino acids required for growth of fastidious bacteria (cysteine, glutamine), and glucose. These supplements are available commercially under the trade names IsoVitalex (BD Biosciences) and GCHI Enrichment (Remel Laboratories, Lenexa, KS). The disadvantage of using chocolate agar for primary

isolation of *Haemophilus* species is that hemolytic properties of several species (*H. haemolyticus*, *H. parahaemolyticus*, *H. pittmanniae*) cannot be determined. Because *Haemophilus* species are common inhabitants of the upper respiratory tract (including both hemolytic and nonhemolytic species), many laboratories have adopted *Hemophilus* isolation agar for recovery of these organisms from respiratory specimens.

***Haemophilus* Isolation Agar.** These commercially available media contain beef heart infusion, peptones, yeast extract, and defibrinated horse blood (5%) containing both X and V factors. Bacitracin (300 μg/mL) is added to inhibit the other normal respiratory tract microbiota (i.e., staphylococci, micrococci, neisseriae, streptococci). Besides the selective recovery of *Haemophilus* species, hemolytic properties can be determined directly upon primary isolation.

***Staphylococcus* Streak Technique.** Many bacteria and yeasts synthesize and secrete NAD during growth on bacteriologic media. In mixed cultures, *Haemophilus* species that require V factor may grow as pinpoint colonies around the colonies of other microorganisms. This phenomenon is called satellitism (Image Plate 9-1C). Satellitism provides a technique for detecting these organisms in mixed cultures as well

as a presumptive test for genus-level identification. A colony of a possible *Haemophilus* species is subcultured onto a sheep blood agar plate and streaked as a lawn. A single streak of an NAD-producing organism (e.g., *S. aureus*) is made through the lawn inoculum. After overnight incubation in a CO_2-enriched environment at 35°C to 37°C, tiny moist gray colonies of the haemophili may be observed within the hemolytic area adjacent to the staphylococcal growth. X-factor-dependent haemophili will also grow as satellite colonies because hemin and hematin are released from the lysed red blood cells. This method may be used for presumptive identification of *Haemophilus* species when species-level identification is not required or essential (e.g., upper respiratory tract specimens).

Identification of *Haemophilus* Species

Colony Morphology and Cultural Characteristics. On chocolate agar, colonies of *H. influenzae* are smooth and blue-gray in color; heavily encapsulated strains may appear mucoid. Most strains produce convex, smooth, entire colonies of 1 to 2 mm after overnight growth. Some strains will have an "*E. coli*" odor because of the production of indole by some biotypes. *H. parainfluenzae* colonies are usually smaller, light gray, and have a matte appearance on growth media. Oxidase and catalase reactions for these organisms are usually positive, although the oxidase reaction may be delayed.

Identification Procedures. Commonly encountered *Haemophilus* species are identified by hemolytic reactions on horse blood agar and growth requirements for X and V factors. Filter paper disks or strips impregnated with X factor, V factor, or both X and V factors are routinely used to determine these factor requirements (Image Plate 9-1D). The organism to be identified is streaked as a lawn on media deficient in growth factors, such as trypticase-soy agar. It is important when selecting colonies from primary culture plates for this test that none of the chocolate agar or other blood-containing media is transferred to the factor determination plate. Suspending the organism in factor-deficient broth before plate inoculation is one way to reduce carryover of growth factors and consequent false-positive results. The X and V factor disks or strips are placed on the agar surface about 1 to 2 cm apart (Chart 9-1). If a disk/strip containing both factors is also used, the disks may be more widely spaced on the agar surface. The plates are incubated in 5% to 7% CO_2 at 35°C for 18 to 24 hours, and the patterns of growth around the disks/strips are observed. Trypticase-soy agar is the medium of choice for performance of the growth factor determination procedure.[361] Although interpretation of factor requirement tests is generally clear-cut, misidentifications of *H. influenzae* with *H. parainfluenzae* have been reported because of inconsistent results in X factor determinations. Reasons for these inaccuracies include presence of trace amounts of hemin in the basal medium used for the factor determination test, carryover of X factor in inocula taken from growth on

blood-containing medium, and the fastidious nature of some *H. parainfluenzae* strains, with consequent difficulty in reading tests for factor requirement. The δ-aminolevulinic acid (ALA)–porphyrin test circumvents many of these problems as this test is a direct assessment of the ability of *Haemophilus* strains to synthesize protoporphyrin intermediates in the biosynthetic pathway to hemin from the ALA. Strains that require exogenous X factor for growth (i.e., *H. influenzae* and *H. haemolyticus*) cannot synthesize protoporphyrins from ALA and, consequently, are negative with this test. Strains that do not require exogenous X factor for growth (i.e., *H. parainfluenzae* and *H. parahaemolyticus*) possess the enzymes that synthesize protoporphyrin compounds from ALA and are, consequently, ALA–porphyrin test-positive. ALA-impregnated disks (BD Biosciences; Remel Laboratories; Hardy Diagnostics, Santa Maria, CA) or growth media containing the ALA reagent (Remel Laboratories) may also be used to perform the ALA–porphyrin test (Image Plate 9-1F and 9-1G). With the disk method, the impregnated disk is moistened with water and inoculated with colonial growth. After 4 hours' incubation, the disk is observed under ultraviolet light (Wood's light). Brick-red fluorescence on the disk indicates a positive test result; bluish fluorescence constitutes a negative test result. With the ALA agar medium, the organism is inoculated onto the medium and incubated overnight. The next day, growth is examined under a Wood's light for brick-red fluorescence. Phenotypic characteristics for the identification of *Haemophilus* species are presented in Table 9-2.

Tests for hemolysis on horse blood agar, factor requirements, and the ALA–porphyrin test have been incorporated into commercially available sectored-plate systems for identification of *Haemophilus* species. These include the *Haemophilus* ID II Triplate and the *Haemophilus* ID Quad plate (Remel). The triplate is a three-sectored plate containing horse blood agar, agar containing V-factor, and agar containing ALA agar. Following inoculation and overnight incubation, the plate is read by inspecting the blood agar sector for hemolysis, looking for growth in the V-factor sector, and looking for brick-red fluorescent growth in the ALA sector under Wood's light illumination. The quad-plate contains four sectors containing factor-supplemented Müeller–Hinton medium with horse blood, X-factor-enriched medium, V-factor-enriched medium, and medium containing both X and V factors, respectively. Based on hemolysis and the pattern of growth in the remaining quadrants, an identification can be obtained.

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TABLE 9-2 Phenotypic Characteristics for Identification of *Haemophilus* Species Isolated From Humans

Species Biotype	Requirement for								Acid Production From						
	HEM HBA	X Factor	V Factor	ALA	IND	URE	ODC	ONPG	GLU	SUC	LAC	FRUC	RIB	XYL	MANN
Haemophilus influenzae															
Biotype I	–	+	+	–	+	+	+	–	+	–	–	–	+	+	–
Biotype II	–	+	+	–	+	+	–	–	+	–	–	–	+	+	–
Biotype III[a]	–	+	+	–	–	+	–	–	+	–	–	–	+	+	–
Biotype IV	–	+	+	–	–	+	+	–	+	–	–	–	+	+	–
Biotype V	–	+	+	–	+	–	+	–	+	–	–	–	+	+	–
Biotype VI	–	+	+	–	–	–	+	–	+	–	–	–	+	+	–
Biotype VII	–	+	+	–	+	–	–	–	+	–	–	–	+	+	–
Biotype VIII	–	+	+	–	–	–	–	–	+	–	–	–	+	+	–
Biogroup aegyptius	–	+	+	–	–	+	–	–	+	–	–	–	+[w]	–	–
Haemophilus parainfluenzae[b]															
Biotype I	–	–	+	+	–	–	+	V	+	+	–	+	–	–	+
Biotype II	–	–	+	+	–	+	+	V	+	+	–	+	–	–	+
Biotype III	–	–	+	+	–	+	–	V	+	+	–	+	–	–	+
Biotype IV	–	–	+	+	+	+	+	V	+	+	–	+	NA	–	+
Biotype VI	–	–	+	+	+	–	+	V	+	+	–	+	NA	NA	V
Biotype VII	–	–	+	+	+	+	–	V	+	+	–	NA	–	–	NA
Biotype VIII	–	–	+	+	+	–	–	V	+	+	–	NA	–	–	NA
Haemophilus haemolyticus	+	+	+	–	V	+	–	–	+	–	–	+[w]	+	V	–
Haemophilus parahaemolyticus	+	–	+	+	–	+	V	–	+	+	–	+	–	–	–
H. paraphrohaemolyticus	+	–	+	+	–	+	–	+	+	+	–	+	–	–	–
Haemophilus pittmaniae	+	–	+	+	–	–	–	+	+	+	–	+	NA	–	+
Haemophilus ducreyi	+[w]	+	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Biotyping reactions are identical with those of *H. influenzae* biogroup aegyptius, but biogroup aegyptius strains are xylose-negative.
[b] Biotype V strains are identical to *Aggregatibacter segnis*.
+, positive; –, negative; +[w], weak positive; V, variable; NA, not available; HEM, hemolysis on horse blood agar; ALA, aminolevulinic acid porphyrin test; IND, indole; URE, urease; ODC, ornithine decarboxylase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; GLU, glucose; SUC, sucrose; LAC, lactose; FRUC, fructose; RIB, ribose; XYL, xylose; MANN, mannose.

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Serotyping of *Haemophilus influenzae*. The easiest technique for serotyping isolates is slide agglutination. A dense organism suspension is prepared in saline, and single drop of the suspension is placed in each of a series of circles on a glass slide corresponding to the number of sera to be tested, plus a saline control. Type-specific antisera are added to each of the test circles, and the slide is rotated. Rapid (i.e., less than 1 minute) agglutination of organisms by a specific antiserum and the absence of agglutination in the saline control identifies the isolate as a specific serotype. Polyvalent and type-specific antisera for all six *H. influenzae* serotypes are commercially available. Serotyping of clinical isolates by slide agglutination must be carefully performed with reliable reagents that are quality controlled with organisms of known serotype.

A COA culture confirmation test (Phadebact *Haemophilus* Test, Bactus AB) is available for simultaneous identification and serotyping of *H. influenzae* type b from primary culture media. This kit contains a vial of staphylococcal cells sensitized with type b antisera (test reagent) and a second vial of staphylococci sensitized with antisera to type a, c, d, e, and f (control reagent). Colonies from growth media are mixed with each of the two reagents on a cardboard slide. After mixing, the slide is rocked for 30 to 60 seconds. Visible agglutination of the mixture with the type b test

reagent but not the control reagent identifies the isolate as *H. influenzae* type b. A positive reaction in the control reagent indicates that the organism belongs to capsular types a, c, d, e, or f.[511]

Biotyping of *Haemophilus influenzae* and *Haemophilus parainfluenzae*. In his taxonomic study of the genus, Kilian introduced biochemical tests for identifying and characterizing haemophili.[701] Biotypes are determined with three tests: indole production, urease, and ornithine decarboxylase. *H. influenzae* strains could be divided into seven biotypes that were independent of serotype (i.e., type b, non–type b, or nontypeable). Seven *H. parainfluenzae* biotypes have also been described using these methods. Biotyping reactions for *Haemophilus* species are included in Table 9-2.

Biotyping of *Haemophilus* species has demonstrated that specific biotypes are associated with different infections, sources, antigenic properties, and antimicrobial resistance patterns. *H. influenzae* biotype I strains are recovered mostly from CSF, blood, and respiratory tract secretions of infants less than 1 year old.[913,962] *H. influenzae* biotypes II and III are recovered from conjunctival and sputum cultures of children aged 1 to 5 years and from adults greater than 20 years of age. Biotypes II and III are also associated with ocular infections.[22] Biotype IV *H. influenzae* are emergent pathogens in obstetric, gynecologic, perinatal, and neonatal infections. The cryptic genospecies associated with maternal/neonatal infections has biotyping reactions that are identical with *H. influenzae* biotype IV and underscores the shortcomings of phenotypic methods for strain typing. The cryptic genospecies can only be distinguished from biotype IV strains by molecular genetic approaches that are not readily available in clinical microbiology laboratories.

Biochemical and Kit Methods for *Haemophilus* Identification. Carbohydrate fermentation tests can also be used for identification of *Haemophilus* species. These methods bypass technical problems associated with factor requirement tests. Acid production from several carbohydrates (i.e., sucrose, fructose, ribose, xylose, and mannose) can be used to separate *H. influenzae* from *H. parainfluenzae*. A limited battery of tests is also necessary for differentiating and identifying the former *Haemophilus* species *Aggregatibacter aphrophilus, Aggregatibacter actinomycetemcomitans*, and *Aggregatibacter segnis*. Lactose fermentation or *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis is useful for identifying *A. aphrophilus*; other *Haemophilus* species and *Aggregatibacter actinomycetemcomitans* are negative for lactose fermentation and ONPG reaction. *A. segnis* produces weak or delayed acidification of sucrose and fructose, and is negative in all three biotyping reactions. Carbohydrate utilization is determined in heavily inoculated semisolid cystine-tryptic digest agar containing 1% filter-sterilized carbohydrates. Acid production is apparent in 4 to 18 hours. Reagent-impregnated urease and ornithine decarboxylase disks and the spot indole test can be used for rapid biotyping of individual isolates.

Kit identification systems that use modified conventional tests and chromogenic enzyme substrates can also be used to identify and simultaneously biotype *Haemophilus* species. The RapID NH panel (Remel Laboratories), the *Haemophilus-Neisseria* Identification (HNID) panel (Siemens-MicroScan, West Sacramento, CA), the API NH strip (bioMérieux, La Balme-les-Grottes, France), and the Vitek 2 *Neisseria-Haemophilus* card identify these organism within 2 to 4 hours of inoculation (Image Plate 9-2A and 9-2C).[641,916]

Haemophilus ducreyi: *Laboratory Diagnosis of Chancroid*

Specimen Collection and Direct Examination. Cotton, rayon, Dacron, or calcium alginate swabs may be used to collect specimens from chancroid ulcers. Specimens should be collected from the base and undermined margins of the ulcer. For bubo, a needle and syringe may be used to aspirate pus from the bubo through normal, intact tissue. If the bubo has suppurated through the skin, a swab may be used to collect the specimen. However, the organism is often not recoverable from bubo pus. Transport media have not been evaluated for recovery of *H. ducreyi*; so growth media should be inoculated directly. If a transport medium is used, Amies transport medium or Amies with charcoal should be used and the specimen should reach the laboratory within 4 hours of specimen collection. Gram-stained smears of the suppurative exudate from genital lesions of chancroid are usually noncontributory because of low sensitivity (from 5% to 63%) and specificity (51% to 99%). When seen on direct smears, *H. ducreyi* appears as pale-staining, gram-negative coccobacilli, often arranged in clustered groups ("school of fish") or loosely coiled parallel chains ("railroad tracks") (Fig. 9-2). Organisms may be inside and outside of polymorphonuclear leukocytes (PMNs). Since genital lesions and suppurative nodes or abscesses may become superinfected with other bacteria, culture should be attempted. Diagnosis of chancroid is frequently made on clinical and epidemiologic grounds; however, syphilis, genital herpes simplex virus infection, and lymphogranuloma venereum must be included in the differential diagnosis.

Culture. *H. ducreyi* may be difficult to recover in culture, and several media have been evaluated for this purpose. For the

■ **FIGURE 9-2** Gram-stained morphology of *Haemophilus ducreyi*. On Gram-stained smears prepared from lesions of chancroid or from colonial growth, the organisms may appear in loose clusters (i.e., "school of fish" ***[left]***) or as loosely coiled clusters of gram-negative bacilli lined up in parallel (i.e., "railroad tracks" ***[right]***).

highest yield, more than one type of culture medium should be inoculated. GC agar base supplemented with 2% bovine hemoglobin, 5% fetal calf serum, and 1% IsoVitalex, and Müeller–Hinton agar supplemented with 5% chocolatized horse blood plus 1% IsoVitalex enrichment have been used for culture of *H. ducreyi*. In some media formulations, activated charcoal (0.2%) instead of fetal calf serum has been used as an additive to absorb toxic materials from the specimen and the agar. Vancomycin (3 µL/mL final concentration) may be added to inhibit contaminating gram-positive bacteria. Commercial chocolate agar media will generally support the growth of *H. ducreyi*; vancomycin disks may be placed in various quadrants of a chocolate plate to help detect the organisms in mixed cultures. Most strains of *H. ducreyi* have vancomycin minimum inhibitory concentrations (MICs) of 32 to 128 µg/mL, but some strains have MICs as low as 4 µg/mL. Therefore, it may be advantageous to inoculate media with and without vancomycin and to collect multiple appropriate specimens. Media are incubated at 33°C to 35°C in 5% to 7% CO_2 or in a candle jar with high humidity; growth may actually be inhibited at incubation temperatures exceeding 35°C. Better growth may be obtained using a microaerophilic environment, where cultures are placed in a GasPak jar with two carbon dioxide/hydrogen gas-generating envelopes and no catalyst. Cultures are inspected daily for 10 days. Most isolates from clinical specimens produce visible growth in 2 to 4 days.

Colonies of *H. ducreyi* are small, nonmucoid, and gray, yellow, or tan in color. The colonies can characteristically be "nudged" along the agar surface with a bacteriologic loop, are difficult to pick up, and produce a nonhomogenous, "clumpy" suspension in saline. The organisms appear as gram-negative coccobacilli, usually in close association with one another. *H. ducreyi* is catalase-negative and oxidase-positive; the oxidase reaction is usually delayed and develops only after 15 to 20 seconds with the tetramethyl-*p*-phenylenediamine dihydrochloride reagent. Because of the fastidious nature of *H. ducreyi*, growth factor requirements cannot be demonstrated with factor-impregnated disk or strip techniques. The ALA–porphyrin test is negative, indicating that exogenous hemin is required for growth. *H. ducreyi* is biochemically inert, except for positive nitrate reduction and alkaline phosphatase tests. In an evaluation with 25 *H. ducreyi* isolates, Shawar et al. found that all strains produced unique and consistent enzymatic reactions on the RapID ANA (Remel), a 4-hour identification system for clinically significant anaerobic bacteria.[1178] *H. ducreyi* strains are also susceptible to sodium polyanetholsulfonate (SPS) as determined by a disk susceptibility method. Growth characteristics, SPS susceptibility, and aminopeptidase profiles obtained with the enzymatic RapID ANA system may be useful for laboratory identification of *H. ducreyi*. A rapid immunochromatographic strip method for organism identification from culture has also been described.[1009] This assay used monoclonal antibodies directed against the highly conserved outer membrane hemoglobin receptor, HgbA, as the capture antibody. Using this assay, all of 26 *H. ducreyi* isolates tested were correctly identified, while all other bacteria tested were negative. Molecular methods and non–growth-dependent techniques, including direct and amplified nucleic acid probes, have also been developed for identification or direct detection of *H. ducreyi*. Suntoke and colleagues from the NIH developed a sensitive, rapid, and reproducible real-time multiplex polymerase chain reaction (PCR) assay for the direct detection of *H. ducreyi*, *Treponema pallidum*, and herpes simplex virus types 1 and 2 directly in genital ulcers.[1231]

Antimicrobial Susceptibility of Haemophilus *Species*

Until about 1973, antimicrobial susceptibility testing of *H. influenzae* was unnecessary because clinically significant isolates were susceptible to ampicillin, the drug of choice for type b meningitis and bacteremia. By 1974, some strains of *H. influenzae* had become resistant to ampicillin beacuse of acquisition of plasmids carrying genes encoding β-lactamase enzymes. Plasmid-mediated β-lactamases have been found in type b, encapsulated non–type b, and nontypeable *H. influenzae* and in *H. parainfluenzae* strains. These organisms harbor a 3.0 megadalton transposon that carries the gene for a TEM-1 type β-lactamase enzyme. Rare *H. influenzae* strains produce a second type of β-lactamase, called ROB-1.[329] Over the last 25 years, the

prevalence of β-lactamase-positive strains has been increasing throughout the world. In the 2002 LIBRA surveillance project, β-lactamase-positive *H. influenzae* accounted for 32.2% of 2,791 isolates.[658] At that time, more than 99% of the strains were susceptible to amoxicillin clavulanate, regardless of β-lactamase production. Clavulanate inactivates the β-lactamase, rendering the organisms susceptible to the amoxicillin included in this combination antibiotic. *H. influenzae* strains have been isolated that are resistant to ampicillin, but do not produce β-lactamases. In these strains, resistance is due to alterations in cell wall penicillin binding proteins or in permeability of the cell membrane to the agent. These strains also display diminished susceptibility to third-generation cephalosporins and to β-lactam/β-lactamase inhibitor combinations. A 2000 to 2001 *H. influenzae* surveillance study in the United States detected only 9 (0.6%) β-lactamase-negative, ampicillin-resistant strains among 1,434 isolates, suggesting that these strains are relatively uncommon.[683] However, a 2009 report from Japan showed that the proportion of ampicillin-resistant, β-lactamase-positive

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H. influenzae strains decreased from 9.6% in 1999 to 4.8% in 2003, whereas the proportion of ampicillin-resistant, β-lactamase-negative strains increased from 15.4% to more than 30% over the same time period.[505]

Clinical and Laboratory Standards Institute (CLSI) methods for antimicrobial susceptibility testing of *H. influenzae* and *H. parainfluenzae* recommend the use of *Haemophilus* test medium or *Haemophilus* test medium broth for disk diffusion and broth dilution, respectively.[285] *Haemophilus* test medium contains hematin, yeast extract, and NAD; Müeller–Hinton agar is included for the disk diffusion method.[660,661] Breakpoints for susceptible, intermediate, and resistant categories are available for ampicillin, β-lactam/β-lactamase inhibitor combinations, third- and fourth-generation cephalosporins (including oral and parenteral formulations), macrolides, and trimethoprim–sulfamethoxazole (SXT).[285] Only susceptible category breakpoints are available for aztreonam, the carbapenems, and the fluoroquinolones as the absence of resistant strains precludes defining intermediate and resistant categories for these agents at the present time. A rapid test for detection of β-lactamase provides a simple method for testing susceptibility to ampicillin and amoxicillin. Ampicillin/amoxicillin-resistant, β-lactamase-negative strains are also considered resistant to β-lactam/β-lactamase inhibitor combination agents (i.e., amoxicillin clavulanate, ampicillin sulbactam, piperacillin–tazobactam) and oral cephalosporins (i.e., cefaclor, cefetamet, cefonicid, cefprozil, cefuroxime, loracarbef) even though isolates may test susceptible to these agents in vitro. Since the standard recommendations for antimicrobial therapy

of *Haemophilus* infections are well defined, many laboratories do not perform antimicrobial susceptibility testing on *Haemophilus* species. Furthermore, some strains *H. influenzae* and *H. parainfluenzae* do not grow well on *Haemophilus* test medium.

Currently, third-generation cephalosporins (i.e., cefotaxime, ceftriaxone) are the recommended therapy for treatment of systemic *H. influenzae* type b infections because of the excellent activity of these agents in vitro and in vivo.[919] Treatment for systemic infections is usually continued for 7 to 10 days; those individuals with complicated infections (e.g., endocarditis, osteomyelitis) may require 3 to 6 weeks of parenteral therapy. Infections associated with nontypeable *H. influenzae* (e.g., chronic bronchitis, COPD, otitis media) are often treated with ampicillin or amoxicillin if susceptibility to these agents has been documented. Oral macrolides (i.e., azithromycin, clarithromycin) or fluoroquinolones are also prescribed for these infections. The fluoroquinolones have excellent activity against *H. influenzae*; in a recent 25-year susceptibility survey performed in Japan, greater than 95% of *H. influenzae* isolates had fluoroquinolone MICs of ≤0.06 μg/mL.[505] Nontypeable *H. influenzae* that are β-lactamase-negative are also susceptible to the fluoroquinolones, macrolides, and the extended-spectrum oral and parenteral cephalosporins. *H. parainfluenzae* and other respiratory tract *Haemophilus* species are susceptible to third-generation cephalosporins, the quinolones, tetracyclines, and aminoglycosides. Although most strains are susceptible to penicillin and ampicillin, resistant strains, including β-lactamase-producing strains, have been reported.

In parts of the world where chancroid is endemic, rapid and accurate methods for specific diagnoses are lacking; so a syndromic approach, as recommended by World Health Organization, is used for management of sexually transmitted infections. Chancroid can be treated with a variety of agents, including fluoroquinolones, macrolides (e.g., erythromycin, azithromycin), and cephalosporins (e.g., ceftriaxone).[919] These agents show excellent activity against *H. ducreyi* in vitro, while many strains are resistant to tetracyclines and SXT. Recommended treatment regimens for chancroid from the World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC), and the United Kingdom Clinical Effectiveness Group (CEG) include erythromycin (500 mg, PO, for 7 days, tid or qid), azithromycin (1 g, PO, single dose), ceftriaxone (250 mg, IM, single dose), and ciprofloxacin (500 mg, PO, bid for 3 days).[900] Because of treatment failures in HIV-infected patients, there is some concern about the clinical efficacy of single-dose or short-course therapies (e.g., with IM ceftriaxone or PO azithromycin) in individuals with chancroid who are also immunodeficient.[1274] Symptomatic or asymptomatic sexual contacts of individuals diagnosed with chancroid should be treated if sexual contact occurred 10 days or less after clinical presentation of the index patient.

Genus *Aggregatibacter*

Taxonomy

In 2006, *H. aphrophilus, H. paraphrophilus*, and *H. segnis* were reclassified, along with *Actinobacillus actinomycetemcomitans*, into the genus *Aggregatibacter*.[956] *H. aphrophilus* and *H. paraphrophilus* are now recognized as the same species, *A. aphrophilus*; the V factor dependence of *H. paraphrophilus* is considered a variable characteristic of the single species (Image Plate 9-2F). *Aggregatibacter* species are classified in the family *Pasteurellaceae*.

Clinical Significance

Aggregatibacter aphrophilus. *A. aphrophilus* is indigenous to the oral cavity, where it is a minor component of the healthy periodontal microbiota. *A. aphrophilus*, along with *H. parainfluenzae*, is the species in this group that is most commonly associated with endocarditis.[10,327,613,666,1018] Most people who develop native valve endocarditis with this organism have some preexisting heart valve abnormalities such as aortic valve stenosis, but cases of endocarditis have been reported in those without preexisting cardiac abnormalites.[10,613,1018] *A. aphrophilus* may also be associated with endocarditis involving prosthetic valves.[1329] *A. aphrophilus* has been isolated from various head and neck infections (e.g., sinusitis, otitis media, epiglottitis, ventriculitis, epidural and brain abscesses, and meningitis) and pleuropulmonary infections (e.g., pneumonia, empyema, and lung abscess).[6,60,253,592,666,680,967,1273] Hematogenous dissemination of *A. aphrophilus* may also result in septic arthritis, vertebral osteomyelitis, sponydylodiscitis, cellulitis, endophthalmitis, and intra-abdominal infections.[296,613,700,1005,1371] Neutropenia,

malignancy, and cancer chemotherapy are predisposing factors for infections with these organisms. *A. aphrophilus* culturally resembles other fastidious gram-negative bacteria and are part of the "HACEK" group, which includes *Haemophilus* (H), *Aggregatibacter* (A), *Cardiobacterium* (C), *Eikenella* (E), and *Kingella* (K) species (Image Plate 9-2D).

Aggregatibacter segnis. *A. segnis* is found occasionally in dental plaque and the upper respiratory tract. Infections with *A. segnis* are rare and include periodontal infections, bacteremia endocarditis, cholecystitis, acute appendicitis, and pancreatic abscess.[89,192,203,762,934,1210] Further investigation of these fastidious organisms is

needed for a more complete appreciation of their pathogenic potential.

Aggregatibacter actinomycetemcomitans. *A. actinomycetemcomitans* is a part of the normal microbiota of the oral cavity, particularly in the gingival and supragingival crevices. Based on reactions of specific monoclonal antibodies with exposed, cell surface carbohydrate moieties of the cell wall lipopolysaccharide (LPS), this species can be divided into seven serotypes designated a through g.[1236] Serotypes a, b, and c are the most prevalent, comprising more than 80% of oral strains. A recent study of *A. actinomycetemcomitans* recovered from subgingival plaque samples from 161 patients with and without periodontal disease in the Los Angeles found that serotype c was the dominant serotype, comprising 50% of all strains, followed by serotypes a and b.[240] Serotypes d, e, and f were less frequently found or were not detected. A recent study from Brazilian patients also reported that serotypes c and a were the predominant serotypes.[1105] Some patients may be colonized with multiple serotypes. Although it has been suggested that certain serotypes (e.g., serotype b) may be associated with specific forms of periodontal disease, biotyping and rRNA gene probe studies have demonstrated significant phenotypic and genomic heterogeneity among strains belonging to the same serotype.[73] In a study of 311 strains from 189 periodontology patients, 216 stains were serologically typeable and 95 were nontypeable. Amplification of the serotype antigen-specific genes indicated that the nontypeable strains possessed serotype-specific gene sequences, but the genes were not expressed among the nonserotypeable strains.[677] All of these serotypes and lineages have been isolated from patients with periodontal disease, indicating that all have pathogenic potential.

A. actinomycetemcomitans is well recognized as a major cause of aggressive periodontal disease.[422,578] This organism is causally related to early-onset periodontitis and localized aggressive periodontal disease seen in older children and young adults, generally in the 12 to 26 year age group. This disease is characterized by rapid degeneration and destruction of the alveolar bone supporting the first permanent molars and incisors. Subgingival bone loss in these areas results in the development of deep gingival pockets that readily bleed with probing. Progression of the disease can result in bone destruction along the affected root surfaces with the involvement of adjacent teeth. This severe periodontitis may lead to acute periodontal abscesses and subsequent tooth loss. Periodontal disease associated with *A. actinomycetemcomitans* evokes both systemic and localized immune responses, and high titers of serum IgG are produced against *A. actinomycetemcomitans* serotype antigens, which are O-side chain oligosaccharides in the cell wall LPS. The LPS of the organism also induces localized gingival inflammation and allows the organism to establish itself in subgingival plaque. In addition to LPS, *A. actinomycetemcomitans* produces a potent leukotoxin and several other virulence factors that contribute to its role as a periodontal pathogen.

Certain strains of *A. actinomycetemcomitans* are more pathogenic because of the production of more virulence factors. For example, the JP2 clone of *A. actinomycetemcomitans* has a 530 base pair deletion in the promoter region of the leukotoxin operon that results in enhanced leukotoxin production and augmented virulence.[182,280,564,669] These factors act in concert to cause inflammation, localized immunosuppression, inhibition of phagocytic activity, and resorption of bone. Some of these factors stimulate production of serum and crevicular fluid antibodies that may help to limit the periodontal process. In addition to the described virulence factors, failure to stimulate humoral or cell-mediated responses against the organism and/or its products may be partially responsible for progression to severe, generalized periodontal disease. *A. actinomycetemcomitans* is also implicated in the pathogenesis of Papillon–Lefèvre syndrome, an inherited disease characterized by hyperkeratosis of the palms and soles and extensive periodontal destruction resulting in the loss of both primary and permanent teeth.

Epidemiologic studies indicate that periodontal disease associated with *A. actinomycetemcomitans* has racial and genetic components that predispose individuals to these severe oral infections caused by endogenous microorganisms. For example, studies on the geographic occurrence of the highly leukotoxic JP2 clone have demonstrated that colonization with this pathogen is largely restricted to those of African American descent and is absent, for the most part, in non-African populations from Northern Europe.[564,565] Epidemiologic investigations have distinguished two patterns of periodontal disease associated with *A. actinomycetemcomitans*. Localized aggressive periodontitis in northern European Caucasian populations is associated with non-JP2 clonal populations of *A. actinomycetemcomitans* circulating as opportunistic pathogens, while the JP2 clone is the principal cause of aggressive, juvenile-onset periodontal disease in young adults of North or West African descent.[566] Furthermore, individuals infected with the JP2 clone may have diminished responses to therapeutic interventions, which may include deep scaling and root planning, antimicrobial therapy, and surgery, if necessary.[307] Sporadic reports have documented colonization of Caucasians with the JP2 clone.[280]

The immune response to periodontal infection with *A. actinomycetemcomitans* may also play a role in the pathogenesis of disease. Patients with aggressive periodontitis produce abnormally high levels of prostaglandin E2, and the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) in the crevicular fluids surrounding the teeth.[537] These factors are produced by local monocytes and macrophages in response to bacterial products. Cultured monocytes and macrophages from periodontitis patients produce three to six times the amount of these cytokines than similar cells from noninfected patients on exposure to *A. actinomycetemcomitans* LPS.[1176]

These inflammatory cytokines induce bone resorption by osteoclasts, leading to alveolar bone loss, and downregulation of neutrophil chemotaxis. The immune response to microbial antigens may help to limit the periodontal process and failure to stimulate humoral or cell-mediated responses against the organism, and/or its products may be partially responsible for progression to more severe, generalized periodontal disease.[1130]

A. actinomycetemcomitans is also associated with actinomycotic infections, endocarditis, bacteremia, wound infections, and dental infections. The name of the organisms is derived from its recognized "concomitant" isolation with *Actinomyces* species from abscesses and other infections. *A. actinomycetemcomitans* is a cause of subacute bacterial endocarditis.[1010,1043,1321,1342] Native valve endocarditis usually occurs in individuals with antecedent valvular damage because of congenital heart diseases (e.g., congenital aortic stenosis, bicuspid aortic valve disease, atrioventricular septal defect, mitral valve insufficiency due to rheumatic heart disease). Prosthetic mitral and aortic valve endocarditis have also been reported in individuals with porcine and mechanical valves and pacemakers. Localized complications have included pericarditis and paravalvular abscesses.[1353] Predisposing factors in the development of *A. actinomycetemcomitans* endocarditis include poor dentition or recent dental manipulations. *A. actinomycetemcomitans* endocarditis usually follows an indolent course. Fever, weight loss, chills, cough, and night sweats are common, along with a heart murmur. Hepatosplenomegaly and conjunctival or splinter hemorrhages are variably present. Complications include septic emboli, cerebral hemorrhage, coronary arteritis, vasculitis, congestive heart failure, valvular damage requiring prosthetic valve placement or replacement, pyogenic ventriculitis, and death.[666,789,1010,1182,1353]Other infections caused by *A. actinomycetemcomitans* result from contiguous spread of the organism from its habitat in the oral cavity or from hematogenous dissemination during bacteremia and endocarditis. Head, neck, and pleuropulmonary infections have included epidural and brain abscesses, osteomyelitis, cervical lymphadenitis, cellulitis, pneumonia, chest wall/subphrenic abscess, and lung abscess.[102,238,545,549,1006,1223,1321] Hematogenous dissemination of *A. actinomycetemcomitans* has resulted in osteomyelitis, spinal abscess, vertebral discitis, endophthalmitis, septic arthritis, and hepatic and intra-abdominal abscesses.[125,936,1182,1184,1228] Portals of entry for various types of infections have included oral lesions, prior pulmonary infections, skin abrasions, thoracotomy sites, and urinary tract instrumentation.

Cultural Characteristics and Identification of Aggregatibacter

Species

Colonies of *A. aphrophilus* are small after 24 hours' incubation; 48 to 72 hours' incubation is often required before colony morphology can be ascertained and sufficient growth is available for preliminary identification tests. On chocolate agar, colonies are 0.5 to 1 mm in diameter, convex, granular, and have a faint yellowish pigment. A distinct "grade school paste" odor may be detected. Some strains of *A. aphrophilus* require V factor; V-factor-requiring strains will grow on chocolate agar, but not on sheep blood agar. *A. aphrophilus* is catalase- and oxidase-negative, although weak or delayed positive oxidase reaction may be observed with some isolates. The negative catalase reaction is helpful in separating *A. aphrophilus* from *A. actinomycetemcomitans*, which is catalase-positive. Acid is produced from glucose, maltose, sucrose, lactose, and trehalose. The organism hydrolyzes ONPG and does not produce arginine dihydrolase, lysine or ornithine decarboxylase, indole, or urease. Phenotypic tests for identification of *Aggregatibacter* species are presented in Table 9-3 (Image Plate 9-2H and 9-3C).

TABLE 9-3 Phenotypic Characteristics for Identification of *Aggregatibacter aphrophilus*, *Aggregatibacter actinomycetemcomitans*, and *Aggregatibacter segnis*

Characteristic	*A. aphrophilus*	*A. actinomycetemcomitans*	*A. segnis*[a]
HEM SBA	–	–	–
OX	–[b]	–[b]	–[b]
CAT	–	+	V
NO_3 RED	+	+	+
Requires X factor	–	–	–
Requires V factor	V	–	+
IND	–	–	–
URE	–	–	–
ODC	–	–	–
ONPG	+	–	V
Acid produced from:			
GLU	+	+	+[w]
MAL	+	+[c]	+[w]
FRU	+	+	+[w]
SUC	+	–	+
LAC	+	–	–
XYL	–	V	–
MNTL	–	V[d]	–
MANN	+	+	–
GAL	+	V	+[w]
TREH	+	–	–
RAFF	+	–	–

[a] Only quantitative differences in the amount of acid produced from carbohydrates may discriminate *A. segnis* phenotypically from stains of *H. parainfluenzae* biotype V (negative for indole, urease, and ODC).
[b] A few strains may produce weak or delayed reactions.
[c] Rare strains may be maltose-negative.
[d] Most strains are mannitol-positive.
+, positive reaction; –, negative reaction; V, variable reaction; +[w], weak positive; HEM SBA, hemolysis on sheep blood agar; OX, oxidase; CAT, catalase; NO_3 RED, reduction of nitrate to nitrite; IND, indole; URE, urease; ODC, ornithine decarboxylase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; GLU, glucose; MAL, maltose; FRU, fructose; SUC, sucrose; LAC, lactose; XYL, xylose; MNTL, mannitol; MANN, mannose; GAL, galactose; TREH, trehalose; RAFF, raffinose.

A. actinomycetemcomitans grows slowly on chocolate and blood agars, with visible colonies appearing after 48 to 72 hours (Image Plate 9-3B). Colonies are small, smooth, translucent, nonhemolytic, and have slightly irregular edges. Fresh clinical isolates are adherent to the agar and are difficult to emulsify. With prolonged incubation (i.e., 5 to 7 days), colonies may develop a central density that appears as a four- or six-pointed star. This characteristic is lost on repeated subculture, and the colonies become less adherent. As with *A. aphrophilus*, growth in broth is scant and adherent to the sides of the tube. On Gram's stain, the organisms appear as pale-staining, gram-negative coccobacilli (Image Plate 9-3A). Longer cells may be noted with repeated subcultures. Characteristics for identification of *A. actinomycetemcomitans* include lack of growth on MacConkey and other enteric agars, and positive reactions for catalase production and nitrate reduction. The organism is usually oxidase-negative, although occasional strains may be weakly positive, urease-negative, does not produce indole, and does not require X or V factors. Lysine and ornithine decarboxylase and arginine dihydrolase reactions are negative (Image Plate 9-2G). Most strains strongly ferment glucose, fructose, and mannose; acid production from maltose, mannitol, and xylose varies. *A. actinomycetemcomitans* can be differentiated from *A. aphrophilus* in that the former is catalase-positive, ONPG-negative, and acid is not produced from lactose, sucrose or trehalose.

A. segnis grows on chocolate agar but not on sheep blood agar because of its requirement for V factor. This organism is ALA-porphyrin test–positive and is negative for indole, urease, and ornithine decarboxylase production. In carbohydrate test, acid production from glucose, sucrose, and fructose is weak or delayed, and no acid is produced from lactose, ribose, xylose, or mannose.

Antimicrobial Susceptibility of Aggregatibacter *Species*

In 2010, the CLSI published an approved guideline for antimicrobial susceptibility testing of the fastidious members of the HACEK group.[285] This method uses cation-adjusted Müeller–Hinton broth supplemented with 2.5% to 5% lysed horse blood. The inoculum is a dilution of a direct colony suspension of the organism that is equivalent to a 0.5 McFarland turbidity standard. End points are read after 24 to 48 hours' incubation at 35°C in a 5% CO_2 atmosphere. Agents suggested by CLSI for primary testing of these organisms include ampicillin, amoxicillin clavulanate, ceftriaxone or cefotaxime, imipenem, ciprofloxacin or levofloxacin, and SXT. Breakpoints are available for these agents and several others (e.g., meropenem, azithromycin,

clarithromycin, tetracycline, and rifampin) No intermediate or resistant categories are defined for ceftriaxone and cefotaxime due to the absence or rare occurrence of strains belonging to these "nonsusceptible" categories.

Strains of *A. aphrophilus* and *A. actinomycetemcomitans* are susceptible to third-generation cephalosporins, fluoroquinolones, tetracyclines, and aminoglycosides.[738] Agents such as ceftriaxone, cefotaxime, and ciprofloxacin have been effective in patients with serious central nervous system (CNS) infections, brain abscesses, and endocarditis. Although most strains are susceptible to penicillin and ampicillin, resistant strains, including rare β-lactamase-producing strains, have been reported.[659] The third- and fourth-generation cephalosporins are considered the drugs of choice for empiric therapy of serious infections such as endocarditis. *In vitro* studies indicate that combinations of antimicrobials agents for the treatment of *A. actinomycetemcomitans* endocarditis may be synergistic, additive, or antagonistic; so efficacy of combination therapy cannot be predicted and must be determined for individual strains by either an agar dilution technique or Etest.

Treatment of *A. actinomycetemcomitans*-associated periodontal disease involves subgingival debridement, scaling, root planing, and antimicrobial therapy directed against periodontitis-associated bacteria. Successful treatment partially depends on the eradication and suppression of *A. actinomycetemcomitans* and other bacteria from deep periodontal pockets. For advanced periodontal disease, systemic metronidazole or doxycycline, along with mechanical debridement, is usually prescribed.[798] Of course, the administration of systemic tetracyclines is contraindicated in children; so nontetracycline regimens are used in such instances. A formulation of extended-release doxycycline is also available for use as a topical agents, and a powder composed of microspheres containing minocycline has also been approved by the FDA for the treatment of established periodontitis.[958,1080] This compound releases controlled amounts of antibiotic beneath the gums and is used along with root planing and scaling for the treatment of refractory periodontal disease. Although most strains of *A. actinomycetemcomitans* have remained susceptible to tetracyclines, plasmid-associated, *tetB*-mediated tetracycline resistance in *A. actinomycetemcomitans* has been reported. Since most strains of *A. actinomycetemcomitans* are resistant to metronidazole in vitro, the success of this agent may be due to the broad activity of metronidazole against co-infecting anaerobic periodontal pathogens (e.g., *Prevotella species, Porphyromonas* species, *Tannerella forsythensis*).

Cardiobacterium Species

Taxonomy

Cardiobacterium hominis (formerly CDC group IID), the first member of the genus *Cardiobacterium*, was first described in 1964. This organism is classified in the family *Cardiobacteriaceae,* which also includes the genera *Suttonella* and *Dichelobacter*.[353] In 2004, a second *Cardiobacterium* species, *C. valvarum*, was described.[551] The genus *Suttonella* was created to accommodate *Kingella indologenes*, an organism that was shown to be more closely related to *C. hominis* than to other *Kingella* species or other members of the family *Neisseriaceae*, where *Kingella* species are classified.[353] *S. indologenes* is now classified with *Cardiobacterium* in the family *Cardiobacteriaceae*. A second *Suttonella* species, *S. ornithocola,* was described in 2005.[434] This organism was isolated from necropsy specimens of congested lung tissues of birds belonging to the tit families during an investigation of an unexplained die-off of specific avian species in the United Kingdom. The family *Cardiobacteriaceae* belongs to the order *Cardiobacteriales* in the γ-subdivision of the *Proteobacteria*.

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Clinical Significance

C. hominis is an organism of low virulence and has the unique characteristic of causing endocarditis almost exclusively.[829,1315] The organism is a part of the normal microbiota of the respiratory tract, but, because of its slow growth, it is rarely noted in respiratory tract specimens. *C. hominis* enters the bloodstream and usually infects previously diseased or damaged heart valves (e.g., rheumatic heart disease, ventricular septal defects, aortitis).[781,829] *C. hominis* endocarditis may also develop in individuals with no evidence of previous heart disease.[244,477] About 20% of reported *C. hominis* infections have involved various prosthetic cardiac valves, including aortic homograft tissue valves, porcine xenograft aortic/mitral valves, mechanical aortic/mitral valves, and pacemaker leads.[50,319,829,1185] Patients are usually middle aged to elderly and have poor dentition and/or a history of recent dental procedures. The infection follows a subacute course, with insidious onset, low-grade fevers, and vague symptoms that may linger for several months before a diagnosis is established. *C. hominis* tends to form large, friable vegetations on the heart valves; so the most serious complications include septic embolization, mycotic aneurysms, and congestive heart failure.[312,914]Severe, sepsis-associated thrombocytopenia may be observed in some patients.[66] Isolation of *C. hominis* from specimens other than blood cultures is rare. Francioli and colleagues diagnosed cardiobacterial meningitis in a patient as a complication of endocarditis, and Rechtman and Nadler reported the recovery of *C. hominis* along with *Clostridium bifermentans* from an abdominal abscess in a diabetic

man with adenocarcinoma of the kidney and caecum.[445,1065] In 1998, Nurnberger et al. described a case of pacemaker lead infection and vertebral osteomyelitis due to *C. hominis*.[959] *C. hominis* has also been isolated from peritoneal fluid of a patient on continuous ambulatory peritoneal dialysis and no evidence of endocarditis and from pericardial fluid of a 10-year-old girl.[124,742]

C. valvarum was first described as the cause of bacteremia and endocarditis in a 37-year-old man with congenital bicuspid aortic valve disease that was complicated by a ruptured mycotic aneurysm.[551] The description of this single isolate prompted a search for additional isolates, and four additional strains were found in the Culture Collection of the University of Goteborg in Sweden.[550] These isolates were oral strains recovered from subgingival pockets and a plaque specimen. The 16S rRNA gene sequences of these isolates exhibited 99.3% to 99.6% sequence homology with the original *C. valvarum* isolate. Whole-cell protein electrophoretic profiles, nutritional and growth requirements, and cellular and colonial morphology were also similar. Since the first description of this organism in 2005, four additional cases of native valve endocarditis[154,241,603,1294] and two reports of prosthetic valve endocarditis due to *C. valvarum* have been reported in the literature.[480,594] A fourth report dealt with identification of the organism and contained no patient information.[1029] Among the four patients with native valve endocarditis, three had tricuspid or bicuspid aortic valve involvement and one had mitral valve involvement. Large vegetations and destruction of heart tissue were seen in all cases, and three of the four patients required placement of prosthetic valves. Prosthetic valve endocarditis was reported in a 71-year-old man with a bioprosthetic valve placed 2 years prior because of native aortic valve stenosis and in a 28-year-old woman with a right ventricle-to-pulmonary artery conduit and a porcine pulmonic valve.[480,594] Although replacement of the conduit and prosthetic valve were planned after 6 weeks of chemotherapy in the latter patient, the 71-year-old male developed septic emboli that were detected in the cerebellum by CT and MRI, and expired from septic shock resulting from severe cardiac deterioration. In other instances, *C. valvarum* has also been isolated from subgingival pockets and dental plaque samples.[551]

S. indologenes has been isolated from human corneal infection and was first reported as a cause of prosthetic valve endocarditis in 1987.[646] In 2010, Yang and colleagues reported *S. indologenes* prosthetic valve endocarditis in a 28-year-old Cambodian man with complex congenital heart disease that was complicated by septic pulmonary emboli.[1396] In 2011, Ozcan and colleagues described a fatal case of *S. indologenes* prosthetic valve endocarditis in a 25-year-old man that was complicated by splenic infarction and fatal intracranial hemorrhage.[992]

Cultural Characteristics and Identification

C. hominis and *C. valvarum* are principally isolated from blood cultures, grow slowly, and produce no visible change in the blood culture medium (i.e., turbidity, hemolysis). Gram's stains of positive blood bottles may not reveal the organism; low-speed centrifugation of an aliquot of the medium to remove red blood cells with subsequent high-speed centrifugation to pellet small numbers of organisms may aid in their visualization. On Gram's stains, both organisms may appear gram-variable, with a tendency for the cells to retain the crystal violet dye at the poles. Individual cells may appear swollen at one or both ends, resulting in teardrop-, dumbbell-, and lollipop-shaped organisms. On Gram's stains from blood cultures, cells may aggregate in rosette-like clusters or line up in parallel, picket fence arrangements (Image Plate 9-3D). Cell morphology is influenced by the type of culture medium used; in media containing yeast extract, cells appear as uniform, gram-negative bacilli with rounded ends.

C. hominis and *C. valvarum* grow as very small, glistening, opaque colonies on enriched blood or chocolate agar generally after 48 to 72 hours at 35°C in 5% to 7% CO_2 (Image Plate 9-3E). *C. valvarum* grows more slowly than *C. hominis,* and some strains of both species may show pitting of the agar that is more evident on chocolate agar than on sheep blood agar. Neither species is able to grow on MacConkey agar or other enteric selective or differential agars. Both *C. hominis* and *C. valvarum* are oxidase-positive, catalase-negative, nitrate-negative, urease-negative, and esculin hydrolysis-negative. *C. hominis* is indole-positive, but this may not be noted with the "spot indole" (cinnamaldehyde) reagent, since very small amounts of indole are produced by most strains. *C. valvarum* isolates from reports of patients with endocarditis have been strongly indole-positive (even with the spot indole reagent), while oral strains have been indole-negative.[550] Indole production can best be detected in heart infusion or tryptone broth. These media are heavily inoculated (with a swab) and incubated for 48 hours. Extraction with xylene and use of Ehrlich's reagent rather than Kovac's reagent allow

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detection of small amounts of indole. Both *C. hominis* and *C. valvarum* produce H_2S by the lead acetate strip method. Phenotypic characteristics for the identification of *Cardiobacterium* species are presented in Table 9-4.

C. hominis produces acid fermentatively from glucose, fructose, sucrose, mannose, and sorbitol; acid production from maltose and mannitol is variable, with most strains producing positive reactions. Like *C. hominis*, *C. valvarum* fermentatively produces acid from glucose, fructose, sorbitol, and mannose, but, unlike *C. hominis*, does not produce acid from maltose, sucrose, or mannitol. Neither species is able to ferment

lactose, galactose, raffinose, or xylose. *S. indologenes* produces acid from glucose, maltose, and sucrose, but not from lactose, and is distinguished by its ability to produce indole. This characteristic should be determined in tryptone broth with xylene extraction and addition of Ehrlich's indole reagent. *S. indologenes* may be differentiated from *C. hominis*, which closely resembles, by appearance on Gram's stain, positive alkaline phosphatase activity and failure to produce acid from mannitol and sorbitol. Phenotypically, *C. hominis* is very similar to *S. indologenes*. *C. hominis* and *C. valvarum* can be differentiated from *S. indologenes*, *E. corrodens*, and *Kingella* species by cellular fatty acid analysis. As with other agents of bacterial endocarditis, molecular methods such as broad-range PCR and DNA sequencing have been used successfully for detection and identification of *C. hominis* and *C. valvarum* directly in clinical specimens.[477,914,946,1029]

Antimicrobial Susceptibility

C. hominis can be tested for antimicrobial susceptibility using the methods in the CLSI-approved standard (see Chapter 17).[286]

TABLE 9-4 Phenotypic Characteristics for Identification of *Cardiobacterium* Species, *Eikenella corrodens*, *Kingella* Species, and *Suttonella* Species

Test	*C. hominis*	*C. valvarum*	*E. corrodens*	*K. kingae*	*K. denitrificans*	*K. oralis*	*K. potus*	*S. indologenes*	*S. ornithocola*
HEM SBA	–	–	–	β	–	–	–	–	β
OX	+	+	+	+	+	+	+	+	+
CAT	–	–	–	–	–	–	–	–	+
NO_3 RED	–	–	+	–	+	–	–	–	NA
NO_2 RED	+		–	+	+	–	–	V	–
IND	+	V	–	–	–	–	–	+	–
URE	–	–	–	–	–	–	–	–	–
ODC	–	–	+	–	–	–	–	–	–
ESC	–	–	–	–	–	–	–	–	NA
ONPG	–	–	–	–	–	–	–	–	–
DNase	–	–	–	–	–	–	+	–	–
Gas from GLU	–	–	–	–	–	–	–	–	–
Acid produced from:									
GLU	+	V	–	+	+	+	–	+	+
MAL	V^+	V^-	–	+	–	–	–	+	–
FRU	+	+	–	–	–	–	–	+	+
SUC	+	V^-	–	–	–	–	–	+	+
LAC	–	–	–	–	–	–	–	–	–
XYL	–	–	–	–	–	–	–	–	–
MNTL	V^+	V^-	–	–	–	–	–	–	–
MANN	+	+	–	–	–	–	–	+	+
GAL	–	–	–	–	–	–	NA	–	–
TREH	–		–	–	–	–	NA	–	+
RAFF	–	–	–	–	–	–	NA	–	–
SBTL	+	V	–	–	–	–	NA	–	–

+, positive reaction; –, negative reaction; V, variable reaction; NA, not available; HEM SBA, hemolysis on sheep blood agar; OX, oxidase; CAT, catalase; NO_3 RED, reduction of nitrate to nitrite; NO_2 RED, reduction of nitrite to nitrogen gas; IND, indole; URE, urease; ODC, ornithine decarboxylase; ESC, esculin; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; DNase, deoxyribonuclease; GLU, glucose; MAL, maltose; MANN, mannose; FRU, fructose; SUC, sucrose; LAC, lactose; XYL, xylose; MNTL, mannitol; GAL, galactose; TREH, trehalose; RAFF, raffinose; SBTL, sorbitol; V^+, variable reaction, most strains positive; V^-, variable reaction, most strains negative.

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Despite the availability of these methods, antimicrobial susceptibility testing of *C. hominis* and *C. valvarum* is difficult because of their fastidious requirements and slow growth rates. From the literature, strains of *C. hominis* are susceptible to most antimicrobial agents, including penicillin, ampicillin, cefazolin, third-generation cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and tetracycline; susceptibility to the macrolides and aminoglycosides is variable.[738] The currently recommended therapy for native valve endocarditis due to *C. hominis* is ceftriaxone, ampicillin sulbactam, or ciprofloxacin for at least 4 weeks.[81] Patients with native valve endocarditis due to *C. hominis* may be successfully treated with antibiotics alone in some instances, but some have required partial valve resection or valve replacement

because of hemodynamic compromise, embolic phenomena, or progressive cardiac failure. The same agents may be used for prosthetic valve endocarditis, but therapy may need to be extended beyond 6 weeks' duration. Although reported, β-lactamase-producing, penicillin-resistant strains of *C. hominis* are rarely encountered.[808] Strains of *C. valvarum* have been tested for antimicrobial susceptibility by Etest methods. Both blood and oral isolates of *C. valvarum* are susceptible to penicillin, ampicillin, ticarcillin/clavulanate, cephalosporins of all generations, fluoroquinolones, carbapenems, tetracyclines, and SXT.[550,551] Patients who have developed *C. valvarum* endocarditis also had similar complications, and survivors have also required surgical intervention and valve replacement.

Eikenella corrodens

Taxonomy

Originally, the genus *Eikenella* (CDC group HB-1) defined isolates considered as facultative strains of *Bacteroides corrodens,* an anaerobic gram-negative rod. Based on genotypic and phylogenetic studies, the species epithet of *B. corrodens* was changed to *Bacteroides ureolyticus* (urease-positive) and the facultative isolates were placed in genus *Eikenella* as the sole species, *E. corrodens*. Based on 16S rRNA sequencing and rRNA–DNA hybridization, *E. corrodens* is related to *Neisseria* species and is in the family *Neisseriaceae* in the β-subgroup of the *Proteobacteria*.

Clinical Significance

E. corrodens is a part of the normal microbiota of the mouth and upper respiratory tract. In the oral cavity, this organism plays a role in some types of periodontal disease. A high prevalence of *E. corrodens* is found in subgingival plaque samples from patients with periodontal disease, whereas prevalence is low in individuals with no periodontal disease.[1227] Among those with periodontal disease, the highest prevalence of *E. corrodens* is found in people under 20 years of age with progressive periodontitis, followed by those with postjuvenile periodontitis, prepubertal periodontitis, rapidly progressive periodontitis, and adult periodontitis. If left untreated, severe periodontitis may become refractory to treatment.[1227] In healthy persons, the prevalence of the organism in deep gingival specimens decreases with advancing age. *E. corrodens* is part of the mixed bacterial microbiota recovered from periodontal and gingival specimens, periapical abscesses, and root canal infections.

E. corrodens has been isolated from a wide variety of infections and clinical specimens.[1011,1181] This organism has been isolated from various types of head and neck infections, including ocular infections (i.e., periorbital cellulitis, corneal ulcers,

endophthalmitis, and lacrimal abscesses), mastoid and submandibular abscesses, and thyroid abscesses.[74,251,577,585,662,663,784,1139,1275,1403] Pleuropulmonary infections involving *E. corrodens* include lung abscesses and empyemas. These occur in settings of immunosuppression, propensity for pulmonary aspiration, and underlying lung disease.[611,704,1366] By extension from periodontal, middle ear, or sinus infections, *E. corrodens* can gain access to the CNS, resulting in meningitis, brain or paraspinal abscesses, subdural empyemas, and osteomyelitis.[53,70,685] Bacteremia and endocarditis usually occur in immunocompromised hosts, intravenous drug abusers, and individuals with previous valvular damage and a history of recent dental work or oral surgery.[572,826,974]Endocarditis has also been diagnosed in healthy people with no recognized risk factors.[1328] Prosthetic valve endocarditis and infections of indwelling vascular prostheses with *E. corrodens* have been reported. *E. corrodens* bacteremia with or without endocarditis may also be diagnosed in patients with rheumatoid arthritis and hematologic malignancies.[409] *E. corrodens* is a recognized cause of subcutaneous abscesses, soft tissue cellulitis, and bacteremia in intravenous drug users.[47,501,974] These infections result from the use of saliva for cleansing of the skin or dissolving the narcotics in saline prior to drug injection. *E. corrodens* can cause cellulitis and osteomyelitis of the hand resulting from "clenched fist injuries" (i.e., fist fights), chronic nail-biting, and human bites.[150,559,1237] Traumatic implantation of the organism into subcutaneous tissue may enable extension of infection into bones and joints, resulting in osteomyelitis and/or septic arthritis.[150,233]Cultures obtained from these infections often grow *E. corrodens* along with other facultative organisms (α- and β-hemolytic streptococci [including the *Streptococcus anginosus/constellatus/intermedius* group], *Staphylococcus aureus*, coagulase-negative staphylococci, enteric gram-negative bacilli) and oral obligate anaerobes. Osteomyelitis of the spine caused by *E. corrodens* has been reported as a result of hematogenous dissemination from an infected site (e.g., severe periodontal disease) or as a complication of spine surgery.[41,775,1055,1267] In 2007, an unusual case of *E. corrodens* cervical spine epidural and retropharyngeal abscess was diagnosed in a 72-year-old man who had swallowed a fish bone.[649] The bone had transversed the esophagus and had lodged in the C3 to C4 intervertebral disk space. Additionally, *E. corrodens* osteomyelitis of the jaw was reported as a complication of radiation therapy for a squamous cell carcinoma of the tonsils.[941] *E. corrodens* may also be isolated in pure or mixed culture from various types of wound infections and abscesses, including necrotizing fasciitis.[889,990,1110,1418] Bacteremia from localized wound infections may result in hematogenous infections and abscesses at many sites, including joint spaces (i.e., septic arthritis), the vertebrae (i.e., discitis, paraspinal abscess), and the female genital tract (i.e., chorioamnionitis).[397,1055] *E. corrodens* can survive in the

gastrointestinal tract and may be isolated

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from abdominal, splenic, hepatic, and pancreatic abscesses and peritoneal infections along with facultative and anaerobic intestinal microbiota.[67,662,1061,1221] *E. corrodens* has also been recovered from gynecological infections, including chorioamnionitis, amniotic fluid infection, endometritis, vulvar abscess, and cervicitis associated with in situ colonization of intrauterine devices.[39,476,637,730,994] In 2007, an *E. corrodens* urinary tract infection was reported in an 83-year-old women with recurrent anal prolapse and chronic lymphocytic leukemia.[602]

Cultural Characteristics and Identification

E. corrodens grows on blood and chocolate agar, but does not grow on MacConkey agar (Image Plate 9-3G). Colonies are small (0.5 to 1.0 mm) after 48 hours. About 50% of isolates may "pit" the agar during growth; both pitting and nonpitting variants may be observed in the same culture. A pale yellow pigment (observed best on a white swab swept through growth on a chocolate agar plate) is usually produced, and most strains have an odor of sodium hypochlorite (i.e., "Clorox" bleach or a heavily chlorinated swimming pool). On Gram's stain, the organisms appear as regular, slender, gram-negative bacilli or coccobacilli with rounded ends (Image Plate 9-3F).

Biochemical characteristics of pitting and nonpitting *E. corrodens* strains are fairly uniform. *E. corrodens* is oxidase-positive and catalase-negative, although rare strains may be weakly catalase-positive. The organism reduces nitrate to nitrite and does not require X or V factors, although hemin is necessary for aerobic growth. Nonpitting isolates may be mistaken for *Haemophilus* species and, when tested with X and V factor disks, display growth around the X but not the V disk. Indole and urease are not produced, and most strains are positive for lysine decarboxylase and ornithine decarboxylase (Image Plate 9-3H). Unlike the other HACEK organisms, *E. corrodens* is asaccharolytic. Kit systems that address fastidious gram-negative organisms are able to correctly identify *E. corrodens*. *E. corrodens*-specific DNA probes, as well as probes for other periodontal pathogens, have been designed by analysis of 16S rRNA, identification of unique nucleotide sequences, and chemical synthesis of probe molecules that are complementary to these species-specific sequences.[1094,1280] Phenotypic characteristics for identification of *E. corrodens* are shown in Table 9-4.

Antimicrobial Susceptibility

Antimicrobial susceptibility testing of *E. corrodens* can be performed using the CLSI-approved standard for susceptibility testing of fastidious bacteria (see Chapter 17).[286]

E. corrodens strains are susceptible to ampicillin, amoxicillin, second- and third-generation cephalosporins, tetracyclines, and fluoroquinolones, and azithromycin and are resistant to the penicillinase-resistant penicillins, clindamycin, vancomycin, erythromycin, metronidazole, and aminoglycosides.[814,883] Penicillin susceptibility varies from strain to strain. Most isolates are variably susceptible to the first-generation cephalosporins, with cefazolin being the most active. *E. corrodens* is susceptible to cefoxitin, ceftriaxone, cefepime, ciprofloxacin, and carbapenems.[738,1181] Rare β-lactamase-positive strains have been reported.[744]

Kingella Species

Taxonomy

Members of the genus *Kingella* are classified in the family *Neisseriaceae* in the γ-subgroup of the *Proteobacteria* and currently include four species: *K. kingae*, *K. denitrificans*, *K. oralis*, and *K. potus*. The former species *K. indologenes* was demonstrated to be more closely related to *Cardiobacterium* species than to the other *Kingella* species; so this organism was renamed *Suttonella indologenes* and transferred to the family *Cardiobacteriaceae* in 1990.[353] *K. kingae*, the type species of the genus, was formerly a member of the genus *Moraxella,* and *K. denitrificans* was formerly called CDC group "TM-1." In 1993, Dewhirst and colleagues described a new *Kingella* species that was recovered from human dental plaque of a patient with adult periodontitis.[352] This new species was named *Kingella oralis*. This organism constitutes 0.4% of the total microbiota in dental plaque from periodontally healthy individuals and about 4.6% of the microbiota in dental plaque from adult and juvenile periodontitis patients.[239] The fourth *Kingella* species, *Kingella potus*, was isolated in 2005 from an infected wound of a zookeeper who, 3 days earlier, had sustained the bite of a kinkajou (*Potus potus*).[766]

Clinical Significance

Although part of the normal microbiota of the upper respiratory and genitourinary tracts of humans, *K. kingae* is increasingly being recognized as an important human pathogen.[373,1390] Since its initial description, *K. kingae* was considered to be a rare cause of infection in patients with endocarditis. However, during the last 10 to 20 years, *K. kingae* has emerged as a significant pathogen in pediatric patients, causing primarily bacteremia and skeletal infections.[1378] This organism colonizes the upper respiratory tract of infants after about 6 months of age, and the rate of colonization increases to about 10% to 12% between 6 months and 2 years of age. After this time,

colonization rates begin to decline. The prevalence of the organism in the respiratory tract is highest among children aged 6 months to 4 years, which parallels the ages having the highest attack rates for invasive disease.[1385] A large study performed in Israel found that invasive *K. kingae* disease occurred most frequently in previously healthy children during the first 2 years of life.[374] Studies in day care center populations have demonstrated that *K. kingae* can be transmitted from child to child by the respiratory route.[699,1199,1383] A study performed at a day care center over an 11-month period found that, among 48 attendees, about 28% carried *K. kingae* in the upper respiratory tract at any one time and that 73% of these children had at least one positive pharyngeal culture over the study period.[1382] Molecular analysis of respiratory tract isolates of *K. kingae* recovered from a large cohort of carriers showed geographic clustering of isolates with identical genotypes, indicating spread of the organism between siblings, playmates, and schoolmates.[1392] This study also demonstrated that invasive strains of *K. kingae* were maintained within a community over several years by respiratory carriage and transmission among close contacts. The portal of entry for the organism into the bloodstream is likely via breaches in the oropharyngeal mucosa.

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Bone and joint infections are the most common clinical manifestations of *K. kingae* infection in children.[224,245,264,769] These infections present as bacteremia, septic arthritis, osteomyelitis, discitis, tenosynovitis, and dactylitis.[126,811,1168] With the availability of effective vaccines against *H. influenzae* type b, *K. kingae* has replaced *H. influenzae* as the most common gram-negative bacterium causing osteoarticular infections in children less than 3 years of age.[912] Most infants with systemic *K. kingae* infections present with low-grade fevers; viral upper respiratory tract infections and stomatitis are also frequently present.[33] In most cases, *K. kingae* septic arthritis is an acute infection, with patients usually presenting within 3 days of the appearance of signs and symptoms. These children are usually febrile, and the involved joints are swollen, are tender, and display decreased ranges of motion. Blood cultures from these patients are frequently negative. *K. kingae* septic arthritis is usually monoarthritic, with the most frequently involved joints being the knee, hip, and ankle.[373] Osteomyelitis due to *K. kingae* is usually an indolent infection that follows a subacute course, with patients usually presenting 7 to 10 days after the appearance of symptoms. *K. kingae* osteomyelitis usually affects the femur, other long bones, the tibia, ulna, radius, and calcaneus bones.[373] Patients with osteomyelitis of the long bones usually are unable to bear weight on the affected limb, and there is local tenderness over the involved bone. Hematogenous seeding of the intervertebral disk spaces leads to spondylitis and

intervertebral discitis.[126] Disk space infections may involve the lumbar, thoracic, lumbosacral, or thoracolumbar spaces. Pediatric osteoarticular infections of the lower sternum and the junction between the manubrium and the xyphoid process have also been described.[811] Children with invasive *K. kingae* infections often have significant underlying conditions, such as acute lymphocytic leukemia or congenital heart disease. Although rare, bone and joint infections due to *K. kingae*, including intervertebral discitis and septic arthritis, have also been diagnosed in adults.[395]

K. kingae is also a rare cause of bacteremia and endocarditis.[373,374] *K. kingae* endocarditis primarily occurs in persons with underlying heart disease (e.g., rheumatic heart disease, cardiac malformations) or in those with cardiac prostheses. However, endocarditis in individuals with no previous heart disease has been reported.[158,373,1114,1168,1409] Unlike bone and joint infections, *K kingae* endocarditis occurs more frequently in adults and school-age children.[1378] Complications of *K. kingae* endocarditis occur commonly and include pericarditis, paravalvular abscess, pericardial abscess, embolic phenomena, mycotic aneurysms, cerebral and pulmonary infarcts, septic shock, and congestive heart failure.[719,855] *K. kingae* endocarditis with meningitis has been reported in a woman with systemic lupus erythematosis.[1360] Patients may have poor oral hygiene, pharyngitis, or mucosal ulcerations due to treatment for other conditions (e.g., radiation therapy and chemotherapy). *K. kingae* bacteremia without endocarditis has been reported in immunocompetent adults following dental manipulations. Additional complications of bacteremia and bone infections include meningitis, hematogenous orbital cellulitis and endophthalmitis, soft tissue infection, and corneal abscess.[199,300,1068,1104] The clinical presentation of *K. kingae* bacteremia may mimic systemic neisserial infections (i.e., meningococcemia or disseminated gonococcal infection).[1262] *K. kingae* was also reported as a cause of corneal ulcer in an 11-month-old child, urinary tract infection in a 45-year-old woman with dysuria and postmenopausal bleeding, and peritonitis in a 55-year-old man with advanced hepatic cirrhosis, who performed a paracentesis procedure on himself, resulting in peritoneal infection.[144,903,1059] The other *Kingella* species are rarely isolated from infectious processes. Although *K. denitrificans* has been cultured from the upper respiratory and genitourinary tracts as part of the normal microbiota, it has also been documented as a cause of septicemia and native/prosthetic valve endocarditis.[563] *K. denitrificans* has also been recovered from empyema fluid of a patient with bronchogenic carcinoma, from bone marrow of a patient with AIDS, from the amniotic fluid of a young woman with chorioamnionitis, and from a corneal ulcer.[707,818,893,901] To date, *K. oralis* has not been associated with infections. *K. potus* has only been isolated from the previously mentioned kinkajou bite in a human.[766]

Cultural Characteristics and Identification

Kingella species are plump gram-negative bacilli or coccobacilli that sometimes occur in pairs or short chains. They are oxidase-positive and, unlike *Neisseria* and *Moraxella* species, are catalase-negative. All species grow on chocolate and blood agars and do not grow on MacConkey agar or other enteric media (Image Plate 9-4A and 9-4B). All *Kingella* species and *S. indologenes* are oxidase-positive and catalase-negative; *S. ornithocola* is positive for both oxidase and catalase. All *Kingella* and *Suttonella* species are negative for arginine dihydrolase, lysine and ornithine decarboxylases, urease, esculin hydrolysis, and ONPG hydrolysis. The phenotypic characteristics that are useful for identification of *Kingella* and *Suttonella* species are shown in Table 9-4 (Image Plate 9-4C).

K. kingae is β-hemolytic on sheep blood agar; the hemolytic reaction is "soft," resembling that of group B streptococci, and may only be noted in areas of confluent growth or after the removal of the colony from the agar surface. On prolonged incubation, some strains of *K. kingae* and *K. denitrificans* may "pit" the agar like *E. corrodens*. *K. kingae* produces acid only from glucose and maltose. *K. denitrificans* produces acid only from glucose in supplemented media, rapid carbohydrate degradation tests, and various kit systems. *K. denitrificans* is also prolyl-aminopeptidase-positive, and is able to grow on Thayer-Martin agar. Because of these characteristics, *K. denitrificans* may be misidentified as *N. gonorrhoeae,* particularly in specimens from the oropharyngeal and genitourinary tracts. This organism, unlike the other species in the genus, reduces nitrate to nitrite, and most strains will also reduce nitrite to nitrogen gas on prolonged incubation. *K. oralis* produces acid from glucose only, but may be differentiated from *K. denitrificans* by its failure to reduce nitrate and nitrite and the absence of prolyl-aminopeptidase activity. Unlike other *Kingella* species, *K. potus* is asaccharolytic and DNase-positive. In addition, the colonies of *K. potus* are yellow-pigmented on blood agar. *E. corrodens* may be differentiated from both

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Cardiobacterium and *Kingella* species by Gram's stain morphology, failure to ferment carbohydrates, and positive ornithine and lysine decarboxylase reactions.

Although identification of these organisms is straightforward once they are isolated, the initial recovery of *K. kingae* from clinical specimens, particularly joint fluid specimens, may present some difficulties. Direct inoculation of plates with fluid from joint aspirates is much less sensitive for recovering *K. kingae* than inoculation of blood culture media. Among 100 specimens cultured by both methods, 34 grew significant organisms; 10 of the 11 *K. kingae* isolates that were obtained grew only in the BACTEC blood culture medium and were not recovered on direct plating.[1381] The

discrepancy in the isolation rate of *K. kingae* was not observed for other organisms causing bone and joint infections. *K. kingae* has also been detected in blood and synovial fluids using the BacT/Alert system (bioMérieux, Inc.), BACTEC 660NR, BACTEC 9240, and the pediatric Isolator 1.5 microbial tube (Wampole Laboratories, Cranbury, NJ).[127,778,1391] Other recent studies have confirmed that the routine use of blood culture bottles significantly enhances recovery of *K. kingae* from joint fluid specimens.[912,1380] Improved recovery of *K. kingae* from joint fluid specimens inoculated into blood culture media may be due to the dilution of inhibitory factors in synovial fluid by the culture medium (e.g., broth-based blood culture systems) or by removal of the organisms from the synovial fluid milieu (e.g., Isolator cultures). In any case, inoculation of osteoarticular fluids into blood culture bottles is now the standard for processing of these specimens in the clinical laboratory. Molecular methods (e.g., real-time PCR) have also been developed and used for the direct detection of *K. kingae* in joint and pericardial fluid.[224,245,264,622,855,1298]

Antimicrobial Susceptibility

K. kingae strains are susceptible to penicillin, ampicillin, cephalosporins of all generations, aminoglycosides, SXT, and fluoroquinolones. Some strains may be relatively resistant to erythromycin, and most are resistant to clindamycin and vancomycin.[127,738]Isolates of *K. denitrificans* have antimicrobial susceptibilities similar to *K. kingae*; growth of *K. denitrificans* on Thayer-Martin agar indicates that they are resistant to vancomycin and colistin. *K. kingae* isolates that produce β-lactamase have been described that were resistant to ampicillin, cefazolin, and ticarcillin, but susceptible to combination drugs containing β-lactamase inhibitors (clavulanate and sulbactam).[1212] Among five patients with *K. kingae* infection reported from Iceland in 1997, three of the five isolates produced β-lactamase enzymes and were resistant to penicillin and ampicillin.[127] Susceptibility of *Kingella* species can be determined using the broth dilution method described in the CLSI document on susceptibility testing of fastidious bacteria.[286]

On antimicrobial susceptibility testing, the single *K. potus* isolate from the kinkajou bite wound was susceptible to penicillin, ampicillin, ceftriaxone, cefotaxime, ciprofloxacin, imipenem, and meropenem, but was resistant to erythromycin, clindamycin, tetracycline, gentamicin, and trimethoprim. The patient responded to treatment with ciprofloxacin and metronidazole.

Capnocytophaga Species

Taxonomy

Capnocytophaga species were originally described by Prevot in 1956 at the Pasteur Institute and were studied further at the CDC during the early 1960s. These facultative, capnophilic, fusiform gram-negative bacilli had been isolated from the human oropharynx and clinical specimens and were given the name CDC group DF-1. DF was an abbreviation for "dysgonic fermenter," referring to the poor fermentative capabilities of these organisms in medium not supplemented with serum. Researchers from the University of Massachusetts and Boston's Forsyth Dental Center published a series of articles on these organisms and addressed their possible role in human periodontal disease. These organisms were named *Capnocytophaga* species, reflecting their "consumption" or requirement for CO_2 for growth.[768] The type species was named *Capnocytophaga ochracea,* and related isolates from the human oropharynx were named *Capnocytophaga sputigena* and *Capnocytophaga gingivalis*. In 1994, two new human *Capnocytophaga* species—*Capnocytophaga granulosa* and *Capnocytophaga haemolytica*—were identified in human dental plaque specimens, and, in 2008, two additional species—*Capnocytophaga leadbetteri* and *Capnocytophaga* genospecies AHN8471—were described among 62 oral isolates from children.[448,1394]

CDC group DF-2 was originally isolated in 1976 from blood and spinal fluid cultures of a patient who had become symptomatic after suffering a dog bite.[142] In 1989, DF-2 and some "DF-2-like" organisms were placed in the genus *Capnocytophaga* based on phenotypic characteristics and DNA relatedness studies. CDC group DF-2 was subsequently named *Capnocytophaga canimorsus* (Latin for "dog bite"), and the "DF-2-like" strains were called *Capnocytophaga cynodegmi* (Greek for "dog bite").[170]Although these organisms are phenotypically different from *Capnocytophaga* species isolated from humans, they are similar with respect to Gram's stain morphology, cellular fatty acid composition, gliding-type motility, and cultural conditions for growth. Chemotaxonomy has been used to differentiate *Capnocytophaga* species from CDC group DF-3 (i.e., *Dysgonomonas*) and aerotolerant strains of *Leptotrichia*.[118] Based on 16S rRNA analysis, human and animal *Capnocytophaga* species are classified in the phylum *Bacteroidetes*, class *Flavobacteria*, and family *Flavobacteriaceae* along with the genera *Flavobacterium, Chryseobacterium*, *Elizabethkingia, Myroides*, and *Weeksella* species.

Clinical Significance

Capnocytophaga species are a part of the normal oropharyngeal microbiota and can behave as opportunistic pathogens under the appropriate circumstances. In the oropharynx, *C. ochracea*, *C. sputigena*, *C. gingivalis*, *C. granulosa*, *C. haemolytica,* and *C. leadbetteri* can be isolated from gingival crevices, periodontal pockets, and

both supragingival and subgingival plaque specimens, but their role in the development of periodontal disease is controversial.[278,279,1394] Survey studies suggest that *Capnocytophaga* species are more prevalent in patients without periodontitis or dental caries than

in those with severe caries or with treatable or refractory periodontal disease.[293,676,1085] As with the HACEK bacteria, head and neck infections with *Capnocytophaga* species may occur by extension from an oral focus to involve the sinuses and the CNS. Wang and colleagues reported a brain abscess due to *C. ochracea* in a 7-year-old boy with recent histories of multiple tooth extractions.[1322] Sepsis due to oral *Capnocytophaga* species is a recognized cause of infection in immunocompromised patients, particularly those with hematologic (e.g., acute/chronic myelogenous leukemia, aplastic anemia, Hodgkin's disease, myeloblastic leukemia, acute lymphocytic leukemia, adenocarcinoma, multiple myeloma, Hodgkin's disease) and solid organ malignancies (e.g., endometrial carcinoma).[147,834,854,891] Bacteremic episodes in these patients coincide with periods of profound neutropenia (especially acute myelogenous leukemia or acute lymphocytic leukemia) and with administration of cytotoxic chemotherapy.[473,479] Mucositis and oral ulcerations are characteristically found in profoundly immunosuppressed patients, thereby establishing an efficient entry route for the organism into the bloodstream.[499] Occasionally, *Capnocytophaga* species may also be isolated from blood of immunocompetent hosts. In 2007, Desai reported severe sepsis and purpura fulminans in a previously healthy 49-year-old man.[348] Rare cases of *Capnocytophaga* pneumonia have been reported in compromised patients as a result of aspiration.[479] Rarely, *Capnocytophaga* species may be recovered from the lower respiratory tract, lung abscesses, wound infections, joint fluid, bone, conjunctival, corneal, and vitreal specimens. *Capnocytophaga* species have been isolated from female genital tract infections as causes of intrauterine, intra-amniotic, and perinatal infections (e.g., endometritis, amnionitis, chorioamnionitis); abortion; preterm birth; congenital bacteremia; and neonatal sepsis.[13,367,802] Endocarditis, cervical lymphadenitis, empyema, lung abscess, sinusitis, vertebral osteomyelitis, iliopsoas abscess, pyonephrosis, liver abscess, and osteomyelitis involving *Capnocytophaga* species have been reported in both immunocompromised and nonimmunocompromised patients.[230,379,539,705,1330] In 1995, the first instance of *Capnocytophaga* species (*C. sputigena*) as a cause of continuous ambulatory peritoneal dialysis-related peritonitis was documented in a 73-year-old man with end-stage renal disease.[405] In 2000, *C. granulosa* was isolated from

an abscess in an immunocompetent patient.[383]

C. canimorsus is the principal human pathogen associated with dog bites and close contact with dogs.[464,1285] Cultural and molecular studies of dogs have shown that 21% to 86% of canines have *C. canimorsus* and/or *C. cynodegmi* in their mouths, but these figures are probably underestimates.[358,830,1232,1286] Although most serious infections have occurred as a consequence of dog bites, several case reports describe infections resulting from dogs licking abrasions, skin wounds, or minor burns.[807,1112] The most serious clinical manifestations occur in individuals with underlying diseases or conditions that predispose them to severe infection with the organism. These conditions include hepatic disease secondary to alcoholism (e.g., cirrhosis), previous splenectomy related to other medical circumstances, Hodgkin's lymphoma, hairy cell leukemia, pulmonary fibrosis, malabsorption syndrome, renal disease, diabetes mellitus, COPD, peptic ulcer disease, Waldenstrom's macroglobulinemia, other malignancies, and the use of systemic or topical corticosteroids. The frequently noted association of systemic *C. canimorsus* infection with asplenia suggests that the reticuloendothelial system (RES) plays an important role in containing the spread of the organism.[376,1127,1226] Although this infection is usually seen in individuals with underlying diseases as outlined above, as many as 40% of patients have no predisposing conditions that place them at higher risk of infection. The mortality associated with severe *C. canimorsus* infection may be as high as 20% to 30%.

Major clinical features of *C. canimorsus* infection include wound infection with cellulitis, meningitis, bacteremia with septic shock, renal failure, hemorrhagic skin lesions suggestive of meningococcal disease, pneumonia with empyema, and both native and prosthetic valve endocarditis.[226,334,455,567,640,780,981,1112,1137] Fulminant *C. canimorsus* sepsis may clinically resemble overwhelming meningococcal disease, with the development of disseminated intravascular coagulation (DIC), purpura fulminans, rapidly evolving hemorrhagic skin lesions, symmetrical peripheral gangrene, and the Waterhouse–Friderichsen syndrome.[190,350,640,897,1313] The clinical presentation of *C. canimorsus* infection can mimic other serious disease syndromes, including tularemia, plague, and hantavirus infection. The disease may progress extremely quickly; in one report, the patient developed DIC that resulted in death over a 4-hour period. Unusual presentations and complications of *C. canimorsus* sepsis have included adult respiratory distress syndrome, myocardial infarction, abdominal symptoms, hemolytic-uremic syndrome, musculocutaneous mononeuropathy, thrombotic thrombocytopenic purpura without DIC, mycotic abdominal aneurysm, vertebral osteomyelitis/discitis, and dialysis-related peritonitis.[424,717,898,942,1255] There is a report of meningitis caused by *C. canimorsus* that was retrospectively diagnosed in a patient who had undergone a CT-guided myelogram. Investigation revealed that the patient, the radiologist, and the radiology technician had prolonged contact with dogs at home, and a procedural

breach in infection control may have resulted in iatrogenic infection of the patient.[1090] *C. canimorsus* was isolated from a prosthetic knee joint infection in a man with Waldemstrom's macroglobulinemia and as the cause of acute tenosynovitis in a patient with systemic lupus erythematosus (SLE), which may be yet another risk factor associated with this organism.[751,779] In 2008, the isolation of *C. canimorsus* from a brain abscess in a patient who had sustained a recent dog bite was reported.[1277] Eye infections, including angular blepharitis, chronic corneal ulcers, corneal ulcer with perforation, and endophthalmitis caused by *C. canimorsus,* have also been reported.[915,999,1030]In one instance, the patient had sustained a scratch on the cornea from his dog that was treated with, among other things, topical prednisone. Interestingly, infections caused by *C. canimorsus*, including keratitis and sepsis with bacteremia, have occurred in individuals who sustained bites and scratches from domestic cats.[255,875] In a 2010 prevalence study, *C. canimorsus* and *C. cynodegmi* were found in 57% and 84% of the oral cavities of cats, respectively.[1232] In 2006, the first documented *C. canimorsus* infection in a nonhuman species occurred in a 2-year-old healthy male pet rabbit that had been bitten on the head by a dog.[1293]

C. cynodegmi is not isolated as frequently as *C. canimorsus*, and in vitro studies suggest that this organism may not be

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as virulent as *C. canimorsus*. However, several case reports in recent years indicate that this organism may be as pathogenic as its relative *C. canimorsus*. In 2005, Khawari and colleagues described the first instance of fatal sepsis and meningitis due to *C. cynodegmi* in a splenectomized woman who had been bitten on the hand by her pet dog 24 hours before presentation to the emergency department.[698] This organism was recovered from cellulitis of the ankle in a patient with rheumatoid arthritis who had suffered a cat bite; this patient was on long-term corticosteroid and anti-TNF-α therapy.[484] *C. cynodegmi* was isolated as a cause of cellulitis, pneumonitis, and bacteremia in a man with insulin-dependent diabetes mellitus, who had sustained a bite from a stray dog.[1144] *C. cynodegmi* was more recently described as the cause of peritonitis in a 67-year-old man with end-stage renal disease who was on continuous ambulatory peritoneal dialysis.[1023] His only animal exposure was to his neighbor's cat. *C. cynodegmi* may also be a pathogen in animals. *C. cynodegmi* was isolated from lung tissues of a Rottweiler dog with bronchitis and pneumonia due to a retained foreign body and from a bronchoalveolar lavage (BAL) specimen of a 10-year-old cat with a pulmonary carcinoma.[429,1369]

Cultural Characteristics and Identification

Human oral *Capnocytophaga* species are slow-growing, with colonies becoming visible after 48 hours' incubation and developing a characteristic morphology after this time. All species require a CO_2-enriched environment for growth. Colonies of the organism are yellow, tan, or slightly pinkish and have marginal finger-like projections (gliding motility) appearing as a film surrounding the central area of the colony (Image Plate 9-4D). The central part of the colonies also has a moist, mottled appearance. *Capnocytophaga* species grow on blood and chocolate agar, but not on MacConkey agar (Image Plate 9-4F). Good growth may also be observed on modified Thayer-Martin agar because of the resistance of the organism to vancomycin, colistin, and trimethoprim. The organisms are gram-negative, are fusiform in shape, and appear straight or slightly curved (Image Plate 9-4E and 9-4G). Pleomorphism, with swollen or large coccal cells, is seen in older cultures. All species are catalase-negative, are oxidase-negative, and produce acid from glucose, maltose, sucrose, and mannose, but not from ribose, xylose, mannitol, or sorbitol. Indole and urease are not produced. The organism is negative for both lysine and ornithine decarboxylase, as well as arginine dihydrolase. *Capnocytophaga* species produce characteristic, uniform profiles on cellular fatty acid analysis that allow genus-level, but not species-level, identification. Species are identified by an expanded battery of tests (e.g., nitrate and nitrite reduction, starch and dextran hydrolysis, etc.). *C. haemolytica* is β-hemolytic on sheep blood and is the only human hemolytic species. *C. granulosa* strains form intracellular granular inclusions that stain with carbol fuchsin when the organisms are grown anaerobically in peptone-yeast glucose broth under anaerobic conditions. These last two species can also be differentiated from the previously described human *Capnocytophaga* species by determination of various aminopeptidase activities. Phenotypic characteristics for the identification of *Capnocytophaga* species are presented in Table 9-5.

C. canimorsus is usually recovered from blood cultures, wound cultures, aspirates from cellulitis, and CSF. *C. cynodegmi* has been recovered from dogs' mouths and from several types of human clinical specimens, including localized dog-bite wounds, cellulitis, blood, BAL specimens, and peritoneal fluid. In cases of high-grade bacteremia, the bacteria may actually be observed on Wright–Giemsa-stained smears of peripheral blood.[1313] The organism has been recovered in several types of blood culture media, and growth is generally slow. In most reports, blood cultures become positive 3 to 7 days after collection. The organism grows slowly on blood and chocolate agar incubated at 35°C in a CO_2 incubator with increased humidity. Poor growth on routine sheep blood agar has been attributed to the use of a trypticase-soy base; better growth is seen when a heart infusion base is employed. The organisms also grow better in a medium supplemented with cysteine, and addition of rabbit serum to the medium also enhances growth. Commercial supplemented chocolate

agars containing IsoVitalex (which contains cysteine) or other similar enrichments are satisfactory. Pinpoint colonies appear after 2 to 4 days of incubation. After further incubation, colonies appear larger, circular, smooth, and convex. The colonies are nonhemolytic and have a shiny, spreading edge consistent with the gliding motility of the organism. Colonies may have a yellowish or pinkish hue. On Gram's stain, the bacteria appear as medium-to-long, slightly curved bacilli with tapered or spindle-shaped cells. Like the other fastidious bacteria discussed thus far, no growth is observed on MacConkey agar. Both *C. canimorsus* and *C. cynodegmi* are catalase-positive and oxidase-positive; these two reactions differentiate them from the human oral *Capnocytophaga* species, which are oxidase- and catalase-negative. Both canine species are arginine dihydrolase-positive, ONPG-positive, and negative for lysine and ornithine decarboxylase, urease, indole, and nitrate reduction. *C. canimorsus* ferments glucose, maltose, and lactose, but not raffinose and inulin. *C. canimorsus* and *C. cynodegmi* are differentiated by carbohydrate utilization tests, with the latter organism producing acid from a wider variety of sugars. Table 9-5 shows the biochemical reactions of *C. canimorsus* and *C. cynodegmi* along with the general biochemical characteristics of the other *Capnocytophaga* species discussed previously. Broad-range PCR has been used to diagnose *C. canimorsus* osteoarticular and meningeal infections.[284,506,886,1326]

Antimicrobial Susceptibility

Human oral *Capnocytophaga* species are generally susceptible to ampicillin sulbactam, tetracycline, linezolid, imipenem, and other β-lactam/β-lactamase inhibitor combinations, but are resistant to polymyxin, colistin, and trimethoprim. Susceptibility to the penicillins, cephalosporins, aztreonam, erythromycin, fluoroquinolones, aminoglycosides, and metronidazole varies from strain to strain.[479,656,657] *Capnocytophaga* strains that are resistant to β-lactams produce novel β-lactamase enzymes that confer resistance to extended-spectrum cephalosporins and penicillins.[553,854] These β-lactamase-producing strains are highly resistant to penicillin, amoxicillin, and cefazolin; addition of clavulanate results in a 64-fold decrease in the amoxicillin MIC for over 90% of the β-lactamase-producing strains. These strains are also less

TABLE 9-5 Phenotypic Characteristics for Identification of *Capnocytophaga* Species

Characteristic	*C. ochraceus*	*C. gingivalis*	*C. sputigena*	*C. haemolytica*	*C. granulosa*	*C. leadbetteri*	*Capnocytophaga* genospecies AHN8471	*C. canimorsus*	*C. cynodegmi*
HEM SBA	–	–	–	β (lost on subculture)	–	–	–	–	–
OX	–	–	–	–	–	–	–	+	+
CAT	–	–	–	–	–	–	–	+	+
Growth, MacConkey agar	–	–	–	–	–	–	–	–	–
NO_3 RED	–	V	–	+	–	–	–	–	V
IND	–	–	–	–	–	+	–	–	–
URE	–	–	–	–	–	–	–	–	–
ADH	–	–	–	–	–	NA	NA	+	+
ESC	V	–	V	+	–	–	V	V	+
STA	+	V^+	V	+	+	V	+	NA	NA
Acid produced from:									
GLU	+	+	V	+	V^+	V^w	+	+	+
MAL	+	+	+	+	NA	NA	NA	+	+
SUC	+	+	V	+	+	–	+	–	+
LAC	+	V	V	+	+	V	V	+	+
RAFF	+	V^+	V	+	–	–	V^+	–	+
XYL	–	–	–	–	–	–	–	–	–
CELL	V^+	V	V	–	–	–	V	V	V
GAL	+	V	V	+	V	–	+	+	V
GLYG	+	+	–	+	–	–	+	+	V

+, positive reaction; –, negative reaction, V, variable reaction; V^+, variable reaction, but most strains are positive; V^w, variable reaction, but positive reactions are weak; NA, data not available; HEM SBA, hemolysis on sheep blood agar; OX, oxidase; CAT, catalase; NO_3 RED, reduction of nitrate to nitrite; IND, indole; URE, urease; ADH, arginine dihydrolase; ESC, esculin; STA, starch hydrolysis; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; RAFF, raffinose; XYL, xylose; CELL, cellobiose; GAL, galactose; GLYG, glycogen.

susceptible to cefuroxime, cefotaxime, and ceftazidime than β-lactamase-negative strains. Imipenem appears to be active against both β-lactamase-positive andβ-lactamase-negative *Capnocytophaga* isolates.[656] *Capnocytophaga* species are usually resistant to aminoglycosides (i.e., gentamicin, tobramycin, amikacin, netilmicin), trimethoprim, colistin, and vancomycin, although some strains may be susceptible. Gomez-Garces et al.[499] reported a patient with fatal bacteremia caused by a ciprofloxacin-resistant β-lactamase-producing strain of *C. sputigena* that was resistant to all β-lactams (except cefoxitin) and aminoglycosides, but was susceptible to tetracycline, erythromycin, clindamycin, aztreonam, and imipenem. Generally, immunocompromised and granulocytopenic patients are empirically treated with a combination of a β-lactam antibiotic and an aminoglycoside, and cidal levels of antimicrobials are usually required for an optimal therapeutic response. Therefore, rapid recognition of the salient features of this organism by the microbiologist is important. The intrinsic resistance of *Capnocytophaga* species to aminoglycosides and

the production of β-lactamase enzymes by some clinical isolates, which can be detected by the nitrocefin test, should be reported to the physician as soon as possible.

Slow growth of *C. canimorsus* on agar medium and failure of some strains to grow in certain types of broth media have precluded studies of the antimicrobial susceptibility of this organism. Unstandardized studies using disk diffusion procedures designed for more rapidly growing bacteria have found that *C. canimorsus* strains are susceptible to most antimicrobials, including penicillins, third-generation cephalosporins, fluoroquinolones, tetracycline, erythromycin, and imipenem. The organism demonstrates resistance to the aminoglycosides, aztreonam, and SXT, but results for these agents may be method dependent.[1301] Antibiotics that are generally more active against gram-positive organisms, such as vancomycin, clindamycin, erythromycin, and rifampin, were also active against *C. canimorsus*. These data support the clinical efficacy observed with penicillin (mean penicillin MIC = 0.04 μg/mL +/− 0.01 μg/mL for all strains tested).

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Dysgonomonas Species

Taxonomy

The genus *Dysgonomonas* was created in 2000 to accommodate the former CDC group DF-3 as *Dysgonomonas capnocytophagoides* and a second species, *D. gadei*, which had been isolated from an infected human gall bladder.[596] Members of the genus are gram-negative, facultatively anaerobic coccobacilli that form a phylogenetic cluster within the family *Porphyromonadaceae* of the order *Bacteroidales*. Biochemically, these organisms resemble certain *Capnocytophaga* species, but 16S rRNA sequencing and chemotaxonomic analyses indicate genetic relatedness to *Bacteroides, Prevotella, and Porphyromonas* species, particularly *Bacteroides distasonis* and *Bacteroides forsythus.*[1289]

Clinical Significance

The first two reports of *D. capnocytophagoides* in association with human disease were published in 1988. In one instance, *D. capnocytophagoides* was isolated in pure culture from stool specimens of an elderly woman with common variable hypogammaglobulinemia, and, in the second instance, from blood cultures of a profoundly granulocytopenic man with acute lymphocytic leukemia.[68,1312] In 1991, Gill and coworkers at the National Cancer Institute screened stool specimens from 690

patients and found 11 with moderate to heavy growth of *D. capnocytophagoides*.[488] Of these 11 patients, 4 had a history of prolonged diarrhea and were treated; diarrhea had been documented in the other seven patients, but the organisms cleared spontaneously. Blum and colleagues also recovered *D. capnocytophagoides* from the stool of eight patients during a year-long period.[140] All patients were immunocompromised or had severe underlying disease. Among these patients, the clinical spectrum of *D. capnocytophagoides* ranged from chronic diarrhea with a clinical therapeutic response to an asymptomatic carrier state. Other investigators have also noted the association of enteric *D. capnocytophagoides* infection with both HIV infection and common variable hypogammaglobulinemia.[570,588] This organism has also been isolated from soft tissue, blood cultures, abscesses, decubitus ulcers, and the urinary tract of compromised patients.[88,555,880,1157] In 2002, a third *Dysgonomonas* species,*Dysgonomonas mossii*, was isolated from abdominal drainage fluid, and two other isolates were obtained from other human clinical specimens.[765] In 2006, *D. mossii* was also recovered from multiple specimens obtained from a surgically placed intestinal drainage tube of a patient recovering from resection of pancreatic cancer.[853] A fourth species, *Dysgonomonas hofstadii*, was isolated from a wound, following abdominal surgery, taken from a 72-year-old man in Helsinki, Finland.[763]

Cultural Characteristics and Identification

Dysgonomonas species are nonmotile, facultatively anaerobic gram-negative coccobacilli. All species grow on blood and chocolate agars, but do not grow on MacConkey agar (Image Plate 9-4H). *Dysgonomonas* species are oxidase-negative and catalase-variable. All species ferment glucose with no gas formation, and none of the species reduce nitrate or produce urease, gelatinase, or H_2S. All species are arginine dihydrolase-negative, and do not produce lysine or ornithine decarboxylases. Phenotypic characteristics of *Dysgonomonas* species are shown in Table 9-6.

D. capnocytophagoides grows slowly, with pinpoint colonies appearing after 24 hours' incubation. *D. capnocytophagoides* has been recovered from stool specimens using cefoperazone–vancomycin–amphotericin (CVA) blood agar incubated at 35°C in 5% to 7% CO_2. In these cases, nonselective blood agar should also be inoculated, as some strains fail to grow on this selective *Campylobacter* medium. The organism has also been recovered on blood agar containing kanamycin and vancomycin following anaerobic incubation. After 48 to 72 hours, the colonies are gray-white, smooth, and nonhemolytic, and some strains produce a sweet odor during growth. Oxidase and catalase reactions are negative, but indole is produced in tryptone broth and esculin is hydrolyzed. This bacterium produces acid fermentatively from glucose, xylose, and maltose; most strains also produce acid from sucrose and lactose, but do not produce acid from mannitol. Some "DF-3-like" strains have also been described that are

consistently sucrose-negative and slightly hemolytic.[325] *D. gadei* and *D. mossii* can be differentiated phenotypically from *D. capnocytophagoides* by acid production from mannitol, inositol, sorbitol, raffinose, and trehalose (Table 9-6). Gas-liquid chromatography (GLC) is also useful for identification, since all strains consistently demonstrate 12- and 13-methyltetradecanoate, with minor amounts of tetradecanoate and hexadecanoate in their cell wall.[325,1312]

Antimicrobial Susceptibility

Antimicrobial susceptibility of *D. capnocytophagoides* isolates has been determined by disk diffusion, broth dilution, and Etest methods. *D. capnocytophagoides* isolates are resistant to penicillin, ampicillin, ampicillin sulbactam, aztreonam, aminoglycosides, cephalosporins of all generations, erythromycin, fluoroquinolones, and vancomycin. Most isolates are susceptible to SXT and are variably susceptible to piperacillin, clindamycin, tetracyclines, and imipenem. Patients respond clinically to therapy with SXT, clindamycin, or tetracycline.

Streptobacillus moniliformis

Taxonomy

Streptobacillus moniliformis is the only member of the genus *Streptobacillus,* and its present taxonomic position is uncertain. Based on 16S rRNA sequencing studies, *S. moniliformis* is currently classified in the proposed family "*Fusobacteriaceae*" in the proposed class "*Fusobacteria*" of the phylum *Fusobacteria*.

Epidemiology

Individuals who are at risk for infection with *S. moniliformis* include animal laboratory workers and those individuals who live in urban areas where rats may be prevalent. Additionally,

TABLE 9-6 Phenotypic Characteristics for Identification of *Dysgonomonas* Species

Characteristic	*D. capnocytophagoides*	*D. gadei*	*D. mossii*
HEM SBA	–	–/α	–
OX	–	–	–
CAT	–	+	V
MOT	–	–	–
Growth, MacConkey agar	–	–	–
NO_3 RED	–	–	–
IND	V	V	+
ESC	+	+	+
ADH	–	–	–
ACET	–	–	–
URE	–	–	–
GEL	NA	–	–
PAL	+	+	+
Gas from glucose	–	–	–
Acid produced from:			
GLU	+	+	+
MAL	+	NA	+
FRU	NA	+	+
SUC	+	+	+
LAC	+	+	+
MNTL	–	–	$+^w$
MANN	+	+	+
SBTL	–	–	NA
INOS	–	–	$+^w$
ADON	–	–	NA
ARAB	+	+	NA
MEL	+	+	NA
RAFF	+	+	V
RHAM	NA	+	$+^w$
SAL	NA	+	+
TREH	–	+	+
XYL	+	+	+
β-GUR	–	+	–
NAGA	–	+	+
α-FUC	–	+	+

+, positive reaction; –, negative reaction; $+^w$, weak positive reaction; NA, data not available; HEM SBA, hemolysis on sheep blood agar; OX, oxidase; CAT, catalase; MOT, motility; NO_3 RED, reduction of nitrate to nitrite; IND, indole; ESC, esculin; ADH, arginine dihydrolase; ACET, acetoin (VP); URE, urease; GEL, gelatin hydrolysis; PAL, alkaline phosphatase; GLU, glucose; MAL, maltose; FRU, fructose; SUC, sucrose; LAC, lactose; MNTL, mannitol; MANN, mannose; SBTL, sorbitol; INOS, inositol; ADON, adonitol; ARAB, arabinose; MEL, melibiose; RAFF, raffinose; RHAM, rhamnose; SAL, salicin; TREH, trehalose; XYL, xylose; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-glucosaminidase; α-FUC, α-fucosidase.

other urban or rural dwelling domesticated animals that prey on rodents may also become infected and are able to transmit the infection to humans via bites, scratches, or contact with animal urine and excrement. *S. moniliformis* may also cause infections in koala bears, turkeys, and monkeys.[1284]

Clinical Significance

Streptobacillus moniliformis is a fastidious gram-negative bacillus that is normally found in the upper respiratory tracts of wild rats, laboratory rats and mice, and domesticated rodents (e.g., guinea pigs, gerbils, ferrets) and may cause disease in these animals.[463] The organism may also be carried by animals that catch or feed on rodents, such as dogs and cats. As the natural reservoir for *S. moniliformis*, bites from these reservoir hosts or exposure to their urine or feces can transmit the organism to humans. Consequently, *S. moniliformis* infection in humans is termed *rat-bite fever*. Infection may also be transmitted via ingestion and is called Haverhill fever when the bacterium is acquired by this route. The latter name comes from Haverhill, Massachusetts, where this organism was recovered from blood cultures of several patients during a local outbreak of the disease involving ingestion of milk contaminated with rat excrement.[745,1175] Since then, outbreaks of Haverhill fever from ingestion of contaminated water and turkey meat have been reported. At present, most cases involve bites from rats or exposure to rodents, and many of these infections occur in children.[87,664] Infection has also been documented following receipt of several scratches from a rooster.[375] After an incubation period of 7 to 10 days following the infection, there is an abrupt onset of high fever, chills, headache, muscle aches, vomiting, and other constitutional symptoms.[391,463] The incidence and severity of pharyngitis and vomiting is greater when infection is acquired by the oral route.[745] A few days after disease onset, a maculopapular rash usually appears on the distal extremities, with petechiae appearing on the plantar soles and the palms. In some cases, the maculopapular rash may be hemorrhagic or pustular.[87] About 50% of patients may develop severe joint pain, migratory, nonsymmetric polyarthritis, or septic arthritis.[345] Cutaneous and subcutaneous abscesses with pitting edema have also been described in association with this infection.[1261] The disease may resolve spontaneously with no residual symptoms, or may develop into a chronic, periodically febrile condition. Complications related to systemic dissemination via the bloodstream include native and prosthetic valve endocarditis, myocarditis, pericarditis, pneumonia with pleural effusion, septicemia, brain abscess, amnionitis, prostatitis, pancreatitis,

cutaneous and genital tract abscesses, and death.[87,242,357,413,718,1033,1108,1121] Arthritic symptoms may persist for years after resolution and/or treatment of the infection. Without a clue as to the origin of infection, rat-bite fever or Haverhill fever may resemble rheumatoid arthritis, staphylococcal or streptococcal sepsis, acute rheumatic fever, disseminated gonococcal infection, meningococcemia, secondary syphilis, typhoid, brucellosis, leptospirosis, acute enteroviral infection, Lyme disease, ehrlichiosis, anaplasmosis, Rocky Mountain spotted fever, and other rickettsial infections.[391,463] Diagnosis may also be problematic because cases have been reported in patients with no history of rodent bites or direct exposure to rodents.[428] Conversely, review of 45 cases occurring in California over the last 30 years revealed that rat-bite fever was suspected in 75% of the cases where a tentative diagnosis was given.[513] Because of the fastidious nature of the organism and the difficulties involved with culture, retrospective diagnoses of *S. moniliformis* infection have been made on occasion by serologic techniques, including detection of serum agglutinins and complement fixing antibodies against the bacillary form of the organism. However, serologic tests for this infection are not readily available, and most serologic approaches have been used to assess presence of the organism in laboratory rodent colonies.

Cultural Characteristics and Identification

S. moniliformis is a fastidious, pleomorphic gram-negative bacillus that tends to form long, thin, filamentous single cells. These thin cells are about 1 μm in diameter, but may be over 100 μm in length and may fold into loops and coils.[463] Occasionally, these organisms may stain gram-variable. On prolonged incubation, bulbous- or sausage-shaped swelling may appear along the filament, causing the organism to resemble a string of beads. On enriched, serum-containing media, the organisms appear as regular, thin, gram-variable fusiform bacteria with rounded or pointed ends. The organism may also lose its cell wall and exist as an "L-form, which may be related to the lower glucosamine and muramic acid content of the cell wall of the organism. In fact, there is genetic, phenotypic, serologic, and structural evidence suggesting that this organism is taxonomically related to the mycoplasmas and ureaplasmas, which also lack a typical bacterial cell wall.[1373] *S. moniliformis* is microaerophilic, and growth in liquid medium usually requires supplementation with serum (10% to 20%), blood, or ascites fluid.

Diagnosis of rat-bite or Haverhill fever is made by recovery of *S. moniliformis* from blood cultures, synovial fluid, CSF, skin lesions, and abscess material.[87,391,463] Because the organism is inhibited by the anticoagulant SPS, blood (10 mL) must be anticoagulated with citrate (10 mL sodium citrate, 2.5%) prior to processing.[745,1174] The organism has also been successfully recovered in SPS-containing blood culture

media with resins (e.g., BACTEC Peds Plus, BD Biosciences) and in BACTEC Plus Aerobic Anaerobic/F bottles (BD Biosciences), which do not contain SPS. The citrated blood cells are sedimented by centrifugation, and the packed cells are inoculated onto agar medium (heart infusion agar) containing 10% to 20% sterile decomplemented horse serum and 0.5% yeast extract. The inoculum is gently spread over the agar surface. A broth medium of similar ingredients (heart infusion broth with 10% to 20% horse or rabbit serum, defibrinated blood or ascites, and yeast extract) is also inoculated with the packed cells. Isolation of *S. moniliformis* has also been accomplished using a biphasic medium of trypticase-soy broth containing a trypticase-soy agar slant. Medium is incubated at 35°C in a candle jar or a CO_2incubator. Other specimens (citrated joint fluid, aspirates, abscess material, etc.) may be cultured in the same manner. Loridant and colleagues reported successful recovery of *S. moniliformis* from blood culture media by inoculation of shell vials containing monolayers of ECV 304 human endothelial cells. The organisms showed cytopathic effects on the cultured

cells after 24 hours, with subsequent successful subculture on agar media.[806]

In broth medium, the organism grows as small "puff balls" near the bottom of the tube or bottle and overlying the red cells and stroma. Accumulation of acid in broth media may kill the organism; so frequent subculture is necessary to maintain viability. On serum-enriched agar medium, growth may appear within 2 to 3 days, or may require a week or more. Colonies are small (1 to 2 mm in diameter), convex, gray-white, smooth, and buttery in consistency. Microscopic L-phase variants form spontaneously under or around the existing colonies; the typical "fried egg" morphology with a dense center similar to the morphology of *Mycoplasma* species can be observed with a dissecting microscope. Identification of *S. moniliformis* is accomplished by observing the typical gram-negative, filamentous morphology on Gram's staining and by performing biochemical identification tests in serum-supplemented medium. Carbohydrate utilization tests may be performed in nutrient broth containing 1% filter-sterilized carbohydrates and 0.5% sterile horse serum. These tests should be incubated for 3 weeks before the final interpretation, although reliable and reproducible results have been obtained when cultures were incubated for only 1 week before test interpretation. Rapid identification of the organism may also be accomplished by fatty acid profile analyses using GLC, by detection of enzymatic activities for various aminopeptidases and glycosidases using the API-ZYM strip, and by protein patterns obtained by polyacrylamide gel electrophoresis (PAGE).[310,384,1117] Fatty acid methyl esters detected by GLC include hexadecanoic acid (C16:0), linoleic acid (C18:2), and octadecanoic acids (C18:1, C18:0).[384,1117] PAGE techniques may

also be useful for strain typing of clinical isolates.[310] The reactions of *S. moniliformis* in various biochemical identification tests are shown in Table 9-7.

Broad-range PCR-based amplification of a part of the 16S rRNA gene followed by amplicon sequencing has been developed for direct detection of *S. moniliformis* in rodent colonies, turkey flocks, and human clinical speciemens.[112,151] This method was used to diagnose rat-bite fever in a symptomatic 11-year-old boy who developed fever, vomiting, rash, and arthritis after being bitten by his pet rat. Cultures from blood, CSF, and urine were negative, but fluid from a cutaneous blister was positive by the amplification/sequencing assay.[112] 16S RNA sequencing has also been used for the detection of *S. moniliformis* in joint fluid specimens and to confirm the identity of isolates recovered from joint fluid cultures.[237,930,1319]

Antimicrobial Susceptibility

In vitro antimicrobial susceptibility data indicate that *S. moniliformis* is susceptible to penicillin, ampicillin, extended-spectrum and penicillinase-resistant penicillins, cephalosporins, erythromycin, clindamycin, tetracycline, rifampin, imipenem, and vancomycin.[384,1373] The organism is intermediate in susceptibility to aminoglycosides and ciprofloxacin, and is generally resistant to colistin and SXT. The drug of choice for treatment of mild-to-moderate disease is intravenous penicillin G, with oral tetracyclines as an alternative for patients with penicillin allergy. Other antimicrobial agents (e.g., ceftriaxone, clindamycin, erythromycin) have also shown some clinical efficacy. Parenteral therapy may be followed by course of oral therapy in severe disease. Combination therapy with intravenous penicillin plus an aminoglycoside may be warranted for treatment of native valve endocarditis.

TABLE 9-7 Phenotypic Characteristics for Identification of *Streptobacillus moniliformis*

Characteristic	Reaction
Oxidase	−
Catalase	−
Nitrate reduction	−
Indole	−
Urease	−
Esculin hydrolysis	+

Production of H_2S (lead acetate)	+
Alkaline phosphatase	+
Gas production from glucose	−
Acid production from:	
Glucose	+
Maltose	+
Fructose	+
Sucrose	V
Lactose	V
Xylose	−
Mannitol	−
Mannose	+

+, positive reaction; −, negative reaction; V, variable reaction.

Simonsiella Species

Simonsiella species are fastidious, aerobic gram-negative bacilli that have the unusual morphologic characteristic of existing in multicellular arrangements measuring 20 to 50 µm by 4 to 8 µm. These arrangements consist of 6 to 8 cells, with the long axis of the individual bacilli at right angles to the axis of the cell group. The cell group exhibits gliding motility and forms pale yellow colonies on blood agar after 18 to 24 hours at 37°C. The genus *Simonsiella* contains the single species *S. muelleri,* which is found in the human oral cavity. Two former *Simonsiella* species—*S. crassa* and *S. steedae*—have been reclassified in the genus *Alysiella* as *A. crassa* and in the new genus *Conchiformibium* as *C. steedae*, respectively.[1376] These organisms are found in the oral cavities of sheep (*A. crassa*) and dogs/cats (*C. steedae*). *S. muelleri* was isolated from a gastric aspirate from a neonate, but has not been implicated as a human pathogen.[1348] The genera *Alysiella*, *Simonsiella*, and *Conchiformibium* are classified in the family *Neisseriaceae.*[1113]

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Pasteurella and *Mannheimia* Species

Taxonomy and Characteristics of the Genus Pasteurella

The taxonomic structure of genus *Pasteurella* is complicated, and taxonomists are now attempting to unravel the relationships among members of the described genera and the proper taxonomic assignment of several "species" to *Pasteurella* and other genera.[273] Prior to the mid-1980s, the genus *Pasteurella* included *P. multocida, P. aerogenes, P. ureae,* and *P. pneumotropica* based on phenotypic characteristics. Subsequent additions to the genus occurred during the mid-1980s, when the delineation of genera in genus *Pasteurella* was based on DNA hybridization studies, with strains showing greater than 50% homology being included in the same species with little regard for phylogenetic origin and phenotypic characteristics.[926–928,1148,1202] *Pasteurella*"species" described by these investigators included *P. dagmatis, P. canis, P. stomatis, P. gallinarum, P. anatis, P. langaaensis, P. bettii, P. lymphangitidis, P. mairii, P. trehalosi, P. testudinis, P. avium,* and *P. volantium*. At the same time, other investigators used DNA hybridization and phenotypic methods to demonstrate that *P. ureae* was not closely related to any of these new "*Pasteurella*" species.[404] Subsequently, examination of 16S rRNA sequences showed that the older species of *Pasteurella*—[*P.*] *pneumotropica*, [*P.*] *aerogenes*, [*P.*] *ureae*, and species within the [*P.*] *haemolytica* group—were not closely related to the type species of *Pasteurella* (*P. multocida*) or to each other and probably belonged in genus *Actinobacillus* or in new genera.[354] With the exclusion of these existing "species" from the "genus" *Pasteurella*, specific epithets for these organisms are written with the genus name in brackets as indicated. *Pasteurella* species sensu stricto, [*Pasteurella*] species incertae sedis, and members of the genus *Mannheimia* are presented in Box 9-4.

BOX 9-4

Pasteurella Species Sensu Stricto, [*Pasteurella*] Species Incertae Sedis, and *Mannheimia* Species

Species/Subspecies	Habitat and Clinical Significance in Humans
Pasteurella Species	

P. multocida subsp.*Multocida*	Respiratory tract of nonhuman mammals; clinical isolate from infections in humans
P. multocida subsp.*septica*	Same as above
P. multocida subsp.*gallicida*	Same as above; also associated with fowl cholera
P. dagmatis	Respiratory tracts of dogs and cats; animal bite wounds and systemic infections in humans
P. canis	Respiratory tracts of dogs and calves; dog-bite wounds in humans
P. stomatis	Respiratory tract of dogs; dog-bite wound infections
[*Pasteurella*] Species Incertae Sedis	
[*P.*] *aerogenes*	Normal flora in the intestinal tract of swine; human infection following a pig bite
[*P.*] *pneumotropica*	Respiratory tracts of guinea pigs, rats, hamsters, cats, and dogs; rarely isolated from humans
[*P.*] *bettyae*	Human Bartholin's gland and finger abscesses; may be associated with genital ulcer disease; postpartum bacteremia; pneumonia and pleural effusion in a patient with AIDS
[*P.*] *caballi*	Equine isolate causing pneumonia, wound infection; human wound infections reported
[*P.*] *mairii*	Abortion in sows; sepsis in piglets
[*P.*] *langaaensis*	Respiratory tract flora of chickens and other fowl
[*P.*] *lymphangitidis*	Bovine lymphangitis
[*P.*] *testudinis*	Pathogen in some species of desert tortoises
Former [*Pasteurella*] Species Incertae Sedis	
Avibacterium gallinarum	Formerly [*Pasteurella*] *gallinarum*; respiratory tracts of chickens and hens; two reported cases of bacteremia in humans
Gallibacterium anatis	Formerly [*Pasteurella*] *anatis*; intestinal tract flora of ducks
	Formerly [*Pasteurella*] *trehalosi*; septicemia in

	adolescent lambs
Mannheimia Species	
M. haemolytica	Pneumonic infections in cattle; mastitis in ewes; septicemia in goats and sheep; upper respiratory tract flora of ruminants; rare human infections reported

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M. granulomatis	Progressive granulomatous disease (panniculitis) in cattle; bronchopneumonia and conjunctivitis in leprine species and deer
M. glucosida	Upper respiratory tract flora in cattle and sheep
M. ruminalis	Normal flora in the rumen of sheep and cattle
M. varigena	Pneumonia, mastitis, and septicemia in cattle; also found as part of the upper respiratory, rumen, and intestinal flora of cattle; respiratory tracts flora of swine; sepsis, enteritis, and pneumonia in swine
M. succiniciproducens	Isolated from the bovine rumen

Restructuring of the *Pasteurellaceae* began when [*P.*] *ureae* was formally reassigned to the genus *Actinobacillus* as *Actinobacillus ureae.*[929] In 1999, members of the "*Pasteurella haemolytica* group" (including *P. haemolytica* sensu stricto, *Pasteurella anatis,* and *P. granulomatis*) were placed in the new genus *Mannheimia* or reclassified as the previously described species *Pasteurella trehalosi*.[46] Based on 16S rRNA sequencing, amplified 16S ribosomal DNA analysis (ARDRA), quinone typing, and sequencing of the *sodA* genes, which encode the manganese-dependent superoxide dismutase enzyme and provide higher discriminatory power for bacterial systematics, the genus *Pasteurella* sensu stricto now consists of *P. multocida* subspecies (*multocida*, *gallicida*, and *septica)*, *P. canis*, *P. dagmatis*, and *P. stomatis*.[672] The other "*Pasteurella*" species were designated as species incertae sedis. New genera will need to be created to accommodate these other "*Pasteurella* species," including [*P.*] *aerogenes*, [*P.*] *pneumotropica*, [*P.*] *bettyae*, [*P.*] *caballi*, [*P.*]

species," including [*P.*] *aerogenes*, [*P.*] *pneumotropica*, [*P.*] *bettyae*, [*P.*] *caballi*, [*P.*] *anatis*, [*P.*] *mairii*, [*P.*] *testudinis*, [*P.*] *skyensis*, [*P.*] *langaaensis*, [*P.*] *lymphangitidis*, [*P.*] *trehalosi*, [*P.*] *avium*, [*P.*] *volantium,* and [*P.*] *gallinarum*.

Reclassification of Other [Pasteurella] *Species*

[*P.*] *anatis*, [*P.*] *avium*, [*P.*] *volantium*, [*P.*] *gallinarum*, [*P.*] *trehalosi*, [*P.*] *testudinis*, [*P.*] *langaaensis*, [*P.*] *lymphangitidis*, [*P.*] *mairii*, [*P.*] *skyensis*, and [*P.*] *lymphangitidis* are animal species incertae sedis that have not been isolated from humans. In 2003, [*P.*] *anatis* and *avian* isolates classified in the "[*P.*] *haemolytica*" group were reclassified in the new genus *Gallibacterium* as *G. anatis*.[269] Unnamed taxa that had been isolated from other avian species were also analyzed and added to genus *Gallibacterium* in 2009 as *G. melopsittaci*, *G. trehalosifermentans*, and *G. salpingitidis*.[134] In 2005, [*P.*] *avium*, [*P.*] *volantium*, and [*P.*] *gallinarum* were transferred to the new genus *Avibacterium* as *Avibacterium avium, Avibacterium volantium,* and *Avibacterium gallinarum*, respectively, along with "*Haemophilus paragallinarum*" (*Avibacterium paragallinarum*).[138] In 2007, a fifth *Avibacterium* species, *A. endocarditidis*, recovered from chickens with valvular endocarditis, was added to this new genus.[132] Also in 2007, the sheep pathogen [*P.*] *trehalosi* was reclassified in the new genus *Bibersteinia* as *B. trehalosi*.[137] In 2009, genotypic analysis of [*P.*] *testudinis* and other isolates from diseased tortoises resulted in the description of the new genus *Chelonobacter*, which was demonstrated to be a member of the *Pasteurellaceae*. This new genus contains a single species, *C. oris*, that is distinct genotypically and phenotypically from [*P.*] *testudinis*, which remains unclassified at this time.[515] In 2004, other unnamed taxa that had been recovered from parrots and parakeets were placed in the new genus *Volucribacter* as *V. psittacicida* and *V. amazonae*.[267] The genera *Lonepinella, Phocoenobacter, Nicoletella,* and *Basfia* include single species that are found in koala bears (*L. koalarum*), harbor porpoises (*P. uteri*), horses (*N. semolina*), and the bovine rumen (*B. succiniciproducens*) and were described in 1995, 2000, 2004, and 2010, respectively.[437,739,740,983] [*P.*] *langaaensis*, [*P.*] *lymphangitidis*, [*P.*] *mairii*, [*P.*] *skyensis*, and [*P.*] *lymphangitidis* remain as species incertae sedis and await reclassification.

Members of the genus *Pasteurella* sensu stricto share certain phenotypic characteristics.[366] *Pasteurella* species are nonmotile, gram-negative, facultatively anaerobic coccobacilli or rods. Most species are oxidase-positive, are catalase-positive, are alkaline phosphatase-positive, and reduce nitrate to nitrite. Most species also produce acid from glucose, fructose, mannose, and sucrose, but none hydrolyze starch or salicin. Several species also produce urease. All *Pasteurella* species are usually

Pasteurella *Species: Clinical Significance, Identification, and Antimicrobial Susceptibility*

Pasteurella multocida. *P. multocida* is the most frequently encountered *Pasteurella* species from human clinical specimens and is also recovered from animals. *P. multocida* organism can commonly be cultured from the oral cavities of healthy domesticated cats (70% to 90%) and dogs (40% to 66%), and is also found in other animals (e.g., cattle, horses, swine, sheep, fowl, rodents, rabbits, monkeys, lions, panthers, lynx, birds, reindeer, and buffalo). In some animals, this organism causes serious infections (e.g., shipping fever and hemorrhagic septicemia in cattle, fowl cholera, atrophic rhinitis in swine and pleuritis, pneumonia, abscess formation, chronic rhinitis, otitis media, and septicemia in laboratory rabbits). The majority of human infections are wound infections and cellulitis usually resulting from cat bites and scratches.[966,1341] Occasionally, *P. multocida* may be found in the nasopharynges

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of healthy individuals who have frequent animal exposure. Immunocompromised patients, particularly those with liver diseases (e.g., cirrhosis), solid tumors, or hematologic malignancies, may develop bacteremia from localized wound infections, which, in turn, may lead to hematogenous pneumonia, meningitis, or other complications.[1057] This organism has also been found as the cause of cellulitis in a patient with diabetes mellitus.[1410] *P. multocida* may be recovered from a variety of clinical specimens particularly from immunocompromised hosts. Box 9-5 describes infections associated with *P. multocida.P. multocida*, a small gram-negative coccobacillus is oxidase-positive, catalase-positive,

BOX 9-5

Clinical Spectrum of Human *Pasteurella multocida* Infections

Infection	Comments
Wound infections and cellulitis	Local wound infections in humans are associated with cat bites, cat scratches, or dog bites. These infections are characterized by rapid development of pain, erythema, swelling, cellulitis, and purulent or serosanguinous drainage at the site of the wound.[1341] Regional lymphadenopathy occurs in 30%–40% of patients, and systemic signs of infection may or may not be present. Serious localized complications (e.g., septic arthritis, osteomyelitis) most frequently follow cat bites, where the wound may be deep, forceful, and traumatic to underlying tissues. Since most bite wounds are on the hands, bone, and joint complications are usually seen at these sites. Serious wound infections requiring wide excisional debridement (e.g., necrotizing fasciitis) may also develop in patients with underlying diseases (e.g., diabetes) or on immunosuppressive therapy.[235,1410] Occasionally, this organism may be found in wounds that are not associated with animal bites or obvious animal exposure. Wound infections with *P. multocida* have also occurred as a result of a dog or cat licking open or partially healed wounds.[235] The bacterium has been isolated from decubitus ulcers, periocular abscesses, and from postsurgical abdominal and orthopedic wound infections.[301,618]
Bone and joint infections	Bone and joint infections result from implantation of *P. multocida* into bone and joints by traumatic dog or cat bites and scratches, by contiguous spread from an adjacent cellulitis, and, rarely, by hematogenous seeding of the joint space. Infections include septic arthritis, osteomyelitis, or both. *P. multocida* septic arthritis is often associated with preexisting joint disease (e.g., rheumatoid arthritis, corticosteroids, osteoarthritis, implanted prostheses). Infections of total knee and total hip replacements have occurred following dog and cat bites and scratches and licks.[583,584] These infections may necessitate removal and subsequent reimplantation of the prostheses. Osteomyelitis results from either direct implantation at the time of the bite or by extension of cellulitis. Infections are more severe in patients with underlying diseases.[193]
Respiratory tract infections	*P. multocida* can be isolated from the upper and lower respiratory tract, where it may behave as a commensal or as a cause of epiglottitis, pneumonia, empyema, lung abscess, adult respiratory distress syndrome, bronchitis, sinusitis, tonsillitis, and otitis media.[163,491,793,887,982,1357] Individuals with respiratory tract colonization often have a history of occupational or recreational animal exposure. Symptomatic patients usually have some preexisting airway compromise (e.g., COPD, chronic bronchitis, bronchiectasis, lung carcinoma, HIV infection). Symptoms of *P. multocida* pneumonia include an insidious or abrupt onset of fever, malaise, shortness of breath, and pleuritic chest pain. On chest x-ray, lobar consolidation, with mostly lower lobe involvement, is the most common presentation. Complications include development of bacteremia, pleural effusions, and empyema.[847] Upper respiratory tract infection with *P. multocida* may have unusual clinical presentations. *P. multocida* tonsillitis was reported in a woman whose cat habitually licked her toothbrush.[1060] Invasive *P. multocida* sinusitis was described in a kidney transplant recipient whose dog licked her face and ears.[1151]
Endocarditis	*P. multocida* can be a rare cause of both native valve and prosthetic valve endocarditis.[414,461,1296] Patients occasionally have a history of underlying or preexisting heart disease, although a history of cat or dog exposure and underlying disease (i.e., hepatitis, cirrhosis) is commonly found.[461] Infection usually involves the aortic and, less frequently, the mitral valve.[414] In 2007, right-sided endocarditis involving the pulmonary valve due to *P. multocida* was reported to occur in an HIV-negative IV drug user.[508] This patient required homograft replacement of the pulmonary valve because of persistent fever and positive blood cultures. Endocarditis due to *P. multocida* is usually a subacute clinical presentation, as indicated by a 1-week to 4-month time period from onset of symptoms to etiologic diagnosis. The one reported instance of prosthetic valve endocarditis occurred in a 72-year-old woman with a Carpentier–Edwards valve implant for aortic stenosis 3 years prior to the onset of disease.[944] The patient responded to penicillin and ampicillin sulbactam, and valve replacement was not required.

CNS infections	CNS infections caused by *P. multocida* have included meningitis, subdural empyema, and brain abscess.[143,514,932] The clinical presentation of *P. multocida* meningitis is similar to that caused by other agents; the CSF is usually purulent, and gram-negative coccobacilli are observed on Gram's stains. CNS infections have been reported in elderly individuals with underlying disease and in infants.[977] Meningitis in infants has often been associated with nontraumatic exposure to animals via licks from cats and dogs and fomites (e.g., pacifiers) contaminated with cat saliva.[712,932] Vertical transmission has resulted in neonatal bacteremia and meningitis. In these instances, vaginal, blood, or endometrial cultures obtained from the mothers were positive for *P. multocida*.[932] Caregivers of neonates and infants who have animal contact may also transmit the organism. An unusual case report described meningitis and sepsis in twins whose father had slaughtered a sheep for a dinner celebration. Isolates from the twins were identical to those isolated from the father's throat and nares.[525] More recently, sepsis and meningitis was reported in a 44-year-old woman and revealed her habit of kissing her dog and transferring food to the dog with her mouth.[692] Meningitis has also occurred as a complication of *P. multocida* septic arthritis.[767]
Eye infections	*P. multocida* has been isolated in rare instances from patients with conjunctivitis, periocular abscess with cellulitis, keratitis, and endophthalmitis. Purulent conjunctivitis was diagnosed in a poodle owner who did not recall any bites or scratches from the animal.[1251] *P. multocida* keratitis and corneal ulcers usually occur following corneal lacerations from cat or dog scratches.[1234] *P. multocida* endophthalmitis has occurred following penetrating injuries from cat scratches or as an immediate or delayed postoperative complication of cataract or lens implantation surgery.[94,326] These infections may result in loss of visual acuity or the need for enucleation of the eye.
Bacteremia and sepsis	*P. multocida* sepsis and bacteremia may occur by spread of the organism from a localized bite wound, or it may originate from an infected site elsewhere (e.g., cellulitis, arthritis, meningitis, or peritonitis).[1057] In some instances, the initial wound may be so innocuous (e.g., a minor scratch from a dog's paw) that it is ignored until signs and symptoms of sepsis supervene. Bacteremia occurs predominantly in the settings of liver disease (e.g., cirrhosis) or other underlying conditions (e.g., solid neoplasms, hematologic malignancies, lupus, infection).[84,180,194,1057] Profound *P. multocida* sepsis has been seen following animal bites in healthy individuals.[1119] In 2007, *P. multocida* bacteremia was diagnosed in two asymptomatic plateletpheresis donors.[189] The first platelet recipient had a transfusion reaction, and *P. multocida* was recovered from the donor platelet culture. The donor was asymptomatic, but had sustained a cat bite 100 minutes prior to platelet donation. In the second instance, platelet contamination was detected by culture, and the donor, a 74-year-old woman who cared for several feral cats, was diagnosed with *P. multocida* lymphadenitis five days later.
Intra-abdominal infections	*P. multocida* is an infrequent cause of intra-abdominal infections, including postsurgical wound infections, spontaneous bacterial peritonitis, appendicitis, and intra-abdominal/splenic/hepatic abscesses.[500,1058,1240] Patients who develop these infections have underlying diseases such as cirrhosis, solid organ tumors, and/or hematologic malignancies. Clinically, peritonitis caused by *P. multocida* is similar to that caused by enteric bacteria, with abdominal pain, fever, gastrointestinal bleeding, and hypotension. *P. multocida* is recovered from paracentesis fluid on culture, although direct Gram's stains of unconcentrated fluid are often negative. Since most patients with peritonitis usually have concurrent bacteremia, seeding of the peritoneal cavity is believed to occur via the hematogenous route in most cases, although peritoneal infection associated with upper endoscopy has been reported.[713] Although most cases of peritonitis are associated with animal contact, some patients do not recall or have no significant exposure to cats or dogs.[101] Continuous ambulatory peritoneal dialysis–associated peritonitis caused by *P. multocida* is unusual but has been reported.[49,923] In some instances, the organism was introduced into the peritoneal cavity by cat bites or scratches on the peritoneal dialysis tubing.
Obstetric, gynecologic, and urinary tract infections	*P. multocida* may occasionally colonize the female lower genital tract and can cause serious gynecologic or perinatal infections, including meningitis in the newborn. Female genital tract and neonatal infections have included diffuse infection, tubo-ovarian abscess, Bartholin's gland abscess, intrauterine device-related endometritis, intrauterine infection followed by septic abortion, chorioamnionitis with neonatal sepsis, pneumonia, and meningitis.[227,799,1088,1314] Most patients have some sort of underlying disease or immune compromise (e.g.,cranial trauma, neurosurgery, hepatic cirrhosis, postoperative wound infection, cervical carcinoma, or congenital genitourinary tract malformations). Puerperal sepsis, with isolation of the organism from the blood and endocervical discharge, has also been described in a previously healthy woman with nontraumatic exposure to the family cat.[1308] In this instance, PCR "fingerprinting" proved the genotypic identity of the isolates from the patient and from the oral cavity of her cat. Rare urinary tract infections may be caused by *P. multocida*. These are most often cat-associated, and patients usually have underlying renal disease and abnormal urologic anatomy secondary to surgery.[309,794]

ornithine decarboxylase-positive, indole-positive, mannitol-positive, and urease-negative (Image Plate 9-5B and 9-5C). In 2004, Christensen and colleagues demonstrated that bovine isolates of [*P.*] *avium* (*A. avium*) biovar 2 and [*P.*] *canis* biovar 2 were actually strains of *P. multocida* that were negative for ornithine decarboxylase, indole, and mannitol.[265]

P. multocida is not difficult to isolate and identify, although knowledge of the specimen type and an animal exposure history increases the index of suspicion that this organism may be present. *P. multocida* grows well on chocolate and sheep blood agar, forming smooth, gray colonies that are 0.5 to 2.0 mm after 24 hours' incubation in CO_2 (Image Plate 9-5A). The organism is nonhemolytic and does not grow on MacConkey agar, Eosin methylene blue (EMB) agar, or other selective/differential enteric media. Isolates from respiratory tract specimens may be mucoid. A characteristic odor (like *E. coli*, but more pungent) is frequently noted due to the formation of indole. *P. multocida* is oxidase-positive, catalase-positive, ornithine decarboxylase-positive, indole-positive, and urease-negative. The "spot indole" (*p*-aminocinnamaldehyde) reagent is usually strongly positive. *P. multocida* produces acid, but no gas, from glucose, sucrose, and mannitol, but not from maltose or lactose. For typical isolates recovered from likely sources, such as a cat bite or scratch, demonstration of oxidase-positive, gram-negative bacilli that are strongly spot indole-positive and do not grow on MacConkey agar is usually sufficient to identify *P. multocida*. *P. multocida* strains can be identified to subspecies level (i.e., subspecies *multocida*, *septica*, and *gallicida*) based on acid production from sorbitol and dulcitol. Subspeciation is of interest mostly regarding veterinary isolates and usually is not part of the species identification. Phenotypic characteristics for the identification of *Pasteurella* and related genera and species are shown in Table 9-8.

Virulence factors of *P. multocida* that are associated with economically significant animal infections (e.g., atrophic rhinitis in swine) have been actively investigated for years. Some *P. multocida* strains are encapsulated, and five capsular groups—A, B, D, E, and F—have been described.[560] These capsular groups can be divided further by somatic O antigen typing. Most human isolates of *P. multocida* belong to capsular group A and, to a lesser extent, group D. Strains of *P. multocida* that cause atrophic rhinitis produce a chromosomally encoded 145-kDa dermonecrotic toxin that is an essential virulence factor for disease development and progression. Molecular tests have been developed for direct detection of the *toxA* gene in *P. multocida*. The role of the dermonecrotic toxin in human disease is not known.

In 2010, CLSI published guidelines for both microbroth dilution and agar dilution antimicrobial susceptibility testing of *Pasteurella* species.[286] The approved microbroth method uses cation-adjusted Müeller–Hinton broth with lysed horse blood (2.5% to 5%), a direct colony suspension equivalent to a 0.5 McFarland turbidity standard, and incubation in air at 35°C for 18 to 24 hours. Agents to consider for testing include

penicillins, β-lactam/β-lactamase inhibitor combinations (i.e., amoxicillin clavulanate), cephalosporins (specifically ceftriaxone), fluoroquinolones (levofloxacin, moxifloxacin), tetracyclines (specifically tetracycline and doxycycline), macrolides (erythromycin, azithromycin), and SXT. Antimicrobial susceptibility testing indicates that *P. multocida* isolates are broadly susceptible to many antimicrobial agents in vitro. The organism is susceptible to penicillin, ampicillin, amoxicillin clavulanate, broad-spectrum penicillins, extended-spectrum cephalosporins, and tetracyclines. Less activity is noted for first-generation cephalosporins (e.g., cephalothin, cefazolin) and semisynthetic penicillins (e.g., oxacillin, methicillin, nafcillin). Aminoglycosides have moderate to poor activity against *P. multocida* strains in vitro, and the organism is resistant to vancomycin and clindamycin. Azithromycin has greater activity against *P. multocida* than erythromycin. *P. multocida* strains are very susceptible to fluoroquinolones and oxazolidinones, but clinical data regarding the use of chemotherapeutic agents are limited. Animal isolates of *P. multocida* are often tetracycline-resistant due to presence of a transposon-associated *tet(H)* tetracycline resistance determinant. At the present time, penicillin and the expanded-spectrum parenteral cephalosporins remain the agents of choice for treatment of infections caused by *P. multocida*.

Pasteurella dagmatis, Pasteurella canis,* and *Pasteurella stomatis. *P. dagmatis, P. canis,* and *P. stomatis* are members of the oral microbiota of dogs, and *P. dagmatis* is also found in the feline oropharynx. Human infections are associated with bites and scratches from these animals. *P. dagmatis* is a rare cause of bacteremia and endocarditis; in one case, endocarditis was complicated by vertebral osteomyelitis.[349,1111,1211] This patient was a 55-year-old woman who worked in an animal welfare agency and had sustained multiple cat bites and scratches from strays.[1211] *P. dagmatis* bacteremia was also documented in a 50-year-old diabetic man who developed *P. multocida* cellulitis of the toe and bacteremia 1 year earlier; at that time, his infection was traced to his dachshund, who had licked an open foot blister.[410] During a second hospitalization, *P. dagmatis* was isolated from blood cultures; this time the isolate was traced to his Yorkshire terrier! Chronic pneumonia due to *P. dagmatis* and *Neisseria canis* were documented in a 66-year-old man, who was a poodle owner with COPD.[20] Spontaneous bacterial peritonitis and peritoneal dialysis–associated peritonitis caused by *P. dagmatis* have also been reported.[72,1318] In 2009, Guillard and colleagues reported on the recovery of *P. dagmatis* from a dog-bite wound; the organism was misidentified as *P. pneumotropica* by the Vitek 2 system.[524]

Both *P. canis* and *P. stomatis* have been isolated from infected dog-bite wounds.[557,1044] *P. canis* strains are divided into two biotypes: biotype 1 is found in the oral cavity of dogs, whereas biotype 2 has been recovered from calves.[926] In a study

conducted by Holst and colleagues, 28 of 159 strains examined were identified as *P. canis*; all of these were biotype 1 strains from dog-bite wounds.[601] Ten isolates were identified as *P. stomatis*, eight from dog-bite wounds and two from abscesses. In the eight *P. stomatis* wound infections, *P. multocida* and *P. canis* were co-isolated with *P. stomatis*. *P. dagmatis*, *P. canis*, and *P. stomatis* are susceptible to penicillins, cephalosporins, aminoglycosides, tetracyclines, fluoroquinolones, and SXT.

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TABLE 9-8 Phenotypic Characteristics for Identification of *Pasteurella* Species, *Gallibacterium* Species, and *Bibersteinia* Species

Species	HEM SBA	OX	CAT	Growth, Mac-Conkey agar	NO_3 RED	IND	URE	ODC	PAL	ONPG	Requirement for V factor
Pasteurella sensu stricto											
P. multocida subsp. *multocida*	–	+	+	–	+	+	–	+	NA	–	–
P. multocida subsp. *septica*	–	+	+	–	+	+	–	+	NA	–	–
P. multocida subsp. *gallicida*	–	+	+	–	+	+	–	+	NA	–	–
P. dagmatis	–	+	+	–	+	+	+	–	+	–	–
P. canis	–	+	+	–	+	V	–	+	+	–	–
P. stomatis	–	+	+	–	+	+^{w}	–	–	+	–	–
Avibacterium gallinarum	–	+	+	–	+	–	–	–	+	–	–
Pasteurella species incertae sedis											
P. aerogenes	–	+	+	+	+	–	+	V	NA	–	–
P. pneumotropica	–	+	+	V	+	+	+	+	NA	–	–
P. bettyae	–	V	–	V	+	+	–	–	+	–	–
P. caballi	–	+	–	–	+	–	–	V	+	+	–
P. skyensis	–/β^{w}	+^{w}	–	–	–	+	–	+	+	–	+
P. langaaensis	–	+^{w}	–	–	+	–	–	–	+	+	–
P. lymphangitidis	–	–	+	V	–	–	+	–	+	–	–
P. mairi	V	+	V^{+}	V	+	–	+	V^{+}	+	V	–
P. testudinis	+	+	+	V	–	+	–	–	–	V+	–
Gallibacterium anatis	–	+^{w}	+	+	+	–	–	–	+	+	–
Bibersteinia trehalosi	β^{w}	–	V	+w	+	–	–	–	+	–	–

Species	GLU	MAL	SUC	LAC	XYL	MNTL	TREH	MANN	ARAB	SBTL	GAL	INOS	RAFF	DULC
Pasteurella sensu sricto														
P. multocida subsp. *multocida*	+	–	NA	–^{a}	V	+	V	NA	–	+	+	NA	NA	–
P. multocida subsp. *septica*	+	–	NA	–^{a}	+	+	+	NA	–	–	+	NA	NA	–
P. multocida subsp. *gallicida*	+	–	NA	–^{a}	+	+	–	NA	V	+	+	NA	NA	+
P. dagmatis	+	+	+	–	–	–	+	+	–	–	+	–	+^{w}	NA
P. canis	+	–	+	–	V–	–	V	+	–	–	+	–	–	NA
P. stomatis	+	–	+	–	–	–	+	+	–	–	+	–	–	NA
Avibacterium gallinarum	+	+	+	–	V	–	+	+	–	–	+	–	V^{+}	NA
Pasteurella species incertae sedis														
[*P.*] *aerogenes*	+	+	NA	V^{b}	+$^{-}$	–	–	NA	–	–	NA	NA	NA	–
[*P.*] *pneumotropica*	+	+	NA	V^{b}	+	–	+	NA	–	–	+	NA	NA	–
[*P.*] *bettyae*	+	V	–	–	–	–	–	V	–	–	–	–	–	–

[*P.*] *caballi*	+	+	+	+	+	+	–	+	–	–	+	–	+	NA
[*P.*] *skyensis*	+	+	–	+	–	+	+	+	NA	–	–	–	–	–
[*P.*] *langaaensis*	+	–	+	+	–	+	+	+	–	–	+	–	–	NA
[*P.*] *lymphangitidis*	+	V	V	–	–	+	+	+	+	V	+	–	–	NA
[*P.*] *mairi*	+	V	+	V^-	+	V^+	V^-	+	+	V^+	+	V	–	NA
[*P.*] *testudinis*	+	V^+	+	V^-	+	V	V	–	V	V	V^+	+	V	NA
Gallibacterium anatis	+	–	+	+	+	+	+	+	–	–	+	–	$+^w$	NA
Bibersteinia trehalosi	+	+	+	–	–	+	+	+	–	+	–	V	V	–

[a] Rare strains may be lactose-positive.
[b] Most reactions are negative for the indicated organisms.
+, positive reaction; –;negative reaction; NA, data not available; V, variable reaction; V^+, variable reaction, but most strains are positive; V^-, variable reaction, but most strains are negative; $+^w$, weak positive reaction; HEM SBA, hemolysis on sheep blood agar; OX, oxidase; CAT, catalase; NO_3 RED, reduction of nitrate to nitrite; IND, indole; URE, urease; ODC, ornithine decarboxylase; PAL, alkaline phosphatase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
+, positive reaction; –, negative reaction; V, variable reaction; V^+, variable reaction, but most strains are positive; V^-, variable reaction, but most strains are negative; $+^w$, weak or delayed positive reaction; NA, data not available; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; XYL, xylose; MNTL, mannitol; TREH, trehalose; MANN, mannose; ARAB, arabinose; SBTL, sorbitol; GAL, galactose; INOS, inositol; RAFF, raffinose; DULC, dulcitol.

[Pasteurella] *Species Incertae Sedis: Clinical Significance, Identification, and Antimicrobial Susceptibility*

[*Pasteurella*] *aerogenes.* [*P.*] *aerogenes* is found among the oropharyngeal/intestinal microbiota of swine. Rare human infections have resulted from bites, tusk wounds, or secondary to the occupational exposures to pigs and boars.[386] This organism is a cause of abortion and stillbirth in pigs, dogs, and rabbits. [*P.*] *aerogenes* was isolated from the ears and throat of a stillborn child delivered by a women during her 31st week of pregnancy; the organism was also found in vaginal cultures following delivery.[1254] The woman worked on a pig farm in Denmark. Quiles and colleagues reported cervical vertebral osteomyelitis caused by [*P.*] *aerogenes* in a previously healthy 62-year-old man.[1054] [*P.*] *aerogenes* was also recovered from peritoneal dialysate fluid of a 14-year-old girl whose pet hamster had chewed on the dialysis tubing.[452] [*P.*] *aerogenes* grows as smooth, convex, circular, nonhemolytic colonies on sheep blood agar and also grows on MacConkey agar. [*P.*] *aerogenes* is indole-negative and urease-positive, and most isolates produce ornithine decarboxylase. This species is "aerogenic," meaning that gas is produced from glucose during fermentation. [*P.*] *aerogenes* isolates from human specimens have been susceptible to ampicillin, cephalosporins, aminoglycosides, ciprofloxacin, and tetracycline.

[*Pasteurella*] *pneumotropica.* [*P.*] *pneumotropica* is part of the microbiota of the respiratory tract of dogs, cats, rats, and mice. Humans become infected via traumatic exposure via dog and cat bites. Human infections attributed to [*P.*] *pneumotropica* include meningitis, bacteremia with shock, bone and joint infections, wound infection, cellulitis, and upper respiratory tract infection.[450,465,895] Bilateral interstitial pneumonia

due to [*P.*] *pneumotropica* was documented in a 27-year-old person with AIDS who lived with several dogs.[317] [*P.*] *pneumotropica* peritonitis resulting from contamination of dialysis tubing by a pet hamster was reported in a patient receiving continuous ambulatory peritoneal dialysis.[195] [*P.*] *pneumotropica* has been rarely recovered from wound drainage, bone, joint fluid, throat swabs, urine, pleural fluid, and blood. After 24 hours' incubation, colonies of [*P.*] *pneumotropica* on sheep blood agar are variable in size (0.5 to 1 mm in diameter), smooth, convex, and nonhemolytic. They are urease-positive, indole-positive, and ornithine decarboxylase-positive; the last two tests help to differentiate [*P.*] *pneumotropica* from *Actinobacillus ureae*, which is negative for these two reactions. Some strains of [*P.*] *pneumotropica* grow on MacConkey agar. The positive urease test and differential reactions for maltose and mannitol help to differentiate this organism from *P. multocida*.

[*Pasteurella*] *bettyae*. [*P.*] *bettyae* (formerly CDC group HB-5, [*P.*] *bettii*) has been isolated from the human female genitourinary tract and related specimens (i.e., amniotic fluid). In 1989, a cluster of five patients with urethritis, pelvic inflammatory disease, or Bartholin's gland abscesses was identified in Tennessee; [*P.*] *bettyae* was isolated as the etiologic agent.[80] This outbreak suggested that [*P.*] *bettyae* may be a sexually transmitted pathogen. In a study performed in Rwanda, [*P.*] *bettyae* was isolated from 25 (3.6%) of 675 patients (204 women and 471 men) with genital ulcer disease, but from only one of 983 patients without genital ulcer disease.[145] Of 145 men with a urethral

discharge but without genital ulcers, [*P.*] *bettyae* was isolated from one man. In 1996, [*P.*] *bettyae* was reported as the cause of peripartum bacteremia in a 25-year-old woman and as the agent of a fatal pneumonia and pleural effusion in a 40-year-old man with HIV infection.[909,1177] [*P.*] *bettyae* grows on both blood agar and chocolate agar; growth on MacConkey agar is variable. After 24 hours' incubation in a CO_2 environment, colonies are pinpoint, nonhemolytic, smooth, and white. The organism is oxidase variable or delayed, and the catalase reaction is negative. Nitrate is reduced to nitrite, but not to nitrogen gas, and urease is not produced. Acid is formed from glucose and mannose, but not from maltose, sucrose, lactose, mannitol, and xylose. The organism produces indole after overnight incubation in tryptone broth; indole formation may be slight; so xylene extraction of the broth and use of Ehrlich's indole reagent may be required for indole detection. [*P.*] *bettyae* isolates are susceptible to penicillins, cephalosporins, monobactams, carbapenems, fluoroquinolones, aminoglycosides, and SXT. In the Rwanda study, 7 of 24 isolates were ampicillin-

resistant because of β-lactamase production.[145]

[*Pasteurella*] *caballi*. [*P.*] *caballi* inhabits the upper respiratory tract of horses and swine, and has been isolated in pure and mixed culture from equine pneumonia, peritonitis, equine and porcine endocarditis, wounds, abscesses, and genital tract infections.[271,277,1148] Documented human infections caused by [*P.*] *caballi* infection include an inflammatory, fluctuant finger lesion that occurred without any previous traumatic injury in a 28-year-old veterinarian who worked with horses, and from a horse-bite wound infection.[133] [*P.*] *caballi* strains are broadly susceptible to antimicrobial agents; some strains, however, are resistant to penicillin G and sulfonamides.

***Avibacterium* ([*Pasteurella*]) *gallinarum*.** [*P.*] *gallinarum* was reclassified in the new genus *Avibacterium* as *A. gallinarum* in 2005.[138] *A. gallinarum* inhabits the respiratory tract of domestic fowl. The first report of a human infection with *A. gallinarum* appeared in 1995, when it was recovered from blood cultures of a 12-year-old Saudi man who developed endocarditis 10 years after surgical repair of a ventricular septal defect.[16] This isolate was susceptible to penicillin, ampicillin, and cefotaxime; the patient responded to 6 weeks of therapy with the last two agents. The patient did not report any bites, scratches, or contact with any animals. A second report of a human infection appeared in 1999 from Japan, when *A. gallinarum* was recovered from blood cultures of a 34-year-old man with acute gastroenteritis. The isolate was susceptible to penicillins, cephalosporins, macrolides, carbapenems, fluoroquinolones, and aminoglycosides; the patient responded to cefazolin therapy. He had developed gastroenteritis 2 days after eating chicken at a barbecue, and the authors suggested that this meal may have been the source of *A. gallinarum*.[54] The identity of the isolates in these two case reports has been questioned since details on phenotypic characteristics were either not included or the data provided were not sufficient for accurate identification.[451] In 2002, *A. gallinarum* was isolated from blood, urine, and CSF of a 4-day-old neonate as a result of a septic home delivery.[7]

Mannheimia *Species (Formerly the* "Pasteurella haemolytica/Pasteurella granulomatis" *Complex)*

P. haemolytica strains are recognized as prominent pathogens in domesticated animals, causing severe disease and major economic losses in cattle, sheep, swine, and poultry industries. Historically, *P. haemolytica* strains were divided into biotypes designated A (cattle-associated) and T (sheep-associated). Biotype A strains produced acid from arabinose but not trehalose, while biotype T strains produced acid from trehalose but not arabinose. These biotypes were each divided into 17 serotypes: serotypes 1, 2, 5

to 9, 11 to 14, 16, and 17 belonged to biotype A and serotypes 3, 4, 10, and 15 belonged to biotype T. In 1985, *P. haemolytica* sensu stricto was excluded from the genus *Pasteurella* based on DNA–DNA hybridization. DNA–rRNA hybridization confirmed this exclusion from the *Pasteurella, Haemophilus, and Actinobacillus* genera.[926] Genotypic investigations of trehalose-positive "*P. haemolytica*" (biotype T) strains resulted in their reclassification as *Pasteurella trehalosi*, despite marginal genetic affinity with the genus *Pasteurella*. Organisms classified as *Pasteurella trehalosi* have recently been reclassified in the new genus *Bibersteinia* as *Bibersteinia trehalosi*.[137]

Examinations of diverse, trehalose-negative (biotype A) "*P. haemolytica*" strains by phenotypic criteria, multilocus enzyme electrophoresis, ribotyping, and 16S rRNA sequencing resulted in the proposal of a new genus, *Mannheimia*, to accommodate the trehalose-negative isolates of the "*P. haemolytica*" complex and "*P. granulomatis.*"[330,1084] Based on 16S rRNA sequencing, four distinct, related clusters corresponding to five new species were delineated in genus *Mannheimia*: *M. haemolytica* and *M. glucosida* (cluster I), *M. ruminalis* (cluster II), *M. granulomatis* (cluster III), and *M. varigena* (cluster IV).[46] These new species are most closely related to genus *Actinobacillus* (particularly *A. lignieresii*) and *B. trehalosi* ("*P. trehalosi*"). *Mannheimia* species can be separated from the genus *Pasteurella* by failure of the former species to produce acid from mannose and from the animal-associated *Actinobacillus* species by being urease-negative. *Mannheimia* species are mannitol-positive, which separates them from members of the genus *Haemophilus*, which are mannitol-negative. *M. haemolytica* strains are the cause bovine pneumonic pasteurellosis or "shipping fever." This infection is triggered by herd overcrowding, stress, immunosuppression, and concomitant viral respiratory tract infection. *M. haemolytica* strains that cause disease in cattle produce a leukotoxin that is species-specific, having cytolytic activity against ruminant lymphoid cells. The leukotoxin is a 105 to 108 kDa protein that belongs to the RTX toxin family.[1126,1224] At low concentrations, this leukotoxin activates neutrophils, induces formation of inflammatory cytokines, and causes cytoskeletal changes that lead to apoptosis. The toxin also causes pore formation in the cell membranes, leading to cell swelling and lysis.[1224] In vitro, the leukotoxin causes cytopathic changes in the bovine alveoli, where it is associated with membranes of degenerating macrophages and neutrophils. Leukotoxin-negative mutants of *M. haemolytica* have reduced virulence in animal models.[1247]

Members of the former "*P. haemolytica*" complex are rarely isolated from humans; infections have resulted from occupational or recreational animal exposures. Rivera and colleagues

reported an aortic graft infection due to both "*P. haemolytica*" biotype A (*M. haemolytica*) and group C β-hemolytic streptococci in a 50-year-old man.[1091] Yaneza and colleagues reported a patient with endocarditis who had no contact with cattle or other farm animals.[1395] *M. haemolytica* isolates are susceptible to penicillin, ampicillin, amoxicillin clavulanate, macrolides, aminoglycosides, and fluoroquinolones, but are resistant to linezolid. During the study of genus *Pasteurella* systematics using *sodA* gene sequences, an isolate of *M. haemolytica* that was recovered from a sputum specimen from a human was shown to be the first human isolate of *Gallibacterium anatis* (Table 9-8). Another previously undescribed *Mannheimia* species, *Mannheimia succiniciproducens*, was isolated from bovine rumen specimens in 2002.[772]This species was characterized genotypically, but phenotypic characteristics are not available. Phenotypic characteristics that are useful for identification of *Mannheimia* species are presented in Table 9-9.

Genus *Actinobacillus*

Taxonomy

The genus *Actinobacillus* currently includes 22 species and unnamed taxa: 19 of these are associated with the respiratory and genital tracts of animals. *A. pleuropneumoniae, A. equuli* subspecies, and *A. suis* are associated with serious animal diseases, and only two species—*A. ureae* and *A. hominis*—have been isolated from human infections. Based on 16S rRNA phylogenetic analysis, DNA–DNA hybridization data, and phenotypic characteristics, the genus *Actinobacillus* sensu stricto includes *A. lignieresii*,*A. pleuropneumoniae*, *A. equuli* subsp. *equuli*, *A. equuli* subsp. *haemolyticus* (Bisgaard taxon 11), *A. suis*, *A. arthriditis* (sorbitol-positive Bisgaard taxon 9), *A. ureae, A. hominis*, *Actinobacillus* genomospecies 1, *Actinobacillus* genomospecies 2 (sorbitol-negative *A. arthriditis*), and Bisgaard taxa 8 and 26.[266] Based on phylogenetic comparisons of the 16S rRNA, other "species" assigned to the genus *Actinobacillus* may be subcategorized into seven monophyletic groups representing miss-named taxa and do not truly belong to genus *Actinobacillus*. These phyla include the "Rodent group" ([*A.*] *muris*),[131] the "Succinogenes-Seminis group" ([*A.*] *seminis*, [*A.*] *succinogenes*), the "Rossii group" ([*A.*] *rossii*, [*A.*] *porcinus*), the "Capsulatus group" ([*A.*] *capsulatus*), the "Parasuis group" ([A.] indolicus), and the "Delphinicola group" ([*A.*] *delphinicola*, [*A.*] *scotiae*). [*A.*] *minor* (from swine) has no group affiliation, but is closely related to the core *Actinobacillus* genus and to another proposed species, "[*A.*] *porcitonsillarum*."[1260] The revised taxonomy of

genus *Actinobacillus* sensu stricto based on 16S rRNA sequencing is supported by alternate gene sequence comparisons (e.g., sequencing of a 426 base pair fragment of the *infB* gene, which encodes translation initiation factor 2).[955] Members of the genus *Actinobacillus* sensu stricto and *Actinobacillus* species incertae sedis are presented in Box 9-6.

Clinical Significance of Actinobacillus *Species*

Actinobacillus ureae. *A. ureae* is an uncommon commensal of the human respiratory tract and a rare isolate from human infections, including bacteremia, endocarditis, meningitis, bone marrow infection, septic arthritis, pneumonia, conjunctivitis, and peritonitis.[77,335,400, 674,691,709,950,1350] In most cases, an underlying condition is present, such as postsurgical infection, diabetes, periodontal disease, emphysema, and alcohol-associated cirrhosis. Patients with meningitis have been associated with previous skull trauma and with underlying disease, including HIV infection.[674]

Actinobacillus hominis. *A. hominis* is a rare human isolate that has been recovered from sputum and empyema fluid specimens of individuals with chronic lung disease. Wust and colleagues reported on two patients with hepatic failure due to chronic hepatitis B infection and cirrhosis, respectively, in which *A. hominis* septicemia occurred as the terminal event.[1374] In a study conducted in Copenhagen on 36 patients with *A. hominis,* lower respiratory tract infection and/or bacteremia, alcoholism, cardiovascular disease, drug addiction, psychiatric disorders, and COPD were associated conditions.[456]

Other *Actinobacillus* Species. Human infections with animal species of *Actinobacillus* usually occur as a result of animal-related trauma. *A. lignieresii*, *A. equuli*, and *A. suis* have been recovered from human clinical specimens, including horse and sheep bite wounds, joint fluid, blood, and sputum.[71,108,403,1015] The remaining *Actinobacillus* species have not been isolated from human clinical specimens.

Cultural Characteristics of Actinobacillus *Species*

Members of the genus *Actinobacillus* are aerobic, microaerophilic or facultative, gram-negative bacilli or coccobacilli that have an optimal growth temperature of 35°C to 37°C. All members of the genus are nonmotile gram-negative bacilli, urease-positive, indole-negative, ALA-porphyrin test–positive, and phosphatase-positive. All species produce acid from glucose (no gas), maltose, sucrose, fructose, and dextrin, and nitrate is reduced to nitrite and to nitrogen gas. All species in the genus are methyl red-negative and do not produce acetoin, and most species are catalase- and oxidase-

positive. The phenotypic characteristics of *A. ureae* and *A. hominis* are shown in Table 9-10, whereas the characteristics of *Actinobacillus* species isolated from animals are found in Table 9-11.

A. ureae is a pleomorphic gram-negative bacillus; on staining, some strains form distinct filaments. After 24 hours of growth on blood agar in a CO_2-enriched atmosphere, colonies are smooth, 1 mm in diameter, and nonhemolytic. Some isolates may appear mucoid. The organism is oxidase-positive, is catalase-positive, reduces nitrate to nitrite, does not grow on MacConkey agar, and hydrolyzes urea rapidly. Indole is not produced, and the lysine and ornithine decarboxylase, as well as the arginine dihydrolase reactions, are negative. Acid is produced from glucose, maltose, sucrose, and mannitol; no acid is produced from lactose and xylose. The other biochemically similar *Actinobacillus* species may be differentiated from *A. ureae* by acid production from additional carbohydrates and by the abilities of the former species

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TABLE 9-9 Phenotypic Characteristics for Identification of *Mannheimia* Species

Characteristic	*M. haemolytica*	*M. glucosida*[a]	*M. granulomatis*	*M. ruminalis*[b]	*M. varigena*[c]	*Mannheimia* species
HEM BBA	β	β	–	–	β	V
OX	+	+	+	+	+	+
NO_3 RED	+	+	+	+	+	+
PAL	+	+	+	+	+	+
CIT	–	–	–	–	–	–
MOT	–	–	–	–	–	–
URE	–	–	–	–	–	–
IND	–	–	–	–	V	V
ADH	–	–	–	–	–	–
ODC	–	V[d]	–	–	+	V–
Gas from GLU	V	V	V	V	V	V
Acid produced from:						
GLU	+	+	+	+	+	+
MAL	+	+	V	V	V	+
MNTL	+	+	+	+	+	+
SBTL	+	+	+	V	–	V^-
ARAB	–	V[e]	–	–	+	V
ADON	–	–	–	–	–	–
AMYG	–	+	V	–	–	–
ARB	–	+	V	–	–	–
CELL	–	+	V	–	–	–
ESC	–	+	V	–	–	–
GENTB	–	V[f]	V	–	–	V^-
M-INOS	V	+	V	–	V	+
MANN	–	–	–	–	–	–
MEL	–	–	–	–	V	–
SAL	–	+	V	–	–	–
TREH	–	–	–	–	–	–
XYL	+	+	V	V	+	+
β-GAL	V	+	V	+	V	V^+
β-GLU	–	+	+	–	V	–
β-FUC	+	V	–	–	V	V^-
β-XYL	V	V	V	–	V	V
Source	Cattle, sheep	Sheep	Cattle, deer	Cattle, sheep	Cattle, swine	

[a] Divided into 9 biovars: A–I.
[b] Divided into 2 biovars: 1, 2.
[c] Divided into 2 biovars: 1, 2.
[d] Biovars A–E and I are positive; biovars F, G, and H are negative.
[e] Biovars A, C, D, G, and I are positive; biovars B, E, F, and H are negative.
[f] Biovars A, B, D–H are positive; biovars C and I are negative.
+, positive reaction; –, negative reaction; V, variable reaction; V^+, most strains are positive, but rare strains are negative; V^-, variable reaction, but most strains are negative; HEM BBA, hemolysis on bovine blood agar; OX, oxidase; NO_3 RED, reduction of nitrate to nitrite; PAL, alkaline phosphatase; CIT, growth on citrate; MOT, motility; URE, urease; IND, indole; ADH, arginine dihydrolase; ODC, ornithine decarboxylase; GLU, glucose; MAL, maltose; MNTL, mannitol; SBTL, sorbitol; ARAB, arabinose; ADON, adonitol; AMYG, amygdalin; ARB, arbutin; CELL, cellobiose; ESC, esculin; GENTB, gentabiose; M-INOS, myoinositol; MANN, mannose; MEL, melibiose; SAL, salicin; TREH, trehalose; XYL, xylose; β-GAL, β-galactosidase; β-GLU, β-glucosidase; β-FUC, β-fucosidase; β-XYL, β-xylosidase.

BOX 9-6

Members of the Genus *Actinobacillus* Sensu Stricto and *Actinobacillus* Species Incertae Sedis

Actinobacillus Species Sensu Stricto	Animal Habitats and Diseases
Actinobacillus lignieresii	*A. lignieresii* is a commensal of the oral cavity and pharynx of ruminants (cattle and sheep). Disease results from traumatic inoculation of the organisms into submucosal tissues, with formation of granulomas in the head, neck, gums, and tongue ("wooden tongue" disease of cattle). Dissemination via the lymphatics results in granulomas in the lungs. This organism also causes pneumonic and cutaneous infections in sheep and other ungulates.
Actinobacillus equuli subsp.*equuli*	*A. equuli* causes a equine disease called "sleepy foal disease" ("joint ill").[270,363] The foal acquires the organism from the mare, who may carry the organism asymptomatically in the mouth, respiratory, and alimentary tracts. In the foal, nephritis is the principal associated disease. *A. equuli* can also cause bacteremia, endocarditis, peritonitis, lower respiratory tract infection, and genital tract infection, leading to abortion in adult animals.[1] The organism has also been isolated from swine.
Actinobacillus equuli subsp.*haemolyticus*	This organism was originally described as a hemolytic variant of *A. equuli*, and the species was subsequently amended to include two subspecies.[270] This subspecies is also associated with bacteremia, peritonitis, nephritis, osteomyelitis, endometritis, abortion, and arthritis in horses. RTX toxin activity that has been detected in subspecies *haemolyticus*, but not in subspecies *equuli,* is active against equine lymphocytes. Like *A. pleuropneumoniae*, this species is CAMP test-positive.
	A. pleuropneumoniae is an important agent that causes a highly contagious pneumonia and chronic lung disease in pigs.[1124]Disease usually occurs in outbreaks among animals lacking immunity to the organism. The infection is a necrotizing pneumonia associated with severe congestion and hemorrhage into the lung parenchyma. The most significant virulence factor of *A. pleuropneumoniae* is the production of Apx toxins (pxI, ApxII, and ApxIII), which are

Actinobacillus pleuropneumoniae	encoded by RTX (repeat in toxins) genes. The RTX family of cytolysins also includes *E. coli* hemolysin and the leukotoxin of *Mannheimia haemolytica*, and similar genes are also found in *A. suis* and *A. equuli* subsp. *haemolyticus*. The Apx toxins are cytotoxins that are able to lyse porcine macrophages in vitro and in vivo. *A. pleuropneumoniae* requires V factor for growth and, therefore, does not grow on sheep blood agar. A V-factor-independent biotype of this organism has been described that does not cause overt disease in pigs. The organism also produces a hemolysin that is responsible for β-hemolysis on V-factor-supplemented blood agar and CAMP test reactivity with *Staphylococcusaureus*.
Actinobacillus arthriditis	*A. arthriditis* is isolated from the oropharynx of healthy horses and as a cause of septicemia and arthritis in ill horses.[268]
Actinobacillus suis	This species causes septicemia, pneumonia, gastroenteritis, and arthritis in suckling and newly weaned piglets.[1124]
Actinobacillus ureae	This species is a rare cause of respiratory tract infections in humans (see text).
Actinobacillus hominis	This organism is implicated as a rare cause of respiratory tract infections in humans (see text).
"*Actinobacillus porcitonsillarum*"	This newly described V-factor-dependent organism has been isolated from porcine tonsil, lung, and liver tissues. Based on 16S rRNA sequence analysis, this "species" is most closely related to *A. pleuropneumoniae*.[1260]This organism was originally thought to be nonpathogenic, but in 2007, "*A. porcitonsillarum*" was isolated from the hilar lymph nodes and lung tissue of a slaughter pig with granulomatous lymphadenitis and pneumonia.[970] This organism is β-hemolytic and CAMP test-positive.
Actinobacillus genomospecies 1	This genomospecies is a cause of stomatitis in horses.[268]
Actinobacillus genomospecies 2	This unnamed species causes arthritis and septicemia in horses.[268]
Bisgaard taxon 8	This taxon has been recovered from the respiratory tracts of guinea pigs.[136]
Bisgaard taxon 26	This taxon is implicated as a cause of sinusitis and sepsis in ducks.
Actinobacillus Species	

Incertae Sedis	
[*Actinobacillus*] *muris*	[*A.*] *muris* was isolated from the oropharynges of rodents.[136]

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[*Actinobacillus*] *seminis*	[*A.*] *seminis* was originally isolated from rams with epididymitis, and may be a cause of infertility in these animals.[1202]
[*Actinobacillus*] *succinogenes*	This "species" has been isolated from cattle.[523]
[*Actinobacillus*] *rossii*	This "species" was originally isolated from the vaginas of postparturient sows.[272,1202]
[*Actinobacillus*] *porcinus*	This V-factor-dependent organism has been isolated from the respiratory tracts of swine.[904]
[*Actinobacillus*] *capsulatus*	This species is a cause of granulomatous arthritis and sepsis in rabbits, hares, and hamsters.
[*Actinobacillus*] *indolicus*	This V-factor-dependent, indole-positive "species" was isolated from the upper respiratory tract of pigs.[904]
[*Actinobacillus*] *scotiae*	This organism was originally isolated from sea mammals.[438]
[*Actinobacillus*] *delphinicola*	This "species" was originally recovered from sea mammals.[436]
[*Actinobacillus*] *minor*	[*A.*] *minor* is a V-factor-dependent isolate found in the respiratory tracts of pigs.[904]

TABLE 9-10 Phenotypic Characteristics for Identification of *Actinobacillus ureae* and *Actinobacillus hominis*

Characteristic	*A. ureae*	*A. hominis*
HEM SBA	–	–

OX	+	+
CAT	+	−
NO_3 RED	+	+
Requires X factor	−	−
Requires V factor	−	−
IND	−	−
URE	+	+
ODC	−	−
ONPG hydrolysis	−	+
Acid produced from:		
GLU	+	+
MAL	+[a]	+
FRU	+	+
SUC	+	+
LAC	−	+
XYL	−	+
MNTL	+	+
MANN	V	V⁻
GAL	−	+
TREH	−	+
RAFF	−	+

See the footnote of Table 9-9 for the meaning abbreviations used here.
[a] A few strains may produce weak or delayed positive reactions.
+, positive reactive; −, negative reaction; V, variable reaction; NA, data not available.

to grow on MacConkey agar. Molecular methods have been used for diagnosis of *A.*

ureae meningitis. *A. ureae* strains are susceptible to most antimicrobial agents, including penicillin, ampicillin, cephalothin, cefoxitin, tetracycline, SXT, and the aminoglycosides.

A. hominis forms nonhemolytic mucoid and nonmucoid colonies on sheep blood agar. The organism is positive for the oxidase, catalase, urease, and ONPG reactions, but negative for indole. Acid is produced from a wide variety of carbohydrates; these characteristics are helpful for differentiation of *A. hominis* from *A. ureae*.

Brucella Species

Introduction

Brucellosis (infection with *Brucella* species) is worldwide in distribution and has been known historically as undulant fever, Bang's disease, Gibraltar fever, Mediterranean fever, and Malta fever. The organism was first isolated in 1887 by Sir David Bruce, who recovered a suspect organism from splenic cultures of British soldiers dying of Malta fever. Later, it was found that milk, cheese, and other foods made with goats' milk were the sources of infection for these soldiers. Similar organisms were recovered from cattle, pigs, and humans exposed to these animals and their products. Brucellosis became recognized as a zoonosis of great economic importance and concern to the livestock industry in many parts of the world. At present, brucellosis still afflicts humans and animals in many parts of the world, including southern Europe, the eastern Mediterranean region, the Middle East, the Arabian Peninsula, Central Asia, the Indian subcontinent, Mexico, and Central and South America.[1002] Animal brucellosis has been successfully eradicated from several parts of the world, including North America, Northern Europe, and northwest Asia. Infections that present in these areas usually result from travel to endemic areas, exposure to infected animals, or accidental laboratory-acquired infections. Brucellosis

TABLE 9-11 Phenotypic Characteristics for Identification of *Actinobacillus* Sensu Stricto and *Actinobacillus species* Incertae Sedis

Species	HEM SBA	OX	CAT	Growth, MacConkey Agar	NO_3 RED	IND	URE	ODC	ONPG	Requirement for V Factor
Actinobacillus sensu stricto										
A. lignieresii	–	+	V+	V	+	–	+	–	+	–
A. equuli subsp. *equuli*	–	+	V+	V	+	–	+	–	+	–
A. equuli subsp. *haemolyticus*	β	+	+/+^{w}	V	+	–	+	–	+	–
A. pleuropneumoniae	β	V	V	–	+	–	+	–	+	+
A. porcitonsillarum	β	V	–	–	+	–	+	–	+	+
A. arthriditis	–	+	+	V	+	–	+	–	+	–
A. suis	β	V+	V	V	+	–	+	–	V+	–
Actinobacillus genospecies 1	–	+	+	V	+	–	+	–	+	–
Actinobacillus genospecies 2	–	+	+	V	+	–	+	–	+	–
Bisgaard Taxon 8	–	+	+	NA	+	–	+	–	+	+
Bisgaard Taxon 26	β	+	+	NA	+	–	+	–	–	–
Actinobacillus species incertae sedis										
[*A.*] *muris*	–	+	+	–	+	–	+	–	–	–
[*A.*] *seminis*	–	V	+	–	+	–	–	V	–	–
[*A.*] *succinogenes*	–	+	+	NA	+	–	–	NA	NA	–
[*A.*] *rossii*	V	V+	+V	V+	+	–	+	–	+	–
[*A.*] *porcinus*	–	NA	–	NA	+	–	–	–	+	+
[*A.*] *capsulatus*	–	+	+	+	+	–	+	–	+	–
[*A.*] *indolicus*	–	NA	+	NA	+	+	–	–	+	+
[*A.*] *scotiae*	–	+	–	–	+	–	+	V	+	–
[*A.*] *delphinicola*	–	+	–	–	+	–	–	V	–	–
[*A.*] *minor*	–	NA	–	NA	+	–	+	–	+	+

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Species	GLU	MAL	SUC	LAC	XYL	MNTL	TREH	MANN	ARAB	SBTL	GAL	INOS	RAFF	MEL	SAL
Actinobacillus sensu stricto															
A. lignieresii	+	+	+	+	+	+	–	+	V	–	+	–	V	–	–
A. equuli subsp. *equuli*	+	+	+	+	+	+	+	+	V	–	+	–	+	+	–
A. equuli subsp. *haemolyticus*	+	+	+	+	+	V	+	+	–	V	+	–	+	+	–
A. pleuropneumoniae	+	+	+	+	+	+	–	+	–	–	+	–	V	–	–
A. porcitonsillarum	+	NA	–	NA	NA	–	NA	NA	–	–	+	–	NA	NA	NA
A. arthriditis	+	+	+	+	+	+	–	+	V	+	+	–	+	+	–
A. suis	+	+	+	+	+	–	+	+	V^{+}	–	V^{+}	–	+	+	+
Actinobacillus genomospecies 1	+	+	+	+	+	–	–	+	V	–	+	–	V	–	–
Actinobacillus genomospecies 2	+	V	+	+	+	+	–	+	–	–	+	–	+	V	–
Bisgaard Taxon 8	+	+	+	+	+	+	–	+	–	–	+	+	+	+	+
Bisgaard Taxon 26	+	+	+	–	–	V	+	+	–	+	–		–	–	
Actinobacillus species incertae sedis															
[*A.*] *muris*	+	+	+	–	–	+	+	+	–	–	V^{+}	V^{+}	+	+^{w}	+
[*A.*] *seminis*	+	V	–	–	–	V	–	–	V	–	V	V	–	–	–
[*A.*] *succinogenes*	+	+	+	+	+	+	–	+	+	+	+	–	+	–	+
[*A.*] *rossii*	+	V^{-}	–	+	+	+	–	V	+	+	+	+	V^{-}	–	–
[*A.*] *porcinus*	+	V	+	V	NA	+	NA	V	V	V	V	V	V	NA	NA
[*A.*] *capsulatus*	+	+	+	+	+	+	+	+	–	+	+	–	+	+	+
[*A.*] *indolicus*	+	+	+	V	NA	–	NA	+	–	–	+	–	+	NA	NA
[*A.*] *scotiae*	+	–	–	+	–	–	–	–	–	–	V	–	–	–	–
[*A.*] *delphinicola*	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
[*A.*] *minor*	+	+	+	+	NA	–	NA	+	–	–	+	–	+	NA	NA

+, positive reaction; –, negative reaction; V, variable reaction; +^{w}, weak positive; V+, variable, but most strains positive; NA, not available; HEM SBA, hemolysis on sheep blood agar; OX, oxidase; CAT, catalase; ODC, ornithine decarboxylase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
+, positive reaction; –, negative reaction; V, variable reaction; NA, not available; +^{w}, weak positive reaction; V^{+}, variable reaction, but most strains are positive; V^{-}, variable reaction, but most strains are negative; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; XYL, xylose; MNTL, mannitol; TREH, trehalose; MANN, mannose; ARAB, arabinose; SBTL, sorbitol; GAL, galactose; INOS, inositol; RAFF, raffinose; MEL, melibiose; SAL, salicin.

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is transmitted among animals via the gastrointestinal tract, skin, and mucous membranes. Following infection, organisms reach the lymph nodes and bacteremia ensues. In some animals (e.g., *B. abortus* infection in cattle), the organisms proliferate in the uterus and in the mammary glands. Growth of the organism in the chorionic membranes of the pregnant animal leads to abortion. Many animals recover from the infection spontaneously, but continue to shed the bacteria for varying times in urine, vaginal secretions, and milk.

Taxonomy and Epidemiology

Originally, the genus *Brucella* contained six classical species: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae,* and *B. ovis*. In 1985, Verger and colleagues found that isolates belonging to all six species had greater than 90% homology with each other and proposed that all brucellae belonged to a single species (*Brucella melitensis*) and that the recognized species should be regarded as biovars of *B. melitensis* (i.e., *B. melitensis* biovars *melitensis, abortus, suis,* and *canis*).[1300] This proposal was not accepted by the Subcommittee on *Brucella* of the International Committee on Systematic Bacteriology because of biochemical and serologic differences among the species, their divergent host ranges, recognized differences in virulence, and the presence of species-specific genes and proteins. The gravity of *Brucella* infections, the significance of the various agents in agriculture, and the potential for bioterrorism with the various species further supported the decision to retain the traditional classification scheme.[986]Molecular genetic studies (i.e., restriction endonuclease mapping) subsequently revealed gene polymorphisms that were able to differentiate *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* along conventional species lines, although these methods could not delineate biovars within species. One of the polymorphic genes that help to differentiate species within the brucellae is the *omp2* porin gene, which codes for a 36-kDa outer membrane protein that is responsible for determining susceptibility to the dyes used for conventional species identification. *Brucella* species are members of the family *Brucellaceae* in the order *Rhizobiales* in the α2-subdivision of the *Proteobacteria*.[532] The order *Rhizobiales* also contains the families *Bartonellaceae*, *Rhizobiaceae*,*Phyllobacteriaceae,* and *Aurantimonadaceae. Brucella* species are related to *Agrobacterium*, endosymbiotic *Rhizobium* and *Mesorhizobium* species, *Bartonella* species, *Ochrobactrum* species, and *Phyllobacterium* species, which are free-living chemoautotrophic pathogens of tropical plants. Brucellae are most closely related to *O. anthropi* hybridization group 2, for which the new species name *O. intermedium* has been proposed.[1152,1297] The genetic relatedness of these organisms is so close that *O. anthropi* is reactive in a PCR-based assay that is supposedly "specific" for *Brucella* species.[320]

Studies on the genetic relationships of brucellae have revealed other unique features

of the genus. The genomes of the five major *Brucella* species have been completely sequenced, and are similar in size and gene structure.[1217] The average genome size within the genus is about 3.29 Mb and consists of two circular chromosomes in all species except *B. suis* biovar 3, which has a single chromosome.[665] Chromosome 1 is about 2.11 Mb, and chromosome 2 is about 1.18 Mb in size.[340,341] Each chromosome encodes functions that are essential for replication and survival of the organism, hence their classification as chromosomes and not plasmids. *Brucella* species have no classical virulence-associated genes, do not have capsules or pili, and do not produce any known exotoxins. Plasmids and genetic exchange via transformation or conjugation have not been described in *Brucella* species.[1166]

Brucella species are named for their primary host species and are further subdivided into biovars based on serologic agglutination with "smooth LPS (S-LPS)"-associated antigens M and A (see below). *B. melitensis* is found primarily in goats and sheep, and may also be found in cattle because of indirect contact with infected sheep and goat flocks. This species is divided into three biovars (1 to 3). *B. abortus* is pathogenic for cattle, but can infect sheep, goats, canines, horses, and humans. *B. abortus* includes eight biovars (1 to 7, and 9). *B. suis* contains five biovars: Biovars 1, 2, and 3 are found in swine, while biovar 4 is found in reindeer and caribou in the Arctic regions of North America and Russia and biovar 5 causes infections in small ruminants.*B. suis* biovar 1 has also become established in cattle herds in Brazil and Columbia. *B. canis* strains comprise a single biovar and occur in dogs (especially beagles) in the United States, Mexico, Argentina, Spain, China, Japan, and Tunisia.[1417] *B. ovis* and *B. neotomae* each contain a single biovar and are found in rams and wood rats, respectively. Antigenic variation among existing biovars has been demonstrated for every *Brucella* species, establishing that biovar classifications require further study and expansion. *B. melitensis*, *B. abortus*, and *B. suis* are associated with human disease, with *B. melitensis* considered the most virulent species, followed by *B. suis* and *B. abortus*. *B. canis* rarely causes infections in humans, and reported infections have often been laboratory associated. *Brucella microti*, a new terrestrial *Brucella* species, was described in 2008 based on two isolates recovered from clinical specimens of the common vole *Microtus arvalis* in the Czech Republic.[1153,1155] These strains contained the *omp2a* gene found in marine brucellae (see below), but the *omp2b* gene was similar to those found in terrestrial *Brucella* species. This species has also been isolated from mandibular lymph nodes of red foxes in Austria and from soil specimens.[1154,1155] More recently, a new *Brucella* species, *B. inopinata*, was isolated from a breast implant wound of a 71-year-old woman with symptoms of brucellosis.[333,1156] This isolate was initially called strain BO1. In 2010, another isolate, called BO2, was isolated from a lung biopsy specimen of a 52-year-old Australian man with necrotizing pneumonia.[1256] Phenotypic characteristics suggested a *Brucella*

species, and 16S rRNA gene sequencing was identical with *B. inopinata* (strain BO1). These two *B. inopinata* strains form a distinct phylogenetic cluster in the genus *Brucella*. Finally, in 2009, a novel *Brucella* isolate was recovered from uterine cultures of two baboons who had stillbirths.[1147] Phylogenetic characterization of the organisms confirmed their membership in genus *Brucella*, with the closest relative being *B. ovis*.

During the 1990s, several *Brucella*-like bacteria were isolated from carcasses of marine animals, including seals, porpoises, dolphins, otters, and whales.[175,283,432,638] Isolates from these marine hosts showed more than 77% relatedness to the genus *Brucella* by DNA hybridization.[1299] IS*711*, a genetic insertion element that is found in all *Brucella* species,

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was also found in marine mammal isolates, and, based on restriction endonuclease mapping and the detection of multiple copies of IS*711*, the species "*Brucella maris*" was proposed for isolates from marine mammals.[283,287] These strains could also be grouped into biovars based on phenotypic and biochemical characteristics.[638] Other investigators examined the *omp2* gene of these strains and identified polymorphisms at that gene locus that supported and aligned with the biovar designations described by Jahans and colleagues.[288] Recognized *Brucella* species contain two *omp2* genes composed of one copy each of *omp2a* and *omp2b*, or two *omp2a* genes. The marine brucellae carry two *omp2b* gene copies, and sequencing of these genes revealed significant heterogeneity among marine isolates, indicating that these organisms encompass more than a single species ("*B. maris*"), as had been proposed.[283,288]Based on these data and consistent with inclusion of preferential hosts in considering taxonomic classification, at least two new species were proposed for organisms included in "*Brucella maris*": *Brucella pinnipediae*, for isolates from seals, and *Brucella cetaceae*, for cetacean isolates.[288] Isolates obtained from aborted bottlenose dolphin fetuses were also characterized, and the name "*Brucella delphini*" was proposed for these isolates.[890] Serologic surveys of seal, porpoise, dolphin, and whale species have shown that antibodies against these *Brucella* species are widely distributed among marine mammals, and molecular analysis has shown that isolates from whales, seals, porpoises, and dolphins are *Brucella* species but differ from each other and from other extant *Brucella* species in many respects, including LPS structure and outer membrane proteins.[98,175,647] Additional studies involving restriction profiles of the genomes of these bacteria recognized three clonal groups that corresponded to specific organisms hosts—seals, porpoises, and dolphins.[157] In 2007, Foster and colleagues proposed two new species—*Brucella pinnipedialis* and

Brucella ceti—for *Brucella* strains with seals and cetaceans as their preferred hosts, respectively.[435] In these hosts, these brucellae cause CNS, liver, lung, and genitourinary tract disease and abortion.[321,433,638] Characterization of strains from marine mammals by traditional *Brucella* biotyping approaches and novel molecular techniques found that strains originating from cetaceans were in two groups with either dolphins or porpoises as the preferred host.[331] These data were consistent with other studies, showing subgroups within the proposed species *B. ceti*.[157,519,636] Strains isolated from seals were in another group corresponding to the proposed species, *B. pinnipedialis*. In the newest edition of *Bergey's Manual of Systematic Bacteriology*, three new species, *Brucella phocae* (seals), *Brucella phocoenae* (porpoises), and *Brucella delphini* (dolphins), are included.[306]

Evidence also suggests that these new species may have extended pathogenic potential. Rhyan and colleagues inoculated six cattle with a *Brucella* isolate from a harbor seal, and found that all animals seroconverted in tests used for detection of *B. abortus* antibodies. Two of the cattle aborted, and the brucellae were isolated from the stillborn fetuses, indicating that these strains are pathogenic for terrestrial mammals as well.[1083] In addition, there have been reports of human infections (i.e., neurobrucellosis) with strains from marine mammals.[173,873,1204] A laboratory technician working with marine isolates manifested symptoms of brucellosis, which was confirmed by isolation and PCR-restriction fragment length polymorphism (RFLP) identification of a marine *Brucella* species. The individual also demonstrated a serologic response to the organism.[173] Two patients from Peru were diagnosed with neurobrucellosis due to marine mammal brucellae as demonstrated by isolation and confirmation by PCR and sequencing.[1204] In 2006, a man from South Auckland, New Zealand, was diagnosed with spinal osteomyelitis; the isolated organism was initially identified as *B. suis*, but was found to resemble a *Brucella* species originating from bottlenose dolphins by PCR-RFLP and sequencing of the *bp26*, IS*711*, and *omp* genes.[873] Additional work is needed to delineate the phylogenetic relationships among these new species, to ascertain their relationships with currently recognized brucellae, and to determine the virulence factors associated with animal and human infections.

In the United States, the incidence of human brucellosis has declined steadily as a result of control measures implemented in the livestock industry. These measures include vaccination of young animals and slaughter of sick animals or older animals with serologic evidence of infection. At the time that the Federal-State Cooperative Brucellosis Eradication Program and routine dairy pasteurization began in 1945, over 6,000 human cases were reported to the CDC annually. During the 1970s, brucellosis in the United States was attributable to *B. suis* infections among abattoir workers. In 1981, the reported annual domestic incidence of human brucellosis had dropped to 185 cases, and since that time, less than 200 cases per year have been reported. In

2007, for example, 131 cases of brucellosis were reported in the United States, and states with the highest numbers of reported cases were California, Texas, and Florida.[222] Human brucellosis is probably underdiagnosed and underreported, with estimates that at least 25 cases go unrecognized for every case that is diagnosed. Human consumption of sheep or goat milk or contaminated meat containing *B. melitensis* is a principle source of human brucellosis worldwide.[342,1239,1317] Domestically acquired *Brucella* infections have been diagnosed among individuals living in Texas and California who have ingested Mexican cheese prepared from unpasteurized goat's milk.[260] Most cases in the northern states largely represent imported disease acquired through international travel or through the consumption of imported, contaminated food. In the western, south central, mountain, and Pacific states, the disease affects largely Hispanic populations in areas bordering Mexico.[368] With increasing availability and popularity of international travel, many patients with brucellosis that are US residents have visited countries where the organism is endemic in ovine/bovine herds and, consequently, is present in raw, unpasteurized dairy products. Infection partly reflects participation in aspects of foreign cultures that are so appealing to travelers (i.e., "living like the natives do"). Box 9-7 includes a brief discussion on the worldwide epidemiology of brucellosis.

Occupational exposure may occur in abattoir workers, farm workers, veterinarians, ranchers, and meat-packing employees; these individuals acquire infection by contact with infected animals, products of conception, or body fluids from infected animals. Human-to-human transmission is rare, but sexual transmission of *Brucella* has been reported.[840,1375] In 2007, human-to-human transmission was documented in an

BOX 9-7

Global Epidemiology of Brucellosis

Brucellosis is found throughout areas of both the eastern and western hemispheres. In Latin America, Mexico is an area of high endemicity, and the disease occurs mostly in the northern regions bordering the United States, and the northwest and north central regions.[813] *B. melitensis* biovars 1-3, *B. abortus* biovars 1, 2, and 4-6, *B. suis* biovar 1, *B. canis,* and *B. ovis* have been isolated from animals in Mexico. *B. abortus* is found mainly in cattle herds, although coexistence of cattle and goat herds had resulted in some *B. melitensis* infections among cattle. Human brucellosis in Mexico is associated with both occupational exposure and infection via contaminated dairy products, as more than 35% of cow's milk and 85% of goat's milk are consumed

unpasteurized.[813]Among reported human infections in Mexico, 93% are due to *B. melitensis* of caprine origin, 5% are due to *B. melitensis* of bovine origin, 1.5% are caused by *B. abortus* of bovine origin, and 0.5% are caused by *B. suis* of bovine origin. Brucellosis is found throughout Central America, including Guatemala, Belize, Honduras, El Salvador, Nicaragua, Costa Rica, and Panama. Infection rates in cattle herds range from 10% to 25%. Bovine and swine diseases due to *B. melitensis* and *B. suis*, respectively, are found in all Central American countries, and ovine and caprine brucellosis has been found in Guatemala.[908]Guatemala and Costa Rica have the highest disease prevalence, while El Salvador has the lowest prevalence. In South America, brucellosis is not endemic throughout the continent.*B. melitensis* is found predominantly in Peru and west Argentina.*B. abortus* is found in cattle populations in east Argentina and several other countries in South America. During the period from 1994 through 2006, *B. suis* emerged along with *B. melitensis* as the most frequent isolate in Argentina, with a concomitant decrease in the prevalence of *B. abortus*.[809]In Argentina, the estimated prevalence of caprine disease ranges from 20% to 25%, while the prevalence of bovine infection ranges between 11% and 13%.[1133] In Venezuela, the prevalence of brucellosis in cattle and buffalo herds is about 10%, and efforts are underway to control and eradicate the disease.[446]Although little data are available due to under-reporting, Brazil remains at risk for *B. abortus* as this country has the largest commercial cattle population in the world.

In the European Union and western Europe, many countries have successfully eradicated brucellosis, including Sweden, Norway, Denmark, Finland, Austria, the Netherlands, and Great Britain. Germany, Belgium, Luxembourg, and Switzerland are also considered *Brucella*-free countries.[1002]France has successfully eradicated brucellosis, and infections seen there are due to localized outbreaks associated with consumption of dairy products imported from Spain. Spain is considered an endemic area and has a high incidence of brucellosis, but regions within the country have noted substantial, consistent declines in the number of human infections beginning in 1997. Portugal borders the high endemicity regions of Spain, but the rest of Portugal has noted declines in disease since 1999. Disease incidence in Italy showed a steady decline over the past 30 years, but since 2003 this decline has not occurred uniformly throughout Italy. Few infections are reported from northern and central Italy, whereas most infections occur in southern Italy and in Sicily.[846]This disparity in Italy reflects the distribution of wealth and access to health care in northern versus southern regions of the country. Greece, Macedonia, and Albania remain regions of high endemicity, with the last two countries having the highest disease incidence in Europe.[1238]Prior to the 1990s, increases in disease prevalence in Greece were due to the illegal importation of infected animals or dairy products into Greece from Albania. In Greece, cattle herds are infected with *B. abortus* and *B. melitensis*.[1238]By 1995, after internal turmoil and civil wars that resulted in the formation of new countries, the disease was recognized to be highly endemic in Turkey, Croatia, and the

former Yugoslav Republic of Macedonia. The disease is found throughout the Balkan Peninsula, and increases in disease have been seen in Kosovo and Bosnia-Herzegovina.[963] Bulgaria was free of brucellosis from 1958 until 2005, when a reemergence of disease was documented.[1123]

In Asia, the Middle East is a highly endemic area; five of the ten countries with the highest incidence of human brucellosis are in this region. Ongoing wars and famines, lack of infrastructure for surveillance, and unregulated animal transportation across open borders in this part of the world contribute to the lack of disease control and the spread of both animal and human disease.[541] Syria has the highest annual incidence of the disease worldwide, and large numbers of patients becoming infected each year in Saudi Arabia, Turkey, Iran, Afghanistan, Oman, and the United Arab Emirates. In Kuwait, high disease incidence was seen prior to the 1990s due to widespread disease in cattle. During the 1991 Iraqi invasion, large die-offs of livestock resulted in substantial decreases in human infections, but this has not been maintained. Disease control in Jordan, Lebanon, and Israel is improving, although increased numbers of cases have been reported in Palestine in recent years. Among the republics of the former Soviet Union, seven (Kyrgyzstan, Tajikistan, Kazakhstan, Uzbekistan, Armenia, Georgia, and Turkmenistan) are among the top 25 countries with the highest incidence of the disease worldwide, and neighboring Mongolia is ranked second in disease incidence.[1317]Political and social upheaval in this region has resulted in loss of control over livestock populations and has precluded disease recognition and the implementation of interventions that would

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reestablish disease control. In China, the incidence of human brucellosis declined from 1964 through 1994 as a result of a large vaccination program. Since then, disease in humans and animals has increased, with *B. melitensis*, *B. abortus,* and *B. suis* prevailing in various provinces.[347] Even though the prevalence of brucellosis in China is increasing, a national surveillance network has been put in place to quarantine and eliminate infected animals and to vaccinate domesticated animals.[347] Disease incidence in South Korea is increasing as well. On the Indian subcontinent, brucellosis is an increasing and significant public health problem.[836]Residents, particularly in rural areas, live closely with domestic and wild animal populations, and the organisms are widespread among cattle, goats, sheep, pigs, and canines. Large outbreaks have occurred on dairy farms, leading to infected milk, abortion, and stillbirth; dairy personnel have high rates of infection as determined by serosurveys. Although B. *abortus* is found in India,

B. melitensis is the predominant species recovered from humans, goats, sheep, and milk samples.

Brucellosis is endemic in North Africa, and Algeria has the tenth highest annual incidence worldwide.*Brucella* exists throughout sub-Saharan Africa, but there is little data regarding its prevalence, and health networks, ongoing surveillance, and vaccination programs are nonexistent. Outbreaks of brucellosis in Uganda involving transmission from cattle to humans have been recognized, but proceed uncontrolled due to lack of resources. The high incidences of malaria, HIV, and tuberculosis eclipse the importance and impact of brucellosis on the African subcontinent.[844]Human brucellosis has been documented in Nigeria, Kenya, Ethiopia, Tanzania, Uganda, Cameroon, Burkina Faso, Mali, Namibia, Swaziland, Chad, and the Republic of the Congo. Little data are available regarding disease incidence in Egypt, Morocco, Tunisia, and Libya.

obstetrician who performed an emergency cesarean delivery on a woman in her 24th week of pregnancy.[884] A pediatric resident and a neonatologist who had provided care to the premature infant were also subsequently diagnosed with *Brucella* infection. The same organism was recovered from the mother and from her congenitally infected neonate. *Brucella* infections have been transmitted via blood transfusions, and bone marrow and kidney transplants from infected donors.[18,402,731,1408] Neonatal infection can be acquired by the transplacental route, during delivery or via the ingestion of contaminated breast milk.[997]

Brucella species also pose an occupational hazard to laboratory workers because of laboratory accidents, spills, or inappropriate manipulations of specimens or cultures containing *Brucella* organisms.[220] Infections have occurred from sniffing plated cultures, spilling blood culture bottles, mucocutaneous exposure to organism suspensions, aerosols generated from broken centrifuge tubes, and performance of culture work on the open benchtop.[425,1092] In 2000, seven laboratory-acquired infections caused by *B. melitensis* were reported at a medical center in southern Israel.[1389] This outbreak was caused by three different *B. melitensis* biovars, indicating multiple exposures, and the infections occurred over a 3-month period when 10% of 530 positive blood cultures grew *Brucella* species. On investigation, no sources or breaches in laboratory safety were found. Brucellosis was also diagnosed in 7 hospital employees (6 bacteriology technologists and a pathologist) over a 9-year period at a hospital in Riyadh, Saudi Arabia.[881] Infections were diagnosed by serology, along with compatible clinical signs and symptoms. Resulting morbidity included relapse in two patients and complications (septic phlebitis, infected prostheses, epididymo-orchitis, and lumbar spondylitis) in four others. Infections in the

technologists were traced to the handling of *Brucella* cultures. In a report from Italy, 12 laboratorians became infected after accidental breakage of a centrifuge tube.[425] Although the laboratories involved in these accidents used class II biologic safety cabinets for manipulations of specimens and cultures, the risk for laboratory-acquired infections is significant, particularly in laboratories located in endemic regions. Strict adherence to safety protocols and procedures in all laboratories and vigilance and care when handling specimens/cultures is an absolute requirement for prevention of laboratory-acquired infections of any kind. Biosafety level 3 (BSL-3) practices, containment equipment, and facilities are recommended for all manipulation of *Brucella* cultures.[1279]

Virulence of Brucella *Species*

Brucella species are facultative intracellular organisms, and their disease spectrum is partially explained by their ability to evade host defense mechanisms by taking up an intracellular existence. *Brucella* species have no classical virulence-associated genes and do not produce any classical virulence factors such as capsules, pili, fimbriae, exotoxins, cytolysins, or hemolyins.[447,1167] These organisms do not harbor plasmids or lysogenic bacteriophages, and do not exhibit genetic exchange mechanisms. *Brucella* species undergo antigenic variation or "dissociation"; the colonies switch from a "smooth" to a "rough" morphology, which results in loss of virulence and diminished reactivity with *Brucella*-specific antibodies. Antigenic variation results from decreased expression of genes encoding the glycosylation of the polysaccharide moieties of the cell wall LPS. Organisms that are in the smooth phase possess an S-LPS and are resistant to intracellular killing by macrophages, monocytes, and PMNs.[19] The LPS of *B. melitensis*, *B. abortus*, and *B. suis* contains two major antigenic determinants called A (for "Abortus") and M (for "Melitensis"). In addition to providing markers for biovar determinations, these molecules also have a role in organism virulence. Monoclonal antibodies against S-LPS are protective in animal models, and smooth isolates that have lost S-LPS have attenuated pathogenicity for mice. The S-LPS O (somatic antigen) chains from both smooth *B. melitensis* and smooth *B. abortus* strains are composed of polymers of 4, 6 dideoxy-4-formamido-D-mannose (i.e., *N*-formyl-D-perosamine). In *B. abortus* S-LPS, the O chain contains about 100 residues, nearly all of which are α-1,2 linked, with a small

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percentage being α-1,3-linked (A determinants). In *B. melitensis* O chains, α-1,2:α and α-1,3 linkages occur in a 4 to 1 ratio (M determinants). The serodominant A antigen tends to be rod-shaped, the shape being determined by the five consecutive α-1,2-

linked residues, while the serodominant M antigen is "kinked" in shape because the fourth residue is linked to the fifth by an α-1,3 linkage. The common expression of nonterminal α-1,2 linked *N*-formyl-D-perosamine is responsible for the cross-reactivity that exists between S-LPS of smooth *B. abortus* and smooth *B. melitensis* strains, and the cross-reactivity that is seen with other species (e.g., *Vibrio cholerae* 0:1, *Yersinia enterocolitica* O:9, *Escherichia coli* O:157, *Salmonella* O:30, and *Stenotrophomonas maltophilia*). Slide agglutination or enzyme immunoassays (EIAs) with polyclonal or monoclonal antibodies specific for A or M epitopes are used to determine A or M antigen predominance and the serovar designations of *Brucella* strains. Strains of *B. abortus*, *B. melitensis*, and *B. suis* can be A antigen-positive, M antigen-positive, or both A and M antigen-positive. A and M antigenic determinants are not found in *B. canis* or *B. ovis*; these species are characteristically rough, do not display phase variation, and have a restricted host range. The unique structure of the *Brucella* LPS renders this molecule several hundred times less toxic than LPS found in enteric gram-negative bacilli.[750]

Brucella species are transmitted to humans by three principal routes: direct contact with infected animal tissues, ingestion of contaminated meats or dairy products, and inhalation of aerosolized organisms. Once inside the host, the organisms are phagocytosed and begin their adaptation to the intracellular environment. Brucellae produce enzymes (e.g., superoxide dismutase, catalase, peroxidase) that neutralize reactive oxygen intermediates produced within the macrophage to inhibit bacterial multiplication.[478] The S-LPS also functions to inhibit the synthesis of immune mediators (i.e., complement, nitric acid, tumor necrosis factor) and alters the ability of the infected cell to process foreign antigens, thereby influencing the host immune response at the cellular level. S-LPS is also able to inhibit the programmed cell death (apoptosis) of infected cells.[517] The two-component quorum sensing system (*BvsR/BvrS*) of brucellae modulates the expression of genes encoding cell surface proteins that are involved in cell binding and intracellular survival. Specific periplasmic proteins termed *osmoregulated periplasmic glucans* (OPGs) interact with lipids in the host-cell membrane and function to interrupt the phagosome–lysosome fusion cycle.[55,804]Intracellular survival is also mediated in part by the *virB* locus, a 12-gene operon that encodes several proteins comprising the type IV secretion system, which is essential for virulence.[152] The *virB* operon proteins interact with the endoplasmic reticulum of the cell and internalized vesicles containing the bacteria, assisting in neutralizing the vesicular pH and further enabling intracellular bacterial replication.[153] Infected cells in the RES eventually degenerate and release the intracellular organisms. The bacteria, in turn, are phagocytosed by other macrophages and monocytes. The waxing and waning fever pattern seen in brucellosis is associated with the periodic release of bacteria and their components from phagocytic cells. The intermittent

bacteremia results in hematogenous seeding of other organs and tissues, leading to the protean clinical manifestations of human brucellosis.

The clinical spectrum of brucellosis depends on many factors, including the immune status of the host, the presence of other underlying diseases/conditions and the species, and virulence of the infecting organism. Relapses and recurrences of illness are controlled to some degree by a balance between the virulence of the organism and the presence of an intact, functional cellular immune response. As with other intracellular pathogens, humoral antibodies are produced, but cellular immune defense mechanisms are required to contain the intracellular bacteria. The greater virulence of *B. melitensis* and *B. suis* has been supported by in vivo studies with experimentally infected animals and by in vitro work examining phagocytosis, intracellular survival, and lymphocyte responses to the different species. Diseases due to *B. abortus* and *B. canis* are insidious in their onset, but tend to cause milder constitutional symptoms and less severe complications.

Because these organisms are infectious via the aerosol route, as evidenced by laboratory-acquired infections, *Brucella* species are one of several bacteria that are viewed as potential agents of biologic warfare and bioterrorism activity. Brucellae could be effectively delivered by aerosol dispersal and are able to survive in both soil and water for several weeks, thereby providing both immediate and delayed periods of infectivity. The United States investigated *B. suis* as a potential weapon during the 1940s and 1950s, and it is likely that other nations have made similar efforts. Reports of unusual clinical presentations, along with spurious serologic tests for *Brucella* antibodies, have prompted investigations involving the CDC and FBI regarding possible bioterrorist activity with *Brucella* species. Vaccination against brucellae is not available, as the existing *B. abortus* vaccine is licensed for veterinary use only. Post-event prophylactic therapy with doxycycline and rifampin has been effective in preventing disease in exposed individuals.

Clinical Spectrum of Brucellosis

Brucella infections may be difficult to diagnose because of the wide spectrum of clinical manifestations associated with them (Box 9-8).[837,1167] Following an incubation period of about 2 to 3 weeks (range, 1 week to 2–3 months), the onset of symptoms may be abrupt or insidious, developing over days to weeks. Fever, night sweats, chills, and malaise, often accompanied by severe headache, myalgias, and arthralgias, are the nonspecific symptoms seen in most infected patients. Most bacteremic individuals present with a febrile illness alone, or with both fever and arthritis. Lymphadenopathy, splenomegaly, and hepatomegaly may also be present. Cutaneous manifestations and vascular complications (e.g., erythema nodosum–like lesions, maculopapular or papulonodular rashes; deep vein thrombosis) may be noted in some patients. "Undulant fever" is synonymous with brucellosis (especially that caused by *B. melitensis*) because of the periodic fevers that occur over prolonged periods. Fevers occur mostly at night, with normal temperatures maintaining during the day, over 2 to 3 weeks. After this, days may ensue when the patient is afebrile and feels relatively well, only to experience another cycle of fevers. Symptoms wax and wane because of containment of the organisms in granulomas and subsequent release of organisms (or LPS) back into the circulation. Brucellosis assume characteristics of a debilitating,

BOX 9-8

Clinical Spectrum of Brucellosis

Infection	Comments
Osteoarticular infections	Bone and joint involvement (i.e., arthritis, bursitis, sacroiliitis, spondylitis, osteomyelitis) is the most commonly described complication of brucellosis, occurring in about 10% of patients. This is seen most often following bacteremia with *B. melitensis*.[15,1078,1400] Arthritis and sacroiliitis are associated with acute disease in pediatric patients, while spondylitis, vertebral osteomyelitis, osteitis, epidural, and paravertebral abscess are seen more frequently in chronic infections in older individuals and in those with underlying disease (e.g., HIV infection).[15,93,469,1400] Arthritis usually involves the hip and knees, but may also affect the smaller joints. Organisms have been isolated from joint fluids

Osteoarticular infections	processed by lysis-centrifugation.[1384] Patients with spondylitis usually present with fever, malaise, lower back pain, and difficulty walking.[1239,1400] Bone scans, computed tomography, magnetic resonance imaging, and newer methods (e.g., fluid-attenuated inversion recovery [FLAIR] modalities) are often helpful for detecting bone invasion, soft tissue lesions, and complications such as paraspinal/paravertebral abscess.[25,93,831] Brucellar osteomyelitis may also involve both joint and nonjoint prosthetic implants.[1249] In 2006, spinal osteomyelitis and bacteremia due to a marine mammal *Brucella* species were documented in a 43-year-old man in New Zealand.[873]
CNS infections	Neurobrucellosis occurs in less than 5% of patients and presents most commonly as a subacute or chronic meningoencephalitis, but it can present as encephalitis, meningitis, meningomyelitis, or cerebellar ataxia.[9,1248] The CSF will usually show pleocytosis, elevated protein, and low to normal glucose. Patients may appear acutely or chronically ill or may actually appear and feel relatively well (i.e., no fever or nuchal rigidity). Some infected patients may develop papilledema, visual symptoms, peripheral neuropathies, cranial nerve palsies, and abducens/optic nerve involvement.[681]Thrombosis of blood vessels may lead to cerebral/thalamic infarction and hemorrhage, encephalitis, myelitis, and peripheral neuropathy.[9,651]Epidural spinal abscesses, subdural empyema, paraspinal abscesses, intramedullary dermoid cysts, and brain abscesses are rare but do occur occasionally.[290,1020,1141,1186] Infection of the CNS resulting in colonization of a ventriculoperitoneal shunt and the subsequent development of *B. melitensis* peritonitis has also been documented.[35] Culture of brucellae from the CSF is negative more than 75% of the time, although blood cultures may be positive. Molecular methods have been useful for detecting brucellae in CSF specimens, and brucellar CNS disease can also be diagnosed by serologic testing of the CSF.[295,681] Treatment usually involves multiple antimicrobial agents, including aminoglycoside, doxycycline, and rifampin, and must be continued for 8–12 weeks.[9]Some patients have responded to third-generation cephalosporins (i.e., ceftriaxone) combined with doxycycline and rifampin.[681]
	Involvement of the respiratory tract is a rare event with *Brucella*

Respiratory tract infections	lungs or by direct inhalation of the organisms in aerosols.[1001]Clinical manifestations of pulmonary brucellosis include bronchitis, bronchopneumonia, lung abscess, pulmonary nodules, hilar lymphadenopathy, interstitial pneumonitis, empyema, and pleural effusions.[3] Patients present with headache, malaise, myalgia, and usually a nonproductive cough. Chest x-rays show typical lobar pneumonia in 32% and interstitial infiltrates in about 40% of patients, and about 10% of patients may have pleural effusions. While sputum specimens usually fail to grow the organism, it has been cultured from pleural effusion specimens.[1001]Diagnosis is usually made by serology unless other clinical manifestations are present that are more typical of brucellosis (e.g., osteoarticular disease).
Gastrointestinal/hepatobiliary tract infections	Gastrointestinal, hepatobiliary, and hepatosplenic involvement occurs as a manifestation of acute systemic infection in over 70% of patients with brucellosis. Symptoms include abdominal pain, nausea, vomiting, anorexia, and diarrhea or constipation. Infections of long-standing may result in more extensive gastrointestinal pathology, including colitis, enterocolitis, spontaneous bacterial peritonitis, pancreatitis, cholecystitis, splenic abscesses, and splenic infarcts.[294,344,619,743,896,1000,1003,1131]Liver involvement may be reflected only by elevations in liver enzymes, but is usually more extensive, particularly in *B. melitensis* and *B. suis* infections.[619] Infections with these two species are associated with the formation of caseating hepatic granulomas and microabscesses, while *B. abortus* tends to produce noncaseating granulomas in the liver. The management of suppurative complications of chronic hepatosplenic brucellosis requires combined surgical and medical approaches for optimal clinical response.[65,1398] *B. melitensis* peritonitis associated with chronic ambulatory peritoneal dialysis has also been described in several patients in Turkey.[21]Patients are usually treated with a 10- to 12-week course of doxycycline and rifampin and require catheter removal until cure is effected.
	Brucellae may infect the genitourinary tract, usually as a consequence of systemic infection, where they may cause epididymitis, prostatitis, orchitis, and renal granulomas.[79,212,670] While renal involvement is fairly uncommon, glomerulonephritis and pyelonephritis have been reported. Brucellae cause abortion in infected animals by localization in the chorioamniotic membranes of the placenta, but there is little evidence supporting a role for these

Genitourinary tract infections	the placenta, but there is little evidence supporting a role for these organisms in spontaneous abortion in humans.[984] However, the organisms may rarely be isolated from amniotic fluid and placental tissues of humans with brucellosis. Malone and colleagues described maternal brucellosis infection that resulted in preterm labor, chorioamnionitis, placental abruption, and delivery of the infant at 25 weeks' gestational age.[832] *B. abortus* was isolated from maternal blood and amniotic fluid. A 1998 case report details the first account of sexual transmission of *B. abortus*.[1250]

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Cardiovascular infections	Cardiovascular brucellosis is a rare complication, occurring in less than 2% of infected patients.[531,1075]Interestingly, *Brucella* endocarditis is the main cause of death related to this disease.[155,543] Endocarditis may occur with both native and prosthetic valves; aortic valve involvement occurs the most frequently.[11,155,531,623] Complications of *Brucella* endocarditis include septic embolization, mycotic aneurysms, myocarditis, pericarditis, and the necessity for surgical intervention for prosthetic valve placement or replacement.[11,531,623] *B. melitensis* infection of a pacemaker and associated leads has also been reported in a 45-year-old sheep shearer who had just undergone 45 days of therapy for treatment of brucellosis.[337] In 1999, Ying and colleagues described a case of serologically confirmed *B. canis* endocarditis that occurred in a 49-year-old man upon return from Kuwait.[1401]
Ocular infections	Ocular infections are unusual, late complications of *Brucella* infection. Uveitis is the most frequent ocular infection associated with brucellosis.[1102,1103] In a study of 12 patients with brucellar uveitis, culture and serology were performed on vitreous, aqueous, and subretinal humor specimens. Eight patients (66.7%) had positive vitreous fluid serologies for *Brucella* antibodies. Four patients (33%) had negative ocular serologies for *Brucella*, but were serum-positive.[1103]Ocular fluid from 11 patients was cultured, and only one patient had a positive subretinal fluid culture. Ocular *Brucella* infections also include optic neuritis, keratitis,

Miscellaneous infections	endophthalmitis, and infection of the lacrimal glands.[17,104,394] Optic neuritis is associated with loss of vision, fever, temporal headache, and retrobulbar pain. Endophthalmitis results from hematogenous dissemination, and positive cultures have been obtained from both aqueous and vitreous humors. *Brucella* infection transmitted by bone marrow transplantation was reported in an 8-year-old boy who received an allogeneic bone marrow transplant from his HLA-matched sibling.[402] Culture of the infused marrow was positive after 4 days, and the patient subsequently developed fever and had *B. abortus* isolated from blood cultures. The sibling presented on post-transplant day 32 with fever, hepatosplenomegaly, positive *Brucella* serologies (agglutination titer of 1:320), and positive blood cultures with *B. abortus*. Yousif and Nelson reported a patient who developed neurobrucellosis 13 years after receipt of a cadaveric kidney transplant.[1408] Cellulitis and soft tissue infections are uncommon manifestations of brucellosis. The tissues are probably seeded with the organisms during bacteremic episodes, leading to abscess formation. Gluteal, breast, psoas, splenic, and Bartholin's gland abscesses due to *Brucella* species have been reported recently.[123,398,527,1003,1016,1081] Treatment includes draining the abscesses and prolonged antimicrobial therapy. Mucocutaneous manifestations (erythematous papular lesions, purpura, Stevens–Johnson syndrome) can also be part of the clinical presentation.

chronic illness. Patients are often pan cultured to determine an etiology for a "fever of unknown origin." Acute and chronic brucellosis may lead to complications affecting several organ systems (Box 9-8). In immunocompromised hosts on chemotherapy, prolonged febrile neutropenia that fails to respond to broad-spectrum antimicrobial coverage is suggestive of *Brucella* infection, particularly in *Brucella*-endemic regions.

Hematologic abnormalities may be present because of chronic infection of the RES, including the lymph nodes, the bone marrow, and the spleen. Granulomas and abscesses form directly in these tissues or manifest as peripheral hematologic dyscrasias. Small, poorly defined noncaseating granulomas may be found in the bone marrow in about 70% of patients along with nonspecifically reactive histiocytes. Leukopenia, pancytopenia, microangiopathic hemolytic anemia, severe thrombocytopenia, and disseminated intravascular coagulation have all been observed in patients with brucellosis.[1172,1272,1405] Hematologic abnormalities may predominate

in early infection, masking the infectious etiology of the disease and mimicking primary hematologic diseases. Hematologic abnormalities are usually transient and normalize following successful antimicrobial therapy.

Serologic Diagnosis of Brucella *Infections*

Serologic tests are commonly used for the diagnosis of brucellosis. These assays detect antibodies against the O-side chain of the bacterial LPS. The serum agglutination test (SAT) is the most widely used and is the cornerstone of *Brucella* serology.[1370] SAT uses a standardized commercial *B. abortus* antigen (BD Biosciences, Sparks, MD), is performed as a tube dilution test, and detects agglutinating antibodies of both IgG and IgM classes.[61,1404] A standardized volume and concentration of a whole *Brucella abortus* cell suspension is reacted with a standardized volume of serum dilutions ranging from 1:20 to 1:1280. After incubation at 37°C for 24 hours, the agglutination of cells at the bottom of the tubes is inspected visually. The titer is the highest dilution of serum that results in agglutination of 50% of the bacterial cells.[26] Incorporation of 2-mercaptoethanol (0.05M) into the tube test inactivates IgM by disruption of the disulfide bonds of the molecule, providing an IgG-specific agglutinin titer.[191] Upon infection, IgM antibodies are produced during the first 7 to 10 days, followed by a decline in IgM and a switch to IgG production after the second week.[688] The sensitivity and specificity of the SAT depend on the background level of disease in the population and the cutoff value used to determine positive results. The test specificity and predictive value of a positive test are increased by selection of a higher cutoff value in areas of *Brucella* endemicity. However, by selecting a higher cutoff titer, the test sensitivity decreases, and persons with persistent or relapsing disease may be missed. Most patients with active brucellosis will have SAT titers of ≥1:160; these titers subsequently fall with adequate therapy. In areas of high endemicity, the use of a cutoff titer of 1:320 may increase test specificity. However, lower or higher titers can be detected in both active and asymptomatic disease.[812]

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Test sensitivity may be increased by evaluating paired sera (collected about 2 weeks apart) and looking for seroconversion or for a fourfold or greater increase in titer. The SAT using *B. abortus* antigen cannot be used to diagnose infections caused by *B. canis* or *B. ovis* because these organisms exist only in a rough colony form and do not have antigens that cross-react with other *Brucella* species.[64] For these two agents, major outer membrane protein antigens are used for serologic diagnosis. The SAT can also be performed as a microagglutination test in U- or V-bottomed microtiter wells.

The indirect Coombs' anti-human globulin test is used as a complement to the standard SAT and for the detection of blocking or nonagglutinating IgG antibodies that are not detected in the SAT.[26] Standard tube dilutions that are negative for agglutination of whole *B. abortus* or *B. melitensis* cells are centrifuged, and the bacterial cells are resuspended and washed several times. Anti-human IgG is then added to the resuspended cells, and the tubes are incubated at 37°C for 24 hours. Agglutination of the bacterial cells by the human antiglobulin is observed visually. The Coombs' antiglobulin test is useful for detection of chronic or relapsing disease as it detects agglutinating and nonagglutinating antibodies having both high and low affinity for the antigen.[204]

The Rose Bengal agglutination test is a rapid test that was originally designed to screen animal populations, but is also used as an adjunctive test for rapid diagnosis of brucellosis in humans. Positive results are generally confirmed by the SAT. The test is based on the agglutination of whole smooth-phase *B. abortus* cells that are stained with Rose Bengal dye and buffered at a low pH (3.65), which inhibits nonspecific agglutination of the bacterial cells. This test detects both agglutinating and nonagglutinating antibodies. In an evaluation of the Rose Bengal test using sera from 711 patients diagnosed with brucellosis and 270 controls, the overall sensitivity of the test was 92.9%.[1120] Specificity of the assay, however, was dependent on the characteristics of the group studied. Among those patients with no regular exposure to or history of brucellosis, the specificity was 94.3%, but among those patients infected with *Brucella* who had been treated within the previous year, the test specificity fell to 76.9%. In endemic regions, asymptomatic or self-limiting infections are known to occur, and anti-*Brucella* IgG can persist for months after the completion of therapy. Because of this low specificity and the consequent large numbers of false-positives, the use of the Rose Bengal test for screening in endemic areas is not recommended. Use of this test as the sole determinant of active infection would lead to unnecessary treatment with potentially toxic agents. In addition, the S-LPS shows cross-reactivity with other gram-negative bacteria, including *Y. enterocolitica*, *E. coli* O157, and *F. tularensis*, which may further increase the number of false-positive results and decrease test specificity.

A newer development in the serodiagnosis of brucellosis is the BrucellaCapt immunocapture agglutination assay (Vircell SL, Santa Fe, Granada, Spain).[61,204,205,979] This test uses microtiter wells that are coated with antibodies directed against human IgG and IgA. Patient serum is serially diluted and added to each well. After incubation, antigen (a formalinized colored suspension of *B. abortus* bacteria) is added and the plate is incubated for 24 hours. Positive tests show agglutination of the bacterial suspension over the bottom of the well. Negative tests are indicated by the formation of a condensed pellet of organisms at the bottom of the well. In a study of 321 serum samples from 48 patients with brucellosis (including 20

patients with focal disease and 8 patients with 9 relapses over the previous 18 months), the BrucellaCapt assay had a sensitivity of 100% for the diagnosis of initial infections.[204] In treated patients with good clinical outcomes, the titer decreases determined with the BrucellaCapt were pronounced and rapid. Following the rapid decline, 80% of patients remained positive at low titer for 12 months or more post-treatment; most of the slow decreases in titer were seen in patients with chronic or focal disease. In another evaluation, the sensitivity and specificity of the BrucellaCapt were more sensitive than the SAT (96% vs. 73%), but the specificity of the BrucellaCapt was 97.5% compared with the 100% specificity observed with SAT.[205] In patients with relapsing disease, BrucellaCapt titers remained high (titer ≥640) even though the SAT may be high or low.[979] Results with BrucellaCapt are also influenced by the prevalence of disease in the population being studied. An evaluation of the BrucellaCapt in an endemic region in Spain had a sensitivity of 98% in culture-confirmed case using a cutoff of 1:80 and a specificity of 96% when specimens from healthy individuals were used. However, the specificity fell to 63% at the 1:80 cutoff when specimens from individuals living in the endemic region and diagnosed with other conditions were tested.[979] In this situation, raising the cutoff titer increases test specificity, but decreases test sensitivity. The BrucellaCapt detects primarily high-affinity antibodies and is more specific than the Coombs' test, but in patients with chronic or relapsing disease, the small changes that occur in low-affinity antibodies are still best detected by the Coombs' antiglobulin test.[204]

EIAs have also been developed for serologic diagnosis of brucellosis, and these methods are the tests of choice for complicated and chronic infections.[51] EIAs for detecting IgM, IgG, and IgA antibodies against *Brucella* have been described that use *B. abortus* whole cells, smooth LPS, protein extracts, and *B. melitensis* S-LPS as the solid-phase antigen.[61,64,841,985] Ariza and coworkers compared SAT with EIA methods for detection of *Brucella*-specific IgG, IgM, and IgA in 761 sera obtained from 75 patients with brucellosis. The EIA method in this study incorporated *B. abortus* S-LPS as the antigen. EIA was sensitive and more specific than standard serologic tests for brucellosis. Initial IgM titers were higher in patients who presented to their physician earlier in the clinical course, while patients who had been ill for some time tended to have high IgG titers and lower IgM titers. Upon adequate antimicrobial therapy, the serum IgG titers decreased four- to eightfold over the subsequent 3 to 6 months. Subsequent increases in EIA IgG and IgA titers were seen in patients with relapsing disease. The persistence of high levels of IgG or a slower decline in titers following treatment in patients without relapse was associated with the presence of focal infection. Antibrucellar IgM and IgG may also be present during bacteremic periods.[985] In 1993, Goldbaum and coworkers in Argentina characterized an 18-kDa cytoplasmic protein that was present in all smooth and rough *Brucella* species

examined.[493]Sera from patients with active brucella infections reacted with this antigen in an EIA-based assay, while patients who did not have brucellosis or who had inactive disease were negative with this test. This EIA could also differentiate healthy vaccinated cattle from vaccinated cattle

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that had been infected with a wild-type, disease-producing strain. This antigen has been purified by affinity chromatography, and the gene for the antigen has been cloned in *E. coli*, with retention of reactivity of the recombinant molecule with animal and human sera containing anti-*Brucella* antibodies. This molecule has been characterized as a lumazine synthase, an enzyme involved in riboflavin biosynthesis.[494] A commercially available EIA for detection of IgG and IgM (PANBIO, Windsor, Brisbane, Australia) was compared with standard SAT and Coombs' test for the diagnosis of brucellosis in Beirut, Lebanon. The sensitivities of the *Brucella* EIA IgM and IgG assays were 100% and 91%, respectively, while the specificity was 100% for both EIAs.[52] Recently, two lateral flow EIAs for detection of anti-*Brucella* IgM and IgG, respectively, have been evaluated for rapid diagnosis of brucellosis in endemic regions.[552,627]In one study, these lateral flow assays were positive in 91% and 97% of sera from adults and children with brucellosis, respectively, and the agreement of these assays with SAT was 92% using an SAT cutoff titer of ≥1:160.[627]

Al-Shamahy and Wright reported on an antigen-detection EIA assay for brucellosis.[23] The test used monoclonal antibodies against the *Brucella* LPS and was performed on serum specimens. As few as 100 *Brucella* organisms or 10^5 μg/mL LPS could be detected. This assay was challenged with 1,607 sera from random blood donors, 146 patients with brucellosis, 20 patients at risk for *Brucella* infection, and 264 sera from patients with infections other than brucellosis. The sensitivity of the assay was 100% compared with positive blood cultures; the specificity of the assay was 99.5% among random blood donors and 99.2% for patients. These data indicated that antigen detection using this assay was an acceptable alternative to blood culture for the diagnosis of *Brucella* infection. Rapid LA tests have also been designed for the serologic diagnosis of brucellosis. Abdoel and Smits developed and evaluated a LA assay using latex beads sensitized with *Brucella* LPS.[2] The test was evaluated with 45 serum specimens from patients with culture-confirmed brucellosis, 90 samples from culture-negative patients diagnosed with brucella infection, and 281 serum samples from patients with illnesses other than brucellosis. The sensitivity and specificity of the test for detection of anti-*Brucella* antibodies were 89.1% and 98.2%, respectively.

Isolation and Cultural Characteristics

Because of the risk of laboratory-acquired *Brucella* infections, all work with specimens suspected of harboring *Brucella* and all manipulations of *Brucella* cultures should be performed in a class II biologic safety cabinet using precautions for BSL-3 organisms. Procedures that generate aerosols (aspiration of liquid with syringes, using a "vortex" mixer, vigorous bulb pipetting, etc.) should be kept to a minimum. To comply with these guidelines, close communication among the laboratory director, the technologists, and the physicians caring for patients with "possible" brucellosis is necessary. The clinician should alert the clinical laboratory personnel when a diagnosis of brucellosis is being considered so that the laboratory staff can take the necessary safety precautions.

Since *Brucella* species infect the RES, the specimens of choice for recovery of organisms in suspected cases primarily include blood and bone marrow. In a study of 50 patients eventually diagnosed with brucellosis, cultures of blood and bone marrow were positive in 70% and 92% of the patients, respectively.[507] Conversely, a study of 106 cases of brucellosis in Amman, Jordan, found that blood cultures were positive in 44.4% of patients, while bone marrow culture was positive in only 27.7% of patients.[1179] Rates of positive cultures from these specimens are dependent on presentations and clinical conditions of individual patients (i.e., subacute, acute, or chronic disease) and previous antimicrobial chemotherapy. In a study performed in Turkey, blood and bone marrow cultures were collected from 102 patients: 61 had acute infection, 29 had subacute infection, and 12 presented with chronic disease.[628] Blood and bone marrow specimens were cultured using the BACTEC 9050 system. The overall rate of positive blood cultures was 48%, while the positivity rate for bone marrow cultures was 34%. The time to detection in the BACTEC system was 4.2 days for bone marrow specimens and 5.8 days for blood specimens. Among the patients with acute disease, blood and bone marrow cultures were positive in 66% and 46% of the patients, respectively. Among those 28 patients with acute disease and positive bone marrow cultures, 23 had positive blood cultures and 5 had negative blood cultures. Of the 29 patients presenting with subacute illness, blood and bone marrow cultures were positive in 31% and 21%, respectively. Interestingly, among the 12 patients with chronic brucellosis, none had positive blood cultures and only 1 patient had a positive bone marrow culture.[628] Another Turkish study reported that among 30 patients with acute brucellosis, 83.3% had positive bone marrow cultures and 66.6% had positive blood cultures. For 17 patients with subacute disease, bone marrow and blood cultures were positive in 52% and 23.5%, respectively; 1 of 3 patients with chronic disease had a positive bone marrow culture and none of 3 had positive blood cultures for *Brucella.*[993] *Brucella* can also be recovered from pus, tissue, CSF, pleural fluid, joint fluid, and ascites fluid specimens and should also be inoculated into blood culture bottles.[1386]

For isolation of *Brucella* species, blood and bone marrow specimens are inoculated

into aerobic and anaerobic bottles, and because of their slow growth rates, conventional blood cultures should be incubated at 35°C for 4 to 6 weeks with blind subcultures to chocolate and blood agar. Continuously monitored blood culture instruments usually detect positive blood/bone marrow cultures with *Brucella* species after 5 to 7 days of incubation. Yagupsky evaluated the ability of the BACTEC 9240 instrument to detect growth of *Brucella* species and found that 21 (78.8%) of 27 positive cultures were detected by the 9240 instrument within 7 days; the remainder were detected by blind subculture after 2 to 3 weeks of incubation.[1388] Ozturk et al.[995]evaluated the BACTEC 9240 system with blood and bone marrow cultures from 23 patients with brucellosis diagnosed by serology. Blood and bone marrow cultures were positive for 19 (82.6%) and 13 (81.2%) of the patients, respectively. All blood cultures were positive within 7 days, and bone marrow cultures were positive within 4 days.[995] An evaluation of the BACTEC 9120 continuously monitored system found that all of 20 *B. melitensis* were flagged with a mean detection time of 63.87 hours and that 13 (65%) of the 20 isolates were flagged as positive by the BACTEC 9120 within the first 72 hours.[380] Recovery of *Brucella* may also be influenced by the type of

blood culture media used. BACTEC MYCO/F LYTIC medium was formulated to enhance detection of intracellular bacteria by lysis of the blood cells in the bottle. In a side-by-side comparison of the BACTEC MYCO/F lytic medium and the regular BACTEC Peds Plus/F aerobic blood culture bottle, both media had similar sensitivity in detecting positive cultures, but the time to detection was significantly longer with the MYCO/F lytic medium (104 +/− 46.7 hours) than with the Peds Plus/F medium (65.5 +/− 19.9 hours).[1379] Using the BacT/ALERT continuously monitored blood culture system (bioMérieux, Inc., Durham, NC), Solomon and Jackson detected *B. melitensis* in the blood culture of a patient in 2.8 days, and these investigators used seeded blood cultures to show a mean detection time of 48 hours +/− 1 hour in bottles inoculated with 100 cfu of *B. melitensis* per mL.[1209] Ozturk et al.[993] evaluated the BacT/ALERT (bioMérieux, Inc., Durham, NC) continuously monitored blood culture instrument (using a 7-day incubation period) in comparison with conventional blood culture in *Brucella* broth for 4 weeks, with blind subculture every 48 hours. Of 59 positive cultures detected in 100 specimens (50 bone marrow specimens and 50 blood specimens), all 59 were detected with the *Brucella* broth culture, while the BacT/ALERT detected only 30 (50.8%). These investigators suggested that positive cultures were missed by the BacT/ALERT because of the low inoculum, the short (7-day) incubation period, possible toxicity of SPS, and the slow production of CO_2 by

the organism, which is the metabolic product detected by the BacT/ALERT system.[993] A side-by-side comparison of the BACTEC 9240 and the BacT/ALERT reported that all of 17 *Brucella* isolates were recovered with both blood culture systems; the mean time to detection was 2.5 days for the BacT/ALERT and 2.8 days for the BACTEC 9240.[100] The BacT/ALERT standard aerobic, BacT/ALERT FAN, and BacT/ALERT-enhanced aerobic bottles also support rapid growth of brucellae as evidenced by simulated blood culture studies.[1229]

Rapid recovery of *Brucella* species from blood cultures has also been accomplished with the Isolator lysis-centrifugation method.[839,1384,1387,1388] Recovery of *Brucella* using this method takes 2 to 4 days and yields a semiquantitative assessment of the level of bacteremia. The sensitivity of lysis-centrifugation exceeds 90% for acute disease and is about 70% for chronic disease.[380,939]

Continuously monitored blood culture systems have also been evaluated for detection of brucellae in other sterile body fluids. In a study of 1,072 synovial fluids from patients with arthritis, specimens were processed by the Isolator 1.5 Microbial Tube and inoculated into BACTEC 9240 blood culture media. Fifteen specimens were positive for *B. melitensis* by lysis-centrifugation, and 14 of these positives were also detected by the BACTEC 9240 within 3 to 7 days.[1386] The single positive culture missed by the BACTEC instrument had 1.3 cfu of organisms per mL as determined with the the Isolator 1.5 Microbial tube (Wampole Laboratories, Cranbury NJ). Akcam and colleagues compared conventional culture of various body fluid with inoculation of aerobic BACTEC blood culture bottles and recovered five isolates of *B. melitensis* from the BACTEC-processed specimens only.[8] Cetin and colleagues compared results obtained by conventional culture of sterile body fluids with inoculation of BACTEC Peds Plus bottles and recovered two isolates of *Brucella* species from synovial fluid and pleural fluid by the BACTEC method only.[225]

Identification of Brucella *Species*

A presumptive identification of a "possible *Brucella* species" can be made when a slow-growing, faintly staining, minute coccobacillus is recovered from blood or bone marrow cultures of a "compatible" patient, that is, one with a history of possible occupational exposure, travel to an endemic area, or ingestion of uncooked meats or unpasteurized dairy products. *Brucella* species grow slowly on blood and chocolate agars, forming visible colonies in 3 to 5 days (Image Plate 9-5D). *Brucella* species do not grow on MacConkey, EMB, or other "enteric" selective or differential media. Good growth will also be obtained on buffered charcoal yeast extract (BCYE) agar used for isolation of *Legionella* species.[1056]

All *Brucella* species are aerobic and require oxygen for recovery from clinical

specimens. Although production of acid from carbohydrates may be demonstrated under certain conditions, their metabolism is largely oxidative. Strains of *Brucella* are positive for both oxidase and catalase; most brucellae reduce nitrate to nitrite, and some may also reduce nitrite to nitrogen gas. All species are indole- and VP-negative. Conventional identification methods for *Brucella* species include the requirement of CO_2 for growth, production of urease and H_2S (at 4 days, using lead acetate strips), and sensitivity to the dyes basic fuchsin (1:50,000 and 1:100,000), thionin (1:25,000, 1:50,000, and 1:100,000), and thionin blue. Dye sensitivity is used not only to aid in identifying the species, but, in the case of *B. abortus* and *B. suis*, is also helpful for determining the biovar of the organism. *B. abortus* and *B. suis* are subdivided into biovars on the basis of biochemical and serologic differences, while the biovars (actually serovars) of *B. melitensis* are defined solely on the basis of serologic differences, since they are classically resistant to basic fuchsin, thionin, and thionin blue. The recognition of serologically confirmed strains of *B. melitensis* that are thionin sensitive suggests that the conventional identification scheme needs to be modified and that new, less cumbersome identification methods are needed.[305] *B. melitensis*, *B. suis*, and *B. abortus* produce urease; with a heavy inoculum, *B. suis* strains usually will be urease-positive on Christensen's urea medium within 5 minutes. Identification of *Brucella* species has also been accomplished by COA and colony dot-blot using a genus-specific monoclonal antibody that detects the A or M antigens of *B. melitensis*, *B. abortus*, and *B. suis*.[1306] Phenotypic characteristics that are useful for identification of *Brucella* species are shown in Table 9-12.

Brucella species are not included in the data bases of any of the commercially available kit systems for identifying gram-negative organisms except for the Vitek 2 Gram-Negative (GN) Identification card, which includes *B. melitensis* in the database. Inadvertent use of these kits may delay diagnosis and treatment. *Brucella* organisms have been misidentified as *Moraxella phenylpyruvica* by the API 20NE nonenteric identification system, as *Moraxella* species by the MicroScan Negative COMBO type 5 system (Siemens Healthcare, Deerfield IL), and as *Haemophilus influenzae* biotype IV by the HNID panel (Siemens Healthcare).[90,97] In one instance, the technologist who inoculated an API 20NE

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TABLE 9-12 Phenotypic Characteristics for the Identification of *Brucella* Species

Species	Fuchsin	Thionin	Safranin Inhibition	H2S Production	Urease	CO_2 Growth	Tiblisi Phage Lysis	Weybridge Phage Lysis	Biotypes	Host reservoir
B. melitensis	+	+	–	–	+, 24 h	–	–	–	1–3	Goats, sheep, camels
B. abortus	+[a]	–[b]	–	+[c]	+, 24 h	+[d]	+	+	1–6, 9	Cows, camels, yaks, buffalo
B. suis	–[e]	+	+	+[f]	+, 15 min	–	–	+	1–5	Pigs (biotypes 1–3), wild hares (biotype 2), caribou (biotype 4), reindeer (biotype 4), wild rodents (biotype 5)
B. canis	+/–	+	–	–	+, 15 min	–	–	–	–	Canines
B. ovis	V		–	–	–	+	–	–	–	Sheep
B. neotomae	–		–	+	+ 15 min	–	V	+	–	Rodents
B. pinnipedialis	+	+	–	–	+	–	–	+	–	Mink whales, dolphins, porpoises
B. cetaceae	+	+	–	–	+	+	–	–	–	Seals

+, positive reaction, –, negative reaction; V, variable reaction; h, hours; min, minutes
[a] Except biotype 2; [b] Biotypes 1,2 and 4; [c] Except biotype 5; [d] Biotype s 1–4; [e] Except biotype 3; [f] Biotype 1.

strip with a *B. melitensis* isolate subsequently developed brucellosis.[97] The RapID NF Plus system (Remel, Lenexa, KS) for identification of nonfermentative gram-negative bacteria misidentified a gram-negative isolate from two sets of blood cultures of the same patient as *Ochrobactrum anthropi*.[608] On the third admission of this patient to the hospital for surgical repair of a femoral bone fracture, pus from the hip socket grew the same short gram-negative rod that was recovered from the previous blood cultures. The Vitek 2 GN Identification card identified the organisms as *B. melitensis*, with a 97% likelihood, and subsequent ribosomal RNA sequencing and serotyping at the CDC confirmed all the isolates as *B. suis*. A study performed in Macedonia reported recovery of 16 presumptive *Brucella* species from blood cultures that were identified as *B. melitensis* by the Vitek 2 card within 8 hours; differences in substrate profiles among the isolates suggested different strains.[208]

Other novel test systems have been evaluated for their ability to identify *Brucella* species. Preliminary studies on the identification of *Brucella* species with the Biolog carbon substrate utilization identification system indicated that all *Brucella* species oxidized three of the 95 substrates on the panel, and that *B. melitensis*, *B. abortus*, and *B. suis* could be differentiated from one another by differential oxidation of seven additional substrates.[1365] Using the results of seven tests on the biotype 100 (bioMérieux, Marcy-l'Étoile, France), a manually inoculated carbon substrate assimilation gallery, Lopez-Merino et al.[805] were able to identify 85.6% of 92 *Brucella* strains; specificity varied from 97.4% to 100%, depending on the species. These workers emphasized that the biotype 100 must be inoculated in a biologic safety cabinet. Al Dahouk and coworkers evaluated the Taxa Profile (Merlin Diagnostics, Bornheim-Hersel, Germany), a commercial biotyping system, for its potential use in identifying *Brucella* species.[14] This test utilizes three microtiter plates coated with

various substrates. Plate A detects utilization of 191 amines, amides, amino acids, organic acids, and heterocyclic aromatic compounds; Plate C contains 191 different mono-, di-, and tri-polysaccharides and sugars; and Plate E contains 188 substrates that detect various peptidases, proteases, glycosidases, and esterases. The plates were inoculated with 23 reference strains representing all *Brucella* species, and 60 field isolates. After analysis of results with the three plates (570 substrates), a battery of 96 tests were selected for *Brucella* identification and typing (Micronaut), and this single plate was challenged with 113 *Brucella* strains. These organisms showed characteristic profiles and allowed identification of isolates to the species level, although biovar determinations could not be resolved. Finally, Ferreira and colleagues evaluated MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry (Bruker Daltonics, Leipzig, Germany) for its ability to identify brucellae.[420] After creating MALDI Biotyper profiles using type strains of *Brucella* species, the system was challenged with 131 clinical isolates, and blood cultures seeded with these organisms were also tested. All strains, either from agar plates or seeded blood cultures, were identified to the genus level by MALDI-TOF, with lesser correlation for species-level identifications.

The isolation and identification of *Brucella* species are time-consuming, requires considerable microbiologic skill, and must be performed in BSL-3 facilities. Because of the economic impact of brucellosis on livestock and wildlife, several molecular methods have been developed for the identification and biotyping of *Brucella* species isolated from animals or animal products (e.g., milk), including conventional PCR methods, multiplex PCR assays, and "real-time" PCR approaches. Earlier molecular approaches were directed at the detection of these organisms in animals and animal products (e.g., milk specimens), and subsequently assays were developed for detection of organisms in clinical specimens. The challenge for molecular assays for the detection of the genus *Brucella* is that the various species have greater than 90% homology with one another. A variety of gene targets have been used to design primers and probes for genus-, species-, and biotype/biovar-level identifications. Targets for these assays include the16S rRNA, 16S-23S internal transcribed spacer (ITS) region, IS*711*, outer membrane protein genes (*omp2, omp2a*, *omp2b*, *omp31*), *bcsp31*, and *per* genes.[938,1067,1087,1106]

In 1994, Bricker and Halling at the U.S. Department of Agriculture developed a multiplex PCR that was able to identify and differentiate several *Brucella* species and biovars, including *B. abortus* biovars 1, 2, and 4, all three biovars of *B. melitensis*, *B. suis* biovar 1, and *B. ovis.*[177] This assay exploited the presence of IS*711,* an insertion sequence that is unique to *Brucella* species, and is found at species-specific and biovar-specific regions of the chromosome. The species and biovars identified by this assay constitute most of those seen in the United States in animals and in human infections. Conventional identification and typing of field isolates correlated 100% with

the results of the multiplex assay. These workers subsequently augmented their PCR assay with additional primers to allow rapid discrimination between pathogenic strains and the *B. abortus* vaccine strains (S19 and RB51) in previously vaccinated livestock and to detect additional livestock-related isolates.[176,178] This assay is called the AMOS PCR because it detects *Brucella* "**A**bortus, **M**elitensis, **O**vis, and **S**uis," respectively. Redkar and colleagues subsequently designed AMOS "real-time" PCR tests using upstream primers derived from the 3′ end of the IS*711* insertion element, and downstream primers and probes from unique sequences specific to individual *Brucella* species and biovars.[1067] The assay used two adjacent fluorescence resonance energy transfer (FRET) probes that hybridized to the amplicons as they were formed. The assay took 30 minutes and identified all biovars of *B. abortus*, *B. melitensis*, and *B. suis* biovar 1. Ewalt and Bricker described an abbreviated version of the *Brucella* AMOS PCR for more rapid screening in the field.[407] Conventional multiplex PCR assays have also been designed for identification of specific biovars of *Brucella abortus* (i.e., biovars 5, 6, 9, 3, and subgroup 3b) recovered from livestock in Spain and for identification and typing of isolates of *B. suis* causing porcine brucellosis in Spain.[419,965] Rees and colleagues described a single-tube PCR assay that used variable-number tandem repeat (VNTR) analysis for simultaneous identification and strain typing of *B. melitensis*, *B. abortus*, and "*Brucella* species" involved in epidemiologically linked infections in families who became infected in Mexico and subsequently developed disease in California.[1069] Mitka and colleagues evaluated four different PCR assays, each of which used different primer pairs and gene targets (including *bcsp31*, *omp2, omp28*, and *bp26)* using serum, buffy coat,

or whole blood specimens, and all four methods were greater than 95.5% sensitive and 100% specific.[899]

Several other multiplex PCR assays have been designed that identify all described *Brucella* species.[616,803,864] In 2008, a multiplex PCR identification typing system was designed and evaluated with isolates in seven international laboratories on five continents.[803] This test, called the Bruce-ladder, used primers selected to detect species- and/or strain-specific sequences. *Brucella* strains (n = 625) tested were from different geographic locations worldwide and included animal and human clinical isolates. Identification with Bruce-ladder was based on the numbers and sizes of seven PCR amplicons analyzed by electrophoresis in 1.5% agarose gels. Bruce-ladder identifications were species-specific, and all strains and biovars from the same *Brucella* species gave the same amplicon profile. The Bruce-ladder assay was also able to identify *B. abortus* S19,*B. abortus* RB51, and *B. melitensis* Rev. 1 vaccine

strains. The Bruce-ladder assay was also able to identify species and biovars not identified by AMOS PCR, including *B. canis*, *B. neotomae*, *B. pinnipedialis*, *B. ceti*, *B. abortus* biovars 3, 5, 6, 7, and 9, and *B. suis* biovars 2, 3, 4, and 5. The Bruce-ladder was specific for *Brucella* species and DNA from 30 other related strains failed to amplify with the primers for *Brucella*-specific sequences. This method was reproducible in all seven laboratories and could be performed with several different DNA extraction methods and commercial thermocyclers. The assay could be completed in less than 24 hours and performed with whole-cell bacterial lysates. In 2010, a similar multiplex PCR assay was described that used eight primer pairs previously incorporated in the multiplex assay, but added primers specific for *B. microti*.[864] *B. ceti* and B. *pinnipedialis* could be distinguished on gels by a size difference of a 794 base pair amplicon, and the assay also demonstrated a unique amplicon pattern for *B. inopinata*, which produced a polyacrylamide gel pattern similar to *B. abortus*, but with an additional 272 base pair fragment.

Some multiplex PCR assays for *Brucella* identification and typing make use of a technique called single nucleotide polymorphism or SNP analysis. This technique identifies single nucleotide differences between and/or among species. This technique has diverse applications in clinical microbiology, and has been used for identification and typing of *M. tuberculosis*, differentiation of *Burkholderia* species groups, and differentiation of vaccine- and wild-type strains of varicella zoster virus, among others. Scott and colleagues used this technology to develop a multiplex assay in which sequence polymorphisms were identified in *glk*, *trpE*, and *omp25* genes that were specific for the individual species or marine mammal species groups.[1164] Amplification and sequencing of amplicons containing discrete polymorphisms provided unambiguous identifications as one of the six classical *Brucella* species or as one of the marine mammal-associated species. Several other research groups subsequently used this technology to design real-time PCR assays based on SNP technology that enabled identification of *Brucella* species and biovar typing.[453,502] The real-time SNP assay reported by Koylass and colleagues was compared with the "Bruce-ladder" assay described above and found that the SNP assay was more accurate for differentiating *B. canis* and *B. suis* isolates than the "Bruce-ladder" assay.[733]

Several real-time PCR methods for identification of *Brucella* have been developed and published.[439,687,1053,1067] The principal advantages of these approaches include the rapidity of test results and the avoidance of amplicon contamination. FRET probe, hydrolysis probe (i.e., TaqMan), and SYBR Green I detection methods have been used, and amplification targets have included IS*711*, *bcsp31*, *omp*, and *per* genes, and the 16S-23S ITS region.[156,687,1053] Redkar and colleagues described three separate real-time assays specific for identification of *B. abortus* (7 biovars), *B. melitensis* (3 biovars), and *B. suis*. Forward primers for the assay were derived from the IS*711* insertion element, while the reverse primers and FRET probes were selected from

species-specific or biovar-specific chromosomal sequences.[1053] Winchell et al.[1356] designed a real-time PCR using seven different primer sets, including a primer (Bspp) that specifically identified organisms in the genus *Brucella*, and six primer sets that enabled detection of all *Brucella* species, with the exception of *B. inopinatus*. Following amplification, species identification was accomplished by high-resolution melt (HRM)-curve analysis, with each species having a different melting profile. Subsequent testing of strain BO1 (*B. inopinatus*) and BO2 confirmed that these isolates are *Brucella* species (i.e., positive with the Bspp primer), that they are distinct from other brucellae, and that BO2 may actually be a different species than *B. inopinatus* (BO1).

Real-time PCR assays have also been designed for detection of brucellae in a variety of human clinical specimens, including blood cultures, whole blood, and serum. Kattar and colleagues developed and evaluated three real-time PCR assays with LightCycler hybridization probe detection of the 16S-23S ITS, the *omp25* gene, or the *omp31* gene, respectively.[687] Each assay was 100% sensitive and specific, with the ITS target having the lowest limit of detection. The ITS assay was challenged with 340 whole blood specimens (24 from brucellosis patients, 31 with treated brucellosis, and 299 SAT-seronegative patients). Clinical sensitive and specificity and positive and negative predictive values were 66.7%, 99.7%, 94.1%, and 97.6%, respectively. A real-time assay using SYBR Green I and LightCycler (Roche Diagnostics, Mannheim, Germany) methodology was described by Quiepo-Ortuno and coworkers for detection of *Brucella* species in human serum samples.[1052,1053] This assay amplified a 223 base pair region of the *bcsp31* immunogenetic membrane protein, and specificity of the assay was determined by melting-curve analysis and sequencing to verify specificity of the PCR-derived products. After optimization of assay conditions, the test was challenged with 62 serum samples from patients with active brucellosis and 65 negative control sera. This assay was 91.9% sensitive and 95.4% specific for diagnosis of active brucellosis, was reproducible, and took less than 2 hours to perform. This assay was subsequently modified for quantitative detection of *Brucella* species in serum samples, and compared with blood culture results, SAT results, and BrucellaCapt results for 46 patients with brucellosis and 64 control patients (including 36 patients with a history of treated brucellosis, 17 asymptomatic patients at risk for brucellosis, and 11 patients with other conditions).[1052] Forty-four (95.7%) of patients with brucellosis were PCR-positive. Five control patients with positive PCRs included four who had just completed treatment for brucellosis and a fifth who was a veterinary surgeon working in a *Brucella* control/

eradication program. The sensitivity and specificity of the assay were 95.7% and

92.2%, respectively, and the predictive values of positive and negative PCR results were 89.8% and 96.7%, respectively. Corresponding values for blood cultures were 69.6%, 100%, 100%, and 78.8%, respectively.

Molecular methods can also be used not only for identification of of isolates, but also for strain typing of *Brucella* isolates. Since the entire genomes of *B. melitensis* and *B. abortus* have been sequenced, regions of DNA have been found that are useful as unique markers for strain typing. In the genomes of these organisms, regions of DNA have been found that possess a variable number of tandem sequence repeats (VNTRs) that range from 1 to 10 base pairs per repeat and from 1 to more than 30 repeats per locus. The locations and numbers of these VNTRs within the published genome sequences are known. One of the first studies using this technology identified an 8 base pair, tandem repeat sequence in nine loci of *B. abortus*, and these loci were hypervariable among various strains of *B. abortus*. Using PCR, the number of repeat sequences per locus generated strain-specific "fingerprints" that were able to identify *Brucella* species type strains and to differentiate unrelated *B. abortus* isolates recovered from cattle and other animals.[174] LeFlech and colleagues used the VNTR technology to establish a 15-marker, two-panel PCR assay for *Brucella* species.[774] The first panel used eight markers to establish a species identification along classical species lines, while the second panel used seven markers and was able to differentiate among isolates of the same biovar. Whatmore and associate used the same technology and identified 21 VNTRs, ranging from 5 to 40 base pairs in length. Six loci were sufficient to determine species identification, while the remaining 15 could discriminate among isolates of the same species/biovar isolated from different geographical locations.[1345] VNTR analysis was rapid, reproducible, and applicable for epidemiologic investigations involving *Brucella* transmission and for determining relationships between and among *Brucella* isolates from various parts of the world.

Treatment of Brucellosis

The treatment of human brucellosis is an evolving area because of the spectrum of disease, the possibility/likelihood of chronic infection, and the propensity for multiorgan system complications.[837,1206] Despite in vitro susceptibility to many antimicrobial agents, in vitro results do not necessarily translate to clinical efficacies and outcomes in patients. Successful treatment requires prolonged antimicrobial therapy, usually with a combination of agents, and in some cases, surgical intervention may also be indicated. The administration of agents that penetrate and have activity within phagocytic cells is prerequisites for therapy, since brucellae are facultative intracellular pathogens, and therapy needs to be prolonged. Short-course therapy is not effective since most patients will relapse.[1208] Current therapy is a combination of doxycycline (200 mg/day PO, for 6 weeks) plus gentamicin (5 mg/kg/day, IM for 7

days).[1115,1207] The treatment recommended by the WHO includes oral doxycycline (200 mg/day) plus rifampin (600 to 900 mg PO) for a minimum of 6 weeks. The regimen of oral tetracycline (2 g/day for 6 weeks) or doxycycline combined with streptomycin or gentamicin (1 g/day, IM for 2 to 3 weeks) is associated with fewer relapses than the WHO recommendations.[63,838] Treatment of brucellosis with cephalosporin monotherapy, such as ceftriaxone, has resulted in failures and cannot be recommended.[748] The fluoroquinolones generally show good activity against *Brucella* species in vitro, even though the MICs for these agents tend to be higher than for other gram-negative bacilli.[697] Studies indicate that these agents cannot be used for single-agent therapy and have been associated with therapeutic failure rates as high as 25%.[24] A randomized, prospective study evaluated the efficacy of ciprofloxacin versus rifampin plus doxycycline in the treatment of acute brucellosis. After treatment for 45 days, five of the six patients treated with ciprofloxacin relapsed, despite in vitro susceptibility and low MICs of the *B. melitensis* isolates.[749] In a Turkish study, the clinical efficacy of a combination of ofloxacin and rifampin for 6 weeks was comparable to that achieved with the doxycycline–rifampin combination.[12] Fluoroquinolone agents and the newer cephalosporins may show better clinical efficacy when incorporated in combination therapy with other agents. SXT, when used in combination with rifampin or an aminoglycoside, is useful for treatment of children less than 8 years old (tetracyclines are contraindicated).[1206] Older children can receive a modified doxycyline (4 mg/kg/day, PO)–rifampin (10 mg/kg/day, PO) regimen for 6 weeks. For severe pediatric infections, gentamicin (5 mg/kg/day, IM) may be added for the first 5 to 7 days of treatment. For *Brucella* endocarditis, a combination of antimicrobial therapy and surgery (i.e., valve replacement) is usually necessary, although some patients have been responded to antimicrobial chemotherapy alone.[40,543,835,838] Therapy for *Brucella* endocarditis, like that caused by other bacterial agents, requires that bactericidal levels of antimicrobial agents be administered. Doxycycline, rifampin, and SXT have been used successfully for endocarditis in conjunction with valve replacement. For CNS disease, aminoglycoside regimens are contraindicated because these agents do not reach therapeutic levels in the CSF. Combination therapies with two or three drugs (e.g., doxycycline, rifampin, and SXT) that penetrate the CNS and are active against the infecting isolate are recommended.[876] The treatment of multiorgan disease may require that triple-antibiotic therapy be more prolonged and certain types of complications (e.g., spondylitis) respond better to the doxycycline–gentamicin regimen than to the doxycycline–rifampin regimen.[62,1194] During and subsequent to the completion of therapy, patients should be monitored for evidence of relapsing/recurring disease by serology using either the 2-ME modification of the SAT test or by EIA.

Francisella Species

Epidemiology of Tularemia

Tularemia is the disease caused by the fastidious gram-negative coccobacillus *Francisella tularensis.*[393] Reservoirs of the bacterium in nature include rabbits, rodents, squirrels, muskrats, beavers, voles, deer, lemmings, and raccoons. Domesticated animals such as cattle, swine, and horses are fairly resistant to infection, while sheep are relatively susceptible. The organism is transmitted among animals by ticks and biting flies, such as deerflies.[1026] Transovarial transmission

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of *F. tularensis* also occurs in the tick, thereby providing a renewing source of the organism in the environment. Infections in humans are most commonly acquired by bites from infected ticks, deerflies, and mosquitoes or by direct contact with blood or internal organs of infected animals.[1169]Contaminated water sources, aerosols, and animal bites are also sources of infection for humans. Among ticks, at least 13 different species are known to be naturally infected with *F. tularensis*, with the dog tick (*Dermacentor variabilis*), the wood tick (*Dermacentor andersoni*), and the Lone Star tick (*Amblyomma americanum*) being the most common in North America. Disease transmission from tick exposure occurs in the Rocky Mountain regions of the United States and eastwards. In California, Wyoming, and Nevada, biting flies are the principal vectors.[218] In contrast, mosquitoes constitute the main vector for disease transmission in the former Soviet Union, Scandinavia, and surrounding regions.[387] Among hunters, infections have occurred following skinning, dressing, and eating infected animals, including rabbits, hares, beavers, muskrats, birds, and squirrels. These activities may generate aerosols, resulting in airborne transmission of the bacteria. Contaminated water is also an important source of the organism in the environment, and infections in humans have occurred following ingestion of water from wells where infected animal carcasses have been found. A large 1999 to 2000 outbreak of tularemia in Kosovo was traced to contaminated food and water, with rodents being identified as the organism source.[1076] Infections acquired by the airborne route have occurred following inhalation of dust and hay containing rodent feces and carcasses. Recent outbreaks of tularemia in Martha's Vineyard have been associated with landscaping, yardwork, and lawn mowing, and animal infections in the area have been documented in skunks and raccoons.[857] Human infections have also been acquired from exposure to or bites from carnivores, including domestic cats. In these cases, contamination of the mouth or claws of the cat following killing and feeding on prey is the likely mode of organism acquisition, although cats may also

acquire systemic infections.[1283,1332] Human infections have also been traced to infected prairie dogs that were sold as pets.[216] *F. tularensis* is highly contagious—as few as 10 to 50 organisms administered intradermally or via the aerosol route are sufficient to cause infection, and the organism can easily penetrate minute, inapparent breaks in the skin.[393] Tularemia has also been acquired from laboratory accidents that have occurred during processing of infected specimens, isolation and identification of the organism, and manipulation of large numbers of organisms in research settings. Human-to-human transmission does not occur.

Prior to World War II, tularemia was relatively common in the United States, but the incidence has steadily declined during the 1950s, and since 2001, case rates of tularemia are less than 0.05 cases per 100,000 population.[221] Although the disease has been reported throughout the United States, the vast majority of cases now occur in the south and south central states (i.e., Missouri, Kansas, Arkansas, Oklahoma, and Texas). In 2006, over half of the cases reported in the United States occurred in Arkansas, Kansas, Missouri, Nebraska, and Massachusetts.[221] The areas with the largest numbers of cases have shifted slightly northward from the south central regions of the United States, possibly secondary to climate change.[931] Most cases occur during the summer months or midwinter, and correspond to peaks of vector-associated disease and hunting-associated disease, respectively.[634] Males are more commonly infected than females, which is related to the association of this infection with certain occupations (e.g., farmers, large-animal veterinarians, hunters, sheep shearers and herders, hunters and trappers). Incidence rates in the United States are highest in children aged 5 to 10 years and adults 75 years of age and older.

During the last decade, issues have been raised concerning the potential use of *F. tularensis* as an agent of biologic warfare and bioterrorism. Consequently, *F. tularensis* is classified as a category A bioterrorism agent by the CDC.[187,346,1093] In the United States, *F. tularensis* was investigated as a potential biologic warfare agent in the 50s and 60s, prior to the termination of the US offensive bioterrorist program, and it is likely that other countries have investigated this agent as well. At Fort Detrick, animals and both civilian and military human volunteers were exposed to aerosols containing *F. tularensis* to determine the infectivity of the agent delivered in this fashion and to assess the efficacy of vaccines, prophylactic therapies, and treatment modalities.[274]Working groups in the biodefense arena have concluded that organism dispersion via aerosol would be the most likely scenario for a bioterrorist event involving *F. tularensis*, as infection by inhalation would affect the largest population.[346] With this method of dispersion, pneumonic and typhoidal disease would be the most likely clinical presentations (see below). Kaufmann and colleagues at the CDC recently published a "best and worst" case scenario if *F. tularensis* organisms were released as an "aerosol cloud" as a part of a bioterrorist event.[690] If the infective

aerosol dose of *F. tularensis* is 50 to 100 organisms, and assuming an 82.5% attack rate, exposure of 100,000 people would result in 82,500 cases of pneumonic tularemia. With a 6.2% death rate, 6,188 deaths would be expected, with an economic impact of between $465 million and $562 million!

History and Taxonomy

Tularemia was first described in 1911 by McCoy as the cause of a plague-like disease in California ground squirrels during investigations of possible bubonic plague among rodent populations in the San Francisco Bay area following the 1906 earthquake. McCoy and Chapin subsequently isolated the causative agent and named it *Bacterium tularense* after Tulare County, California, the site of their laboratory.[870] From 1912 through 1925, Edward Francis was studying the human disease called "deerfly fever" and made the connection between this illness and the "plague-like disease" described by McCoy in rodents. He investigated the modes of transmission, studied the causative agent, and coined the name "tularemia." This name reflects that of Tulare County, where McCoy and Chapin isolated the organism, and the "bacteremia" that occurs as a manifestation of infection. For his pioneering work on the cultivation of the organism and methods for serologic diagnosis, the associated clinical syndromes, and the recognition of the principal reservoirs and vectors, Francis received the 1959 Nobel Prize in science, and the name of the organism was changed from *Bacterium tularense* to *Francisella tularensis* in his honor. Based on molecular taxonomic techniques, *F. tularensis* is classified in the order *Thiotrichales*,

family *Francisellaceae* in the γ-subdivision of the *Proteobacteria*.[472,1192] Sequencing of 16S genes and whole-genome sequence analysis of several strains of *F. tularensis* indicate that the closest relatives are endosymbionts such as *Wolbachia persica*.[693] The family *Francisellaceae* includes the genera *Francisella* and *Fangia*. Genus *Fangia* includes a single species, *F. hongkongensis*, a gram-negative coccobacillus isolated from seawater in Hong Kong.[761]

Members of the genus *Francisella* are gram-negative, nonmotile, strictly aerobic facultative intracellular coccobacilli. At the present time, the genus *Francisella* includes four species: *F. tularensis*, *F. philomiragia*, *F. noatunensis*, and *F. hispaniensis*. *F tularensis* is divided into four subspecies: *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *Mediasciatica*, and *F. tularensis* subsp. *novicida*.[615,752,988] The last subspecies was formerly a separate species (*F. novicida*), but was reclassified as a subspecies of *F. tularensis* in 2010.[615] *F. noatunensis* was formerly a subspecies of *F. philomiragia* (*F. philomiragia* subsp.

noatunensis), but was elevated to species status in 2009.[888,988] *F. noatunensis* includes two subspecies: *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis*.[988] The organism named *Francisella piscicida* is a later heterotypic synonym of *F. noatunesis* subsp. *noatunensis* and is no longer a valid species name.[988] The four subspecies of *F. tularensis* can all cause tularemia, although they differ in their virulence for humans and rabbits.[474,652] *F. tularensis* subsp. *tularensis* (also called *F. tularensis* subsp. *nearctica* or biovar type A) predominates in North America but is not commonly found in Europe (although this subspecies was recognized in Slovakia), is associated with ticks and rabbits, and is virulent in both rabbits and humans.[536,654] This biogroup is characterized phenotypically by acid production from glycerol and possession of citrulline ureidase.[843,1138] Type A strains have been divided into two genetically distinct subpopulations or clades, termed *A1* and *A2*, by a molecular technique called multiple-locus VNTR analysis (MLVA), and additional molecular typing methods have delineated subtypes within types A1 (i.e., A1a, A1b) and A2 (i.e., A2a and A2b).[653,693,736,1027] Disease due to A1 strains is clinically more severe and occurs primarily in the eastern half of the United States, while disease due to A2 strains is milder and occurs only in the western United States.[412,1025,1218] However, deerfly-associated infections due to both A1 and A2 clades have occurred in Utah.[1025] *F. tularensis* subsp. *holarctica* (formerly called *F. tularensis* subsp. *palaearctica* or biovar type B) has been isolated in Europe, Asia, Japan, and North America, is associated with waterborne infection in rodents, tick, and mosquito vectors, and is intermediate in virulence in humans and minimally virulent in rabbits.[34,634] Disease due to subspecies *holarctica* may be more common in the United States than previously thought.[1276] Subspecies *holarctica* strains are glycerol-negative and do not usually produce citrulline ureidase. *F. tularensis* subsp. *mediaasiatica* was suggested for strains isolated in the central Asian focus of the Soviet Union, and *F. tularensis* biogroup *palaearctica japonica* was suggested for isolates from Japan.[1138] Initially, all four were proposed as separate species by Soviet scientists, but this proposal has not been generally accepted. Central Asian and Japanese strains have been shown to have low virulence for rabbits (like biogroup *holarctica/palaearctica*), but are clearly more closely related to *F. tularensis* biogroup *tularensis* based on 16S rRNA analysis.[1138] Recent data using two insertion sequence elements to type strains by RFLPs revealed that Japanese strains of *F. tularensis* subsp. *holarctica* (*palaearctica*) may actually represent another subspecies.[1252] *F. novicida,* originally isolated from water sources in Utah in 1951, was previously classified as a *Pasteurella* species, and was renamed as *Francisella novicida* in 1959.[752] Genetic studies subsequently determined that *F. novicida* was not a separate species, but constituted another biogroup of *F. tularensis* (biogroup novicida), since

the degree of 16S rRNA similarity was 99.6%.[430,598] *F. tularensis* biogroup novicida was elevated to subspecies status (i.e., *F. tularensis* subsp. *novicida*) in 2010.[615] *F. tularensis* subsp. *novicida*, which is an organism of relatively low virulence, has been isolated from water containing dead muskrats and from humans. *Francisella*-like organisms have been recovered from fish and human infections in recent years, suggesting that the genus may undergo expansion in the future.[961,986,1347]

F. philomiragia includes bacterial strains formerly called "philomiragia bacteria" that were mistakenly included with the yersiniae as "*Y. philomiragia.*" These organisms show genetic and antigenic relatedness to *F. tularensis* subspecies and have similar cell wall fatty acid and ubiquinone constituents. Analysis of the 16S rRNA of these organisms has corroborated the genotypic relationship of *F. tularensis* and *F. philomiragia*.[430,431,598] *F. philomiragia* is considerably less virulent than *F. tularensis* and has been isolated mostly from animals (e.g., muskrats) and water. Unlike *F. tularensis*, *F. philomiragia* is an opportunistic agent, causing pneumonia and systemic infections in patients with chronic granulomatous disease, near-drowning victims, and patients with hematologic malignancies.[828,1187,1338] Over a 12-year period, 14 human isolates of *F. philomiragia* were submitted to the Special Bacteriology Branch of the CDC. Of these 14 isolates, five were from patients with chronic granulomatous disease, five were from individuals who nearly drowned in salt water, two were from unknown human sources, and single isolates were from a patient with Hodgkin's disease and a patient with myeloid metaplasia.[598,1165,1338] These cases have defined the clinical spectrum of *F. philomiragia* as a rare agent of necrotizing pneumonia, bacteremia, and meningitis.[1165,1338] A recent case of *F. philomiragia* adenitis and pulmonary nodules was reported in a 10-year-old boy with chronic granulomatous disease following a facial abrasion from a saltwater crab.[828]

F. noatunensis subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* are well-known pathogens of various fresh- and saltwater fish, including three-line grunt in Japan, tilapia in America and Asia, hybrid striped bass from America, Atlantic Salmon from Chile, and Atlantic cod from Norway.[888,987,988] In these various fish species, these bacteria cause chronic granulomatous infections of the heart, kidney, spleen, and liver. *F. noatunensis* subspecies are most closely related to *F. philomiragia*. The new species *F. hispaniensis* was a blood culture isolate from a patient with bacteremia secondary to acute obstructive pyelonephritis.[615] This isolate was initially thought to be a strain of *F. tularensis* subsp. *novicida*, but analysis of five housekeeping genes and two genes encoding membrane proteins revealed that it was distinct from recognized *Francisella* species and subspecies. In July of 2008, two isolates—one from CSF and one from blood—were recovered from two different patients.[737] The

first isolate was recovered from the CSF of a 15-month-old female with hemophagocytic syndrome and juvenile rheumatoid arthritis, and the second was isolated in a blood culture from an 85-year-old man with end-stage renal disease. When 16S rRNA sequences were analyzed, the two isolates showed 97% identity with *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *novicida,* and *F. philomiragia*, and were 98% similar to sequences found in soil isolates in the genus *Francisella*. All *F. tularensis*-specific tests (e.g., slide agglutination with specific antisera, fluorescent antibody (FA) tests, and PCR analysis) were negative. Investigators proposed that these two isolates, which were genotypically and phenotypically similar, may represent a new *Francisella* species.[737]

In 1997, another possible *Francisella* species was recognized as an endosymbiont in ovarian tissue of female *Dermacentor andersoni* ticks, but has not been detected in male ticks.[940] The relation of this organism, designated the "*D. andersoni* symbiont" (DAS), and *Francisella* species is based on the presence of a specific membrane antigen and 16S rRNA sequences that are similar to those of *F. tularensis*. These endosymbionts are termed FLEs or "*Francisella*-like endosymbionts." These FLEs have been identified in ticks belonging to the genera *Dermacentor*, *Amblyomma*, *Rhipicephalus*, and *Ornithodoros.*[1162,1230] Investigation of obligately endosymbiotic bacteria found in *Paramecium tetraurelia* found that one of these endosymbionts, *Caedibacter taeniospiralis*, formed a novel evolutionary lineage with the γ-*Proteobacteria* and shared 87% similarity in its 16S rRNA sequences with the family *Francisellaceae*.[103] In a study of FLEs from a variety of tick species, Machado-Ferreira and colleagues identified gene sequences of the *F. tularensis* pathogenicity island gene *iglC* and its regulatory gene *mglA* in FLEs.[819] The *iglC* gene encodes a protein in *F. tularensis* that is involved in bacterial escape from the phagolysosome and intracellular bacterial survival. At the present time, FLEs do not appear to be pathogenic in humans, but the recognition of gene sequences in FLEs that are similar to *F. tularensis* virulence-associated genes suggests a pathogenic potential for these unusual agents. Using primers and probes for various regions of the *lpnA* gene of *F. tularensis*, which encodes a lipoprotein, a 233 base pair fragment was identified that was able to discriminate among *F. tularensis* subspecies and FLEs.[406] This method would be useful in field studies of *Francisella* species in environments where FLEs are likely to be present. This molecular approach was useful not only for the detection and differentiation of *Francisella* subspecies and FLEs, but was also helpful for detection of atypical *Francisella* isolates associated with human disease, such as the unusual *F. tularensis* strain FnSp1, which is now *F. hispaniensis*.[615]

Clinical Spectrum of Tularemia

Clinical manifestations of tularemia depend on the mode of acquisition of the organism, the bacterial subspecies involved, the innate virulence of the infecting strain, the immunocompetence of the host, and the extent of organ system involvement.[580]After infection, there is an incubation period of a few hours to 3–5 days, but it may range as far as 3 weeks. The onset is acute, with fever, chills, malaise, headache, sore throat, and sometimes diarrhea. Fevers may wax and wane but usually remain elevated for 4 to 5 weeks in the absence of treatment. During this time, weight loss, lymphadenopathy, and chronic illness develop. Patients who are infected with less virulent strains may have a mild illness that resolves spontaneously, while others develop chronic, debilitating, multisystem infections.

Tularemia was clinically divided into six syndromes: ulceroglandular tularemia, glandular tularemia, oculoglandular tularemia, oropharyngeal tularemia, typhoidal tularemia, and pneumonic tularemia. This classification has been replaced by a binary disease classification recognizing ulceroglandular and typhoidal disease. The distinction between these two forms depends on the involvement of skin/mucous membranes and the location and severity of the associated lymphadenopathy. About 75% of patients present with the ulceroglandular form, while 25% present with the typhoidal form. Ulceroglandular tularemia, which is the most common clinical presentation, now encompasses ulceroglandular, glandular, and oculoglandular syndromes.[1099] The patient presents with a painful lesion at the site of organism entry that later ulcerates, forming a central, punched-out ulcer with a raised periphery. Vesicular ulcers resembling those of herpes simplex and varicella viruses that have been culture-positive for *F. tularensis* have also been described. The location of the lesion depends on whether the infection was obtained from an arthropod or a mammalian vector. In 2% to 12% of cases, the primary lesion is in the oropharynx and results from ingestion of contaminated food or water. Mucous membrane involvement presents as severe granulomatous conjunctivitis, loss in visual acuity, preauricular and submandibular lymphadenopathy, and, in some patients, nonexudative pharyngitis.[210,470] Some patients present with severe headache, tonsillitis, and exudative pharyngitis suggestive of streptococcal pharyngitis, diphtheria, or Vincent's disease. Pharyngeal involvement invariably has associated regional lymphadenopathy that can result in lymph node and retropharyngeal abscess formation. Otitis media and middle ear infections are rare complications.[535,816] Enlarged lymph nodes and abscesses may become fluctuant and suppurate through the overlying skin or become persistent.[276,576] In some case, lymphadenopathy and fever are present, but a skin lesion is not apparent.[815,969] In these cases, the lesion may have healed or may be innocuous and unnoticed. Typhoidal tularemia presents with an abrupt onset of fever, chills, headache, sore throat, vomiting, and abdominal pain and tenderness. No initial skin lesion or

lymphadenopathy is apparent, and the infection is probably acquired by ingestion of contaminated water or food. Typhoidal tularemia is the only form in which diarrhea is a prominent part of the clinical presentation. The diarrhea is usually loose and watery. Almost half of the patients with typhoidal tularemia develop secondary pleuropulmonary disease, with pulmonary infiltrates and pleural effusions. In this form, blood and sputum cultures may be culture-positive, and the mortality rate is usually high. Bloody stools may be seen, particularly in children. Pneumonia is seen in about 80% of patients with typhoidal tularemia and in about 30% of those with ulceroglandular tularemia and results from inhalation of aerosols containing the organism or from hematogenous dissemination.[106,415,416] Radiographic examinations may show involvement of one or more lobes, with pleural effusions, pneumonic infiltrates,

abscess formation, pulmonary nodules, and hilar lymphadenopathy.[487,937] Symptoms include fever, dry cough with minimal sputum production, and pleuritic chest pain. Clinically, pneumonic tularemia resembles tuberculosis, atypical pneumonias due to *Legionella*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, *Coxiella burnetii*, and fungal pneumonia. Pneumonic tularemia tends to be more severe in patients with underlying conditions, such as malnourishment, alcoholism, renal disease, chronic granulomatous disease, and HIV infection.[842] Complications such as hepatosplenomegaly, renal failure, and rhabdomyolysis occur more frequently in these individuals. Pneumonic tularemia is usually seen in adults and is associated with certain occupations (e.g., sheep shearers, animal husbandry workers, laboratorians), farming activities (e.g., threshing), lawn mowing, and brush cutting. This association is likely due to the presence of rodent feces or infected animal remains in hay.[1235]

Clinical tularemia can display an array of presentations in individual patients. Plourde et al.[1036] reported a case of glandular tularemia with some features of typhoidal disease in a child who had sustained an insect bite on the left scapula. Inguinal lymphadenopathy rather than lymphadenopathy near the site of the insect bite was found, and the patient developed abdominal distention and severe, watery diarrhea, both of which are features of typhoidal disease. In another instance, a serologic diagnosis of tularemia was achieved on a 53-year-old woman with a large pericardial effusion who reported close contact with rabbits 2 weeks prior.[747] Chronic infection of a knee prosthesis was reported in a patient with degenerative joint disease 6 months after a tick bite.[304] Meningitis and brain abscess are a rare complication of *F. tularensis* infection.[471,595,1095] Patients with meningitis may or may not have significant nonmeningeal disease (glandular or typhoidal tularemia). CSF findings are

consistent with a bacterial meningitis (i.e., low glucose with high protein), and show a predominance of lymphocytes on Gram's stain of the CSF. A *F. tularensis* ventriculoperitoneal shunt infection was first reported in 1996 in a 5-year-old boy with myelomeningocoele.[1034] Signs and symptoms were limited to the CNS, with no other systemic involvement. Two weeks prior to presentation, the child had been with friends who were skinning a rabbit. Clinical and bacteriologic cure was achieved with intrathecal gentamicin and shunt replacement. LeDoux reported a case of presumptive *F. tularensis* CNS disease in a 61-year-old man whose clinical presentation was predominantly gait ataxia.[771] In 2000, Tancik and Dilliha reported the first published case report of *F. tularensis* native valve endocarditis in a 42-year-old man from Arkansas.[1244] The patient responded to a 4-week course of gentamicin. Severe disease is also associated with compromised host status, as evidenced by a rapidly fatal infection in a neutropenic bone marrow transplant recipient.[1145] *F. tularensis* subsp. *novicida* is a rare pathogen, and only a very few infections have been reported, including the recent description from Thailand of *F. tularensis* subsp. *novicida* bacteremia in a woman undergoing chemotherapy for ovarian cancer.[773]

Detection, Isolation, and Cultural Characteristics

Because this organism can penetrate through small breaks in the skin, it is potentially dangerous to handle specimens or cultures of *F. tularensis* in the laboratory. As with brucellosis, tularemia may not be suspected when a patient presents to the clinician; so considerable time may be spent handling specimens and cultures before this diagnosis is entertained. For laboratories working with clinical materials of human and animal origin, BSL-2 practices, containment equipment, and facilities are recommended, while BSL-3 and Animal BSL-3 practices are required for working with live cultures or performing experimental animal studies.[1279] In the routine clinical microbiology laboratory, all specimens and cultures should be processed in a class II biologic safety cabinet, gloves should be worn during all procedures, and any procedures that may generate aerosols should be avoided. With the threat of bioterrorism, screening tests for *F. tularensis* and other bioterrorism agents must be incorporated into laboratory bench procedures in order to permit rapid presumptive identification of an isolate so that it can be referred to level B laboratories for definitive identification.[1093] Persons with confirmed laboratory exposure should be treated prophylactically.

F. tularensis may be isolated from primary ulcers, lymph node aspirates and biopsies, sputum, bone marrow, and tissue biopsies (e.g., liver, spleen). Specimens should be kept at 4°C to 8°C until processing. Specimens may also be placed in Amies transport medium with charcoal or in Stuart medium at room temperature prior to processing. The organisms have also been isolated from peripheral blood specimens, usually in the setting of preexisting, underlying disease. Positive blood cultures have

been found in patients with typhoidal, pneumonic, and oropharyngeal tularemia, and in the single report of *F. tularensis* endocarditis.[593,951,1046,1276,1320] In case reports describing *F. tularensis* bacteremia, growth of the organism was found after 3 to 7 days' incubation using the BACTEC radiometric system. However, in one patient with endocarditis, *F. tularensis* was recovered after 9 days with the BACTEC system.[1244] *F. tularensis* was isolated after 12 days' incubation from a BACTEC nonradiometric aerobic blood culture bottle (BACTEC NR6A) that was inoculated with a 10 to 20 μL volume of an percutaneous fine needle aspirate of an inguinal lymph node.[179] If tularemia is suspected clinically, broth blood cultures should be incubated beyond the standard 5- to 7-day incubation period used in many laboratories. Blind subculture of blood culture bottles may be advantageous for detection.

F. tularensis is a small, pale-staining, gram-negative coccobacillus. Classically, the organism has a growth requirement for cysteine, cystine, or other sulfhydryl-containing compounds, and the preferred medium for isolation was glucose cysteine blood agar. However, *F. tularensis* will grow on commercially available chocolate agar and modified Thayer-Martin agar, since these hemin-containing medium are supplemented with a growth enrichment (e.g., IsoVitelex, BD Biocience) that contains cysteine, cystine, and other nutrients required by fastidious bacteria. Like the brucellae, this organism is also able to grow on BCYE agar, the medium used for isolation of *Legionella* species.[1340] *F. tularensis* has also been isolated from a biopsy of a cutaneous lesion by the centrifugation shell vial technique using human embryonic lung fibroblasts.[440] Although the organism failed to grow upon subculture on bacteriologic media, the identity of the organism was determined by PCR analysis of the organism

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growing in the shell vial. Atypical strains may lack the cysteine requirement of "classical" *F. tularensis* strains, but they are usually weakly oxidase-positive, catalase-negative, agglutinate strongly in *Francisella* antiserum, and unreactive in other phenotypic tests, including those for biogroup determinations.[122] Cellular fatty acid analysis of these strains revealed the presence of long-chain (i.e., C_{18} to C_{26}) saturated and unsaturated fatty acids, and large amounts of 3-hydroxy-octadecanoate (3OH-$C_{18:O}$), a fatty acid that is unique to *F. tularensis*.[644] These unusual hydroxy fatty acids comprise part of the lipid A structure in the LPS of the organism. Consequently, these organisms may be identified readily by cellular fatty acid analysis using the Microbial Identification (MIDI) System and Library Generation System (LGS) software (MIDI, Newark, NJ). Clarridge and colleagues isolated two nonfastidious *F. tularensis* strains from blood culture of patients with pneumonia.[282] These isolates

grew on both chocolate and blood agar and were identified as *F. tularensis* by phenotypic characteristics, 16S rRNA gene sequencing, and rDNA probe hybridization. These strains also failed to agglutinate in commercial *F. tularensis* antisera. Strains of *F. tularensis* subsp. *novicida* and *F. philomiragia* do not demonstrate a requirement for cysteine.

F. tularensis is obligately aerobic; growth is stimulated by increased CO_2 and may require 2 to 5 days' incubation at 35°C to 37°C before colonies are visible on agar medium. Colonies on chocolate agar are gray, smooth, buttery, and about 2 mm in diameter after 3 days' incubation. *F. tularensis* subsp. *tularensis* grows more slowly than the other subspecies. Colonies of *F. philomiragia* are larger, whiter, and mucoid. *F. tularensis* subspecies are oxidase-negative, are weakly catalase-positive, grow poorly (if at all) on MacConkey agar, and are inert biochemically. *F. philomiragia* isolates are oxidase-positive. Species identification is usually confirmed by agglutination tests using specific rabbit polyclonal antisera. Cultures should be examined over a 10- to 14-day period. Phenotypic characteristics of *Francisella* species are presented in Table 9-13.

Studies using spiked blood cultures and experimentally infected mice initially suggested the utility of PCR as a method for detection and identification of *F. tularensis* in clinical specimens from humans.[362,667,801] These assays were able to detect both *tularensis* and *holarctica* subspecies at sensitivities equal to 1 colony-forming unit/mL of blood. Subsequently, a multiplex PCR assay targeting both the 16S rRNA gene and a 17-kDa lipoprotein gene was successfully developed for direct detection of *F. tularensis* in swabs from ulceroglandular tularemia lesions in Sweden.[654,1193] The lipoprotein gene is conserved among *F. tularensis* strains and does not show sequence similarities with other known prokaryotic sequences published in current gene banks.[654] PCR performed on wound specimens was able to provide a rapid diagnosis in patients with suspected ulceroglandular tularemia who were negative on culture and had not yet seroconverted.[654,682] Subsequent investigations of ulceroglandular

TABLE 9-13 Phenotypic Characteristics for the Identification of *Francisella* Species and Subspecie

Characteristic	*F. tularensis* subsp. *tularensis*	*F. tularensis* subsp. *holarctica*	*F. tularensis* subsp. *novicida*	*F. tularensis* subsp. *mediaasiatica*	*F. philomiragia*	*F. hispaniensis*
Oxidase	–	–	–	–	+	+
Catalase	+	+	$+^{w}$	+	+	$+^{w}$
Motility	–	–	–	–	–	–
Growth on Mac-Conkey agar	V	–	V	NA	V	NA
H_2S in TSI agar	–	–	–	–	$+^{w}$	–
Urease	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–
Acid production from:						
Glucose	+	+	+	–	$+^{w}$	NA
Maltose	+	+	V	–	$+^{w}$	NA
Sucrose	–	–	+	+	$+^{w}$	NA
Glycerol	+	–	+	+	NA	NA
Cysteine/cystine requirement	+	+	–	–	–	–
Citrulline ureidase	$+^{-}$	$-^{+}$	+	+	NA	NA

+, positive reaction; –, negative reaction; V, variable reaction; $+^{w}$, weak positive reaction; $+^{-}$, positive reaction, rare strains negative; $-^{+}$, negative reaction, rare strains positive; NA, data not available.

tularemia in Sweden have further confirmed the clinical and diagnostic utility of PCR.[389] PCR had also been applied to lymph node tissues and aspirates for the diagnosis of oropharyngeal tularemia. During an investigation of an outbreak of oropharyngeal tularemia in Turkey, PCR performed on lymph node aspirates of all seven patients was positive; these tests were positive even though all seven patients had been receiving antimicrobial chemotherapy for over 2 weeks when the tests were performed.[209] PCR has also been applied to the diagnosis of oculoglandular disease, with positive results being obtained from a conjunctival swab and a conjunctival nodule in two patients, respectively; both patients had positive *F. tularensis* PCR results from blood cultures.[679] Two additional patients in this study had positive PCR and cultures from lymph node tissue. In response to the need for epidemiologic tools for investigating outbreaks of tularemia, PCR approaches have also been developed to detect infection directly in experimental animals and in insect vectors, and to provide methods for typing and discrimination of strains.[338,411,474,586,1252] Real-time PCR assays have been developed that identify and distinguish the A1 and A2 clades of *F. tularensis* subsp. *tularensis*.[902]

Several other novel techniques for rapid detection/identification of *F. tularensis* have been developed as a direct result of concerns over the use of *F. tularensis* in

bioterrorism and biologic warfare. Grunow and colleagues developed a standard EIA and a handheld immunochromatographic assay that used monoclonal antibodies against LPS antigen as the capture antibody for detecting *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* directly in veterinary specimens.[520] Antigen-capture EIAs using time-resolved fluorometry have also been developed for detection of several bioterrorism-associated agents in both clinical and environmental samples.[1024] Emanuel and coworkers developed a fluorogenic PCR-based method for detection of *F. tularensis* in infected mice. This assay could be performed using a handheld thermocycler designed for field use; results were available within 4 hours.[396] Multiplex PCR platforms capable of detecting a number of agents have also been developed in response to the threat of bioterrorism. These real-time, bead- or DNA microarray-based amplification assays have been developed and optimized to detect *F. tularensis*, *Y. pestis*, *B. anthracis*, and *B. melitensis* in animals tissues (e.g., cow anthrax, hare tularemia), blood specimens (e.g., blood spiked with serial dilutions of pathogens), and environmental samples.[1195,1259,1355]

Because strains belonging to the different *F. tularensis* biogroups are antigenically homogenous, subspecies are differentiated from one another on the basis of phenotypic criteria or subspecies-specific probes for 16S rRNA.[430] Strains of *F. tularensis* subsp. *tularensis* produce acid in media containing glucose, maltose, or glycerol as a carbon source. These also hybridize with an oligonucleotide probe for subspecies *tularensis*-specific 16S rRNA sequences. Subspecies *holarctica* strains produce acid from glucose and maltose, but not from glycerol, and hybridize with a probe that is specific for subspecies *holarctica* 16S rRNA sequences. Both subspecies *mediaasiatica* and subspecies *palaearctica japonica* strains hybridize with the subspecies *tularensis*-specific probe; however, subspecies *mediaasiatica* strains produce acid from glycerol and sucrose, while subspecies *palaearctica japonica* strains acidify media containing either glucose or glycerol. Serologic methods can distinguish between subspecies *tularensis* and *novicida* strains, but not between subspecies *tularensis* and *holarctica* strains. Citrulline ureidase, an enzyme that correlates with virulence of *F. tularensis*, is found in virulent strains of subspecies *tularensis,* some *holarctica* strains, and *mediaasiatica* strains.[843,1138] The enzyme is not found in attenuated *tularensis* strains and in most subspecies *holarctica* strains with low virulence. Phenotypic testing of these organisms is problematic, since reactions are often delayed or variable.

Treatment of Tularemia

The drug of choice for treatment of tularemia is the aminoglycoside streptomycin, with gentamicin being an alternative agent. Doxycycline has also been used but is associated with higher relapse rates than aminoglycoside therapy, especially if administered early

in the clinical course. The initial clinical response to treatment with tetracyclines is dramatic (i.e., patients defervesce rapidly), but relapse rates after therapy are over 12%, which is twice the relapse rate seen with gentamicin therapy. Intramuscular or intravenous gentamicin and doxycycline are not useful for treatment of *F. tularensis* meningitis owing to poor penetration into the CNS, but patients have responded to intrathecal gentamicin or intrathecal combinations of streptomycin or gentamicin with doxycycline. Intravenous therapy with erythromycin has also been used successfully, but in vitro data suggest that the *F. tularensis* strains are not predictably susceptible. Scheel et al.[1146] found that 14 of 22 Scandinavian strains were resistant to erythromycin and clarithromycin in vitro, and resistance to erythromycin is substantial among strains recovered in Russia.[1281] Fluoroquinolones are also active against type A and type B *F. tularensis* isolates. However, clinical responses to fluoroquinolone therapy in patients with disease due to subspecies *tularensis* are suboptimal compared with infections caused by the less virulent *holarctica* subspecies. In the former cases, combination therapy with gentamicin and a fluoroquinolone may result in a better clinical response.[387,388]

Using the CLSI-recommended broth microdilution method, Urich and Petersen tested 92 *F. tularensis* subsp. *tularensis* (type A) isolates and 77 *F. tularensis* subsp *holarctica* (type B) isolates and found that all organisms tested were susceptible to those agents that are used for the treatment of tularemia, including streptomycin, gentamicin, tetracycline, doxycycline, ciprofloxacin, and levofloxacin.[1281] All strains were in the susceptible range for erythromycin, with type A strains having MICs two doubling dilutions lower than type B strains. Valade and investigators in France tested 71 *F. tularensis* subsp. *holarctica* stains and found that reproducible results could not be obtained for some agents using the broth microdilution method. Using agar dilution, all 71 subsp. *holactica* isolates were susceptible to streptomycin, gentamicin, doxycycline, ciprofloxacin, nalidixate, and chloramphenicol.[1282] While some in vitro studies using broth dilution methods have reported low MICs for β-lactams when tested against *F. tularensis*, other studies using agar dilution have concluded that *F. tularensis* is resistant to β-lactam antimicrobial agents.[83,1146] In a study of 22 animal and human *F. tularensis* isolates using the agar dilution

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method with blood–cysteine–glucose agar, all strains were resistant not only to penicillin and cephalothin, but also to cefuroxime, ceftazidime, aztreonam, imipenem, and meropenem, with MIC of >32 μg/mL for all drugs. Ikaheimo and colleagues used Etest strips on cysteine heart agar plates for testing 20 human isolates and 18 isolates from dead animals. All strains were susceptible to the agents that have been used for

treatment (i.e., streptomycin, tetracycline), but were resistant to β-lactams and carbapenems, including ceftriaxone, ceftazidime, cefepime, imipenem, meropenem, and piperacillin–tazobactam.[621] Other studies have also determined that *Francisella tularensis* subspecies are generally resistant to penicillins and cephalosporins but susceptible to the mainstay drugs used for therapy.[860,1258] Clinical experience with third-generation cephalosporins for tularemia indicates that they are not effective. Cross and Jacobs reported on eight cases of documented failures with outpatient use of ceftriaxone in the treatment of tularemia.[315]

Isolates of *F. philomiragia* are usually susceptible to the aminoglycosides, the fluoroquinolones, and tetracycline, and variably susceptible to amoxicillin clavulanate, rifampin, and erythromycin. Most strains produce β-lactamase enzymes and are resistant to ampicillin, and some strains may be resistant to cefazolin, cefotaxime, and SXT.

Virulence of F. tularensis

The factors that are responsible for the virulence of *F. tularensis* are being studied intensively at this time because of funding increases in bioterrorism programs.[991,1013] The organism produces no identifiable exotoxins, and its virulence stems from its ability to grow to large numbers within host tissues, and to induce a significant inflammatory host response. Upon infection, the organism multiplies locally for 3 to 7 days, then spreads to the regional lymph nodes and disseminates via the lymphohematogenous route to involve multiple organs. An initial inflammatory response ensues, with the accumulation of neutrophils, fibrin, and macrophages. Lymphocytes and macrophages migrate into the infected tissues, engulf the organisms, and form granulomas in multiple sites (i.e., lymph nodes, bone marrow, spleen, liver, and lungs). Organisms remain viable within these macrophages by inhibition of phagosome–lysosome fusion and utilization of iron from host cells. The predominant antibody response in humans is to LPS. The LPS of the organism has a unique structure, induces less cellular toxicity, and does not bind to host molecules involved in triggering proinflammatory responses. Animal studies have demonstrated that immunization with LPS induces protection against *F. tularensis* strains of low virulence, but will not protect against more virulent strains.[299] Natural infection or immunization with the *F. tularensis* live vaccine strain (LVS) engenders long-lasting cell-mediated and humoral resistance to reinfection, with the cell-mediated immune response being the most important protective mechanism. Cell-mediated immunity appears 7 to 10 days after infection, with IgM, IgG, and IgA agglutinating antibodies appearing during the subsequent 2 weeks. Lysis of cells harboring organisms by immune effector cells in the liver and other organs releases organisms from intracellular compartments, thereby enabling them to infect other cells. If left

untreated, suppuration of involved lymph nodes in various tissues results in additional manifestations of systemic disease. Persistence of cell-mediated, protective immunity with a concomitant decline in humoral immunity has been demonstrated in individuals who were naturally infected 25 years earlier.[401] Studies performed with mutants of the LVS strain suggest that the principal virulence factor of *F. tularensis* is the ability to invade and multiply within macrophages, hepatocytes, and other cells of the RES. *F. tularensis* is able to enter host macrophages and, once inside, is able to destabilize the phagosome and escape into the cytosol of the cell, where it then multiplies to high numbers.[1140] Eventually, the bacteria are released by organism-induced apoptosis. *Francisella* organisms are also able to enter neutrophils without triggering an oxidative burst, and to multiply inside the neutrophils as well.[867] Acid phosphatases unique to *F. tularensis* play a key role in the escape of organisms from macrophages and neutrophils. *F. tularensis* contains a pathogenicity island comprising 19 genes that are essential for virulence, and the products of these genes are under current investigation.[935] Pili and secretion systems for virulence factors have also been detected.[544]

The LVS vaccine was developed in Russia via multiple in vivo and in vitro passages of a virulent *F. tularensis* subsp. *holarctica* strain and was sent as a gift to the United States in 1956. The vaccine was subsequently tested on volunteer populations by the US Army.[298,299] Although the vaccine is effective in protecting against low- and high-dose subcutaneous and low-dose aerosol challenge with a virulent type A strain, it does not adequately protect against high-dose aerosol administration of virulent *F. tularensis*. When administered by the aerosol route instead of scarification, greater protection was seen, but some volunteers developed disease from this attenuated strain. Because of the lack of adequate responses in volunteers and the questionable degree of attenuation observed in the volunteers subjected to aerosol inoculation, the vaccine did not achieve licensure in the United States. Investigations with subunit vaccines based on immunoreactive surface proteins and lipoproteins of the organism were disappointing, as the level of protective immune responses were less than that seen following administration of LVS.[497] In addition to attenuated and subunit vaccines developed from LVS and *F. tularensis* subsp. *holarctica*, mutants of *F. novicida* have also been investigated as surrogate organisms for live, attenuated tularemia vaccines. Investigated organisms have included *F. novicida* strains with mutations in the pathogenicity island genes, metabolic pathways genes, acid phosphatase genes, and in genes affecting LPS biosynthesis. Immunization of mice with these various mutants has led only to the demonstration of protective immunity against the homologous strain, and none have been able to confer protective immunity to mice on challenge with either *F. tularensis* type A or type B.[1048,1180] Live mutants of *F. novicida* that are unable to escape from the phagosome following infection do

not appear to function well as live vaccine candidates, suggesting that the vaccine needs to mimic actual infection more closely and replicate outside of the phagosome in order to stimulate humoral or cell-mediated immune responses. Mutants of the LVS strain (*F. tularensis* subsp. *holarctica*) confer immunity against the wild-type LVS strain and variable degrees of protection against heterologous type B and some type A strains.[1013] As with the wild-type LVS,

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mutants of LVS do not appear to confer a high level of immunity when *F. tularensis* type A or B challenge strains are administered via the respiratory route. The use of attenuated strains or mutants of *F. tularensis* subsp. *tularensis* has also been investigated. Wu and colleagues showed that giving a sublethal dose of a virulent type A strain as a booster inoculation following vaccination with LVS provided augmented protection when compared with giving a booster dose of the LVS strain.[1372] In addition to investigations on which vaccine types (i.e., killed, live-attenuated, or subunits) engender the best immune response, vaccine researchers are also examining the routes of vaccine administration (scarification, parenteral, respiratory inhalation) to see which route affords the best protection against challenge with virulent type A and type B strains of *F. tularensis*.[1372]

Afipia Species, *Bosea* Species, and Other α-*Proteobacteria*

Afipia species are fastidious members of the class α-2 *Proteobacteria*, order *Rhizobiales*, family *Bradyrhizobiaceae*.[171,755] These organisms live within free-living amebae belonging to the genus *Acanthamoeba* and can be isolated from them by amebal enrichment and coculture methods. Amebal coculture is a technique in which axenic amebae are used as host systems for isolation of intracellular bacteria directly from clinical or environmental specimens. Bacteria from these specimens enter the amebae and multiply, subsequently leading to lysis of the amebae and the release of the intracellular organisms. Bacterial characterization is accomplished by differential stains, subcultures, and molecular methods (e.g., PCR, sequencing). Amebal enrichment techniques involve culture of environmental amebae on nonnutritive agar media seeded with enteric bacteria (e.g., *E. coli*) as a food source. Following incubation, the agar surface is screened for the presence of motile amebae, followed by subculture onto additional plates of seeded nonnutritive agar. Intracellular bacteria can then be isolated by amebal coculture, adapted to in vitro cultivation on agar media

such as BCYE agar, tested for phenotypic traits, and characterized by molecular approaches such as 16S rRNA sequencing. Amebal coculture and enrichment techniques have been specifically developed to identify bacterial agents in environmental samples and in specimens from patients.[746] Organisms in the genus *Afipia* were first isolated from lymph node aspirates of patients with cat scratch disease (CSD; *A. felis*); additional species were subsequently recovered from human clinical specimens (*A. clevelandensis*, *A. broomeae*, *Afipia* genospecies 1 and 2) and water (*Afipia* genospecies 3).[171] In 1999, LaScola and colleagues recovered *A. felis* from the water supply of a French hospital by amebal coculture, and a subsequent systematic cocultivation survey of water samples from the Timone Hospital Center in Marseilles yielded 68 α-*Proteobacteria* that were divisible into 7 clusters by 16S rRNA sequence comparisons.[753,759] Five clusters were composed of organisms closely related to *A. felis* and various *Afipia* genospecies; some of these subsequently were characterized as new *Afipia* species (i.e., *A. birgiae*, *A. masseliensis*, and *Afipia felis* genospecies A).[755] Another study performed on ICU water samples at four university hospitals in Marseille yielded 64 bacterial strains that were identified as *Afipia* species or close relatives within the *Rhizobiaceae*.[757] In this same study, a serosurvey of 85 ICU patients with hospital-acquired pneumonia at these hospitals was performed, and 11 patients were found to have elevated antibody titers to *Afipia* genospecies 1 (2 patients),*Mesorhizobium amorphae* (3 patients), and two unnamed bacteria (6 patients) that were genotypically related to *Rasbobacterium.* In vitro studies have also shown that *Afipia* species are able to grow within human cells, including HeLa, macrophages, and endothelial cells. Phenotypic characteristics for the identification of *Afipia* and related species are presented in Table 9-14.

The genus *Bosea* includes gram-negative, oxidase-positive, motile environmental bacilli composed of several species (*B. thiooxidans*, *B. minatitlanensi*, *B. massiliensis*, *B. vestrisii*, *B. eneae*, *B. sequanensis*, *B. lascolae*, *B. lupini*, *B. lathyri*, *B. robiniae*).[328,343,756,989,1253] *B. minatitlanensis* and *B. massiliensis* have been isolated from hospital water supplies by amebal cocultivation and characterized after adaptation to growth on BCYE and blood agar. Coculture methods, serology, and molecular techniques have suggested possible involvement of these *Proteobacteria* in ventilator-associated pneumonias. In a study from Sainte-Marguerite Hospital in Marseille, Berger and colleagues screened 210 ICU patients with pneumonia. Both ameba-associated and conventional agents were implicated in 15 patients and included *B. massiliensis*, *Mesorhizobium amorphae*, and several *Legionella*-like amebal pathogens (LLAPs).[113] In another study using amebal cocultivation, LaScola and colleagues tested water from ICU sites weekly for 6 months using amebal cocultivation, and the isolates that were obtained were adapted to growth on BCYE agar.[754] Organisms recovered from water samples included *Legionella anisa*, several

LLAPs, *A. broomeae*, *B. massiliensis*, and *Bradyrhizobium* species. At the same time, serum and BAL specimens for subsequent amebal coculture were obtained from 30 ICU patients. Amebal isolates from these specimens were adapted to growth on BCYE agar and characterized by molecular methods. Sera from patients were tested for antibodies against the ameba-associated bacteria obtained from ICU patients using indirect microimmunofluorescence. Twelve of 30 serum samples indicated host seroconversion to one or more ameba-associated bacteria from ICU water samples; most seroconversions occurred against *L. anisa* and *B. massiliensis*, which were the most common water isolates. The nucleic acids of *L. anisa* and *B. massiliensis* were each detected once (2 patients) in 66 BAL specimens obtained from the 30 patients. Seroconversion to *L. anisa* and *B. massiliensis* was observed 4 weeks and 2 weeks after the BAL specimen was PCR-positive for these organisms, respectively.[754]

*Mesorhizobiu*m and *Bradyrhizobium* species are α-*Proteobacteria* that are closely related to both *Afipia* and *Bosea* species, while *Rasbobacterium* forms a monophyletic subcluster related to *Afipia* and *Methylobacterium* species. *Mesorhizobium* and *Bradyrhizobium* species are found in water but have their specific ecologic niche in soil, where they establish symbiotic relationships with leguminous plants and fix atmospheric nitrogen in exchange for carbohydrates from the associated plants.*Rasbobacterium* was originally isolated in 1997 from a 33-year-old Swedish man with an

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TABLE 9-14 Phenotypic Characteristics for Identification of *Afipia* Species

Characteristic	*A. felis*	*A. felis* genospecies A	*A. clevelandensis*	*A. broomeae*	*A. birgiae*	*A. massiliensis*	*Afipia* genospecies 1	*Afipia* genospecies 2	*Afipia* genospecies 3	*Afipia* geno-species 3 related
Hemolysis, SBA	–	–	–	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$
Motility	+	+	+	+	–	+	+	+	+	+
Growth on BCYE agar, 30°C	+	+	+	+	+	+	+	+	+	+
Growth on BCYE agar, 35°C	+	+	$+^{w}$	+	–	–	+	+	+	–
Growth on BCYE agar, 37°C	+	+	–	+	–	–	+	+	+	–
Growth on BCYE agar, 42°C	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	–	–	+	+	–	–	–	+
Citrate	–	–	–	–	–	–	+	–	–	–
Urease	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	–	–	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–
ONPG hydrolysis	–	–	–	–	–	–	–	–	–	–
H_2S production	–	–	–	–	–	–	–	–	–	–
Acid production from:										
Glucose	–	–	–	–	–	–	+	+	–	–
Maltose	–	–	–	–	–	–	–	–	–	–
Fructose	–	–	–	–	–	–	–	–	–	–
Sucrose	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	+	+	–	–
Mannose	–	–	–	–	–	–	+	+	–	–
Arabinose	–	–	–	–	–	–	+	+	–	–
N-Acetyl-glucosamine	–	–	–	–	–	–	+	+	–	–

+, positive reaction; –, negative reaction, $+^{w}$, weak positive reaction; SBA, sheep blood agar; BCYE, buffered charcoal yeast extract; ONPG, o-nitrophenyl-β-d-galactopyranoside.

influenza-like illness, with subsequent development of persistent fevers, sepsis, and pericarditis.[139] The patient's fevers persisted for several months despite repeated courses of antimicrobial agents, and the patient experienced three relapses with similar symptoms over the subsequent 21 months. The organism was repeatedly isolated from blood and also from pericardial fluid. Of the three isolates recovered from the patient, two could be cultivated on cell-free media, while the third was isolated only by cocultivation in Vero cells. Phenotypically, *Rasbobacterium* is a motile gram-negative bacillus that grows optimally at 25°C to 30°C and is oxidase- and urease-positive.

Bartonella Species

Taxonomy

The genus *Bartonella* currently comprises at least 25 species and subspecies of fastidious gram-negative bacteria that are vector-transmitted and highly adapted to one

or more mammalian reservoir hosts. Older members of genus *Bartonella* were formerly classified in the genus *Rochalimaea* (*Bartonella quintana*, *Bartonella vinsonii*) and the genus *Grahamella* (*Bartonella talpae*, *Bartonella peromysci*, *Bartonella grahamii*, *Bartonella taylorii*, and *Bartonella doshiae*).[129,172] This genus also includes the agents of CSD, *Bartonella henselae* and *Bartonella clarridgeiae,* and several other species found in a variety of mammalian hosts. Genetic analyses by 16S rRNA sequencing and DNA hybridization techniques confirmed that *Bartonella* is the only genus in the family *Bartonellaceae*. This family resides in the order *Rhizobiales*, which is in the α-2 subgroup of the class α-*Proteobacteria*.

Epidemiology of Bartonella *Species*

The discovery of *B. henselae* and *B. quintana* as opportunistic human pathogens, the evidence for *B. henselae* as the agent of CSD and the likelihood of vector-borne transmission of these bacteria among animals and humans prompted extensive studies on the epidemiology of these novel organisms and their roles as emerging human and veterinary pathogens.[166,261,533] Using cultural, serologic, and molecular methods, several new *Bartonella* species have been discovered and described during the last several years. In 1993, a unique isolate named *B. elizabethae* was recovered from a patient with endocarditis.[324] In 1995, Clarridge and colleagues published a description of their laboratory approach for diagnosis of CSD and included presentations of two patients with CSD.[281] While *B. henselae* was recovered from both patients, a second *Bartonella* species was isolated from a kitten that belonged to one of the patients. This isolate was given the name *B. clarridgeiae* in honor of Dr. Jill Clarridge, and in 1997, the first patient with CSD caused by *B. clarridgeiae* was described.[721,764] During a survey to determine the prevalence of *B. henselae* in cats in the San Francisco Bay area, another new *Bartonella* species was isolated from two cats.[372] These two isolates were 97% to 100% related to one another by DNA sequencing, but only 68% to 92% related to the type strain of *B. henselae*. This new species was named *B. koehlerae*. In 2000, a fourth cat-associated species ("*B. weissii*") was isolated from felines in Utah and Illinois; this organism was identical to another species—"*B. bovis*"—which was subsequently found in beef cattle in North Carolina.[119,250,1072] During the period from 1993 to the present, several *Bartonella* species have been described in many vertebrate hosts that are transmitted by a wide range of confirmed or putative insect vectors. These species, their primary hosts, and known or putative vectors of transmission are listed in Box 9-9.

It is now well established that *Bartonella* species constitute a unique group of bacteria that are responsible for unusual infections in wild and domesticated animals and in humans. Infected animals may be asymptomatic or may develop significant disease, including chronic bacteremia. These animals serve as reservoirs and potential

sources of additional animal or human infections. In 1995, a novel *Bartonella* species was described by workers at the College of Veterinary Medicine at North Carolina State University as a cause of canine endocarditis. Phenotypic and genotypic characterization of this agent confirmed its relatedness to the "vole agent," *B. vinsonii*, and this organism was christened *B. vinsonii* subsp. *berkoffii*.[165,172,723] This subspecies represented a previously unrecognized cause of cardiac arrhythmias, endocarditis, myocarditis, syncope, and sudden death in dogs. In 2000, *B. vinsonii* subsp. *berkhoffii* was detected by PCR analysis in valvular tissue as the cause of human endocarditis in a 35-year-old man, who also had contact with several animals, including a dog.[1116] In 1999, another *B. vinsonii* subspecies, designated *B. vinsonii* subsp. *arupensis*, was isolated from blood cultures of a cattle rancher in Wyoming with an acute febrile illness that had a marked neurologic component.[1333]

Serosurveys and blood culture studies among small mammals, ruminants, and other wildlife have resulted in the descriptions of several *Bartonella* species. During a survey to determine potential reservoirs of *Bartonella* species in wild mammals, another new species, designated *B. alsatica*, was isolated from blood cultures of wild rabbits (*Oryctolagus cuniculus*) trapped in the Alsace region of France near the Rhine River.[574] This species was subsequently documented as a cause of endocarditis and axillary lymphadenitis in two humans, both of which had significant contacts with rabbits.[43,1064] The species *B. schoenbuchii*, *B. bovis*, *B. capreoli*, and *B. birtlesii* have been isolated from blood cultures of roe deer, dairy cattle, reindeer, and small mammals, respectively.[119,120,336] Isolation of bartonellae from rodents and other wild mammals has prompted searches for these bacteria in tick populations. Among DNA extracts from 109 *Ixodes ricinus* ticks collected from roe deer in the Netherland, 70% contained 16S rRNA gene sequences for *Bartonella* or species closely related to *Bartonella*.[1158] *Bartonella* gene sequences have also been detected in extracts prepared from *Ixodes* and *Dermacentor* ticks that were collected in California.[232] *Ixodes ricinus* ticks fed on ovine blood infected with cultured *B. henselae* were demonstrated to transmit the bacteria across larval developmental stages. *B. henselae* migrated to the salivary glands of the tick, multiplied, and were actively transmitted from the infected tick to blood, thereby providing experimental evidence that *I. ricinus* is a competent vector for *B. henselae*.[311]

Bartonella infection is widespread and highly prevalent in rodent populations in different localities. The species

BOX 9-9

Bartonella Species, Reservoirs, and Vectors of Transmission

Species	Principal Reservoir	Associated Vector	References
Human Pathogens			
B. bacilliformis	*Homo sapiens*	*Lutzomyia verrucarum* (sand flies)	172
B. quintana	*Homo sapiens*	*Pediculus humanus corporis* (human body lice)	172
B. henselae	*Felis catus* (cats)	*Ctenocephalides felis* (cat fleas), ticks	1335
B. clarridgeiae	*Felis catus* (cats)	*Ctenocephalides felis* (cat fleas)	764
Uncommon or Suspected Human Pathogens			
B. alsatica	*Oryctolagus cuniculus* (rabbits)	Fleas	574
B. elizabethae	*Rattus norvegicus* (rats)	Fleas	172
B. grahamii	*Clethrionomys glareolus* (bank voles)	*Ctenophthalamus nobilis* (fleas)	129
B. koehlerae	*Felis catus* (cats)	*Ctenocephalides felis* (cat fleas)	75, 168, 372
Candidatus "B. mayotimonensis"	Unknown	Unknown	788
Candidatus "B. melophagi"	Unknown	Unknown	822
B. vinsonii subsp. *arupensis*	*Peromyscus leucopus* (white-footed mice)	Fleas, ticks	172, 418, 1333
B. vinsonii subsp. *berkhoffii*	*Canis familiaris* (dogs), *Canis latrans* (coyotes)	Ticks, fleas	164, 172, 234, 723
B. rochalimae	*Urocyon cinereoargenteus* (Gray foxes), *Canis familiaris* (dogs), *Rattus norvegicus* (rats)	Pulex spp. (fleas)	399, 579, 791
B. tamiae	Unknown	Unknown	727
B. washoenesis	*Spermophilus beecheyi* (California ground squirrels)	Fleas	728
Not Recognized as Human Pathogens (Yet!)			
Candidatus "*B. antechini*"	*Antechinus flavipes* (Mardos [small carnivorous marsupial])	Fleas (*Acanthopsylla jordani*), ticks (*Ixodes antechini*)	671
B. australis	*Macropus giganteus* (gray kangaroos)		443
B. birtlesii	*Apodemus* spp. (wood mice)	Fleas	120
B. bovis	*Bos taurus* (domestic cattle)	Ticks, biting flies	119
B. capreoli	*Capreolus capreolus* (roe deer)	Biting flies	119
B. chomelii	*Bos taurus* (domestic cattle)		827
B. coopersplainsensis			530
B. doshiae	*Microtus agrestis* (field voles)	Fleas	129
B. japonica	*Apodemus* spp. (wood mice)		625
B. peromysci	*Peromyscus* spp. (field mice)	Fleas	129
B. phoceensis	*Rattus norvegicus* (rats)	Fleas	529
B. queenslandensis			630
B. rattaustraliani			530
B. rattimassiliensis	*Rattus norvegicus* (rats)	Fleas	529
B. schoenbuchensis	*Capreolus capreolus* (roe deer)	Biting flies	336, 1101
B. silvatica	*Apodemus* spp. (wood mice)	Biting insects	625
B. talpae	*Talpa europaea* (moles)	Unknown	129
B. taylorii	*Apodemus* spp. (wood mice)	*Ctenocephalides nobilis* (fleas)	129
Candidatus "*B. thailandensis*"	*Rodents*		1129
B. tribecorum	*Rattus norvegicus* (rats)	Fleas	575
B. vinsonii subsp. *vinsonii*	*Microtus pennsylvanicus* (meadow voles)	Fleas	172, 723

B. tribecorum was isolated from the blood of two wild rats (*Rattus norvegicus*) that were trapped in the Alsace region of France.[575] A field study of 7 rodent species from 12 different geographic areas in the southeastern United States found *Bartonella* species in blood cultures of 42.2% of 279 rodents tested.[729] These isolates represented 14 phenotypic variants that clustered into 7 phylogenetic groups, 4 of which contained previously described *Bartonella* species. Genetic analyses of isolates from Peruvian rats and from rats (*R. norvegicus*) captured in Louisiana and Maryland revealed gene sequences identical with those of *B. elizabethae*, which has otherwise been recovered only from a patient with endocarditis.[324] A study conducted in Japan found that 26% of 546 small mammals imported into Japan as pets had *Bartonella* bloodstream infections.[626] These animals included Egyptian jerboas, gerbils, spiny mice, and sand rats. Ellis and coworkers suggested that *Rattus* species, and perhaps other rodents, are the reservoir for *B. elizabethae*, and that the presence of antibodies against *B. elizabethae* among inner-city intravenous drug users reflected infection with this or a related *Bartonella* species.[392] In a study of 200 sera from "street people" using a free clinic in downtown Los Angeles, the seroprevalence of antibodies to *B. elizabethae*, *B. quintana*, and *B. henselae* was 13.6%, 12.5%, and 9.5%, respectively.[1200]

The involvement of *B. henselae* and *B. clarridgeiae* in CSD prompted investigations into the prevalence and pathogenicity of these organisms in domestic cats, the reservoir for both organisms. Seroprevalence studies have documented high rates of *Bartonella* infection among cats in various parts of the world. These studies have involved detection of feline bloodstream infections and seroepidemiology. In a study conducted in San Francisco, 41% of blood specimens from 61 impounded or pet cats were culture-positive for *B. henselae.*[714] Among 205 cats living in Northern California, 39.5% had sustained *B. henselae* bacteremia and 81% tested positive for *B. henselae* antibodies, with bacteremic cats having higher antibody titers than nonbacteremic cats.[257] A study conducted in France on 94 stray cats from 10 different cat colonies reported that 53% of the animals were bacteremic with a *Bartonella* species.[573] Of the 50 isolates obtained, 35 were *B. henselae* or a *B. henselae* variant, and 15 were *B. clarridgeiae*. A second French study of 436 domestic cats found that 5 cats (1.1%) were co-infected with *B. henselae* and *B. clarridgeiae*, while 2 cats (0.5%) were co-infected with two variant *B. henselae* strains, showing differences in 16S rRNA gene sequences.[534] Seroprevalence studies conducted on large cat populations in Baltimore, Sweden, and Denmark reported *B. henselae* seroprevalences of 14.7%, 8.3%, and 45.5%, respectively.[254,259,490] Another Swedish study demonstrated that 25% of 292 cats had antibodies to *B. elizabethae*, with the highest

prevalence (46%) being found in cats living in Stockholm.[589] The authors pointed out that *B. elizabethae* was the most common agent found in human patients in the same geographicl region.

Bartonella species, particularly *B. henselae*, have also been sought in cat fleas (*Ctenocephalides felis*), the presumed vector responsible for transmission of bartonellae among cats. A survey of 113 cats from shelters in the Netherlands found that 50% were seropositive for *Bartonella* species and 22% of the cats had positive blood cultures.[115] Seven of 27 DNA extracts of cat fleas removed from these cats contained *Bartonella* DNA. Chomel and coworkers detected *B. henselae* DNA in 34% of 132 fleas removed from 47 cattery cats, 89% of whom were bacteremic with *B. henselae*.[262] Cat fleas removed from bacteremic cats were able to transmit *B. henselae* to specific pathogen-free cats. However, pathogen-free kittens housed together with bacteremic cats without fleas did not become infected with *B. henselae*. It has been shown that *B. henselae* can multiply to high numbers in the cat flea, and the organism can remain viable in flea feces for at least 3 days.[423]

In cats, bacteremia with *B. henselae* or *B. clarridegiae* is usually asymptomatic, and the presence of overt disease appears to be strain-dependent. Kordick and colleagues inoculated 18 specific pathogen-free cats with *B. henselae* or *B. clarridgeiae* and found that despite persistent bacteremia, clinical signs and symptoms were minimal.[720] *Bartonella* DNA could be detected by PCR in the blood, brain, lymph nodes, myocardium liver, and kidneys of infected animals. O'Reilly and coworkers inoculated 9 cats with a virulent *B. henselae* strain and found that all 9 became bacteremic within 14 days' postinfection; one animal remained bacteremic up to 18 weeks' postinfection.[980] All animals developed anti-*B. henselae* antibodies. In another study of 19 cats belonging to 14 patients with CSD, 17 of them had positive blood cultures and 13 cats remained culture-positive for *Bartonella* during the ensuing 12 months.[724] A study of three naturally infected cats over a 2-year period found relapsing bacteremias in all three cats, with intervals of 3 to 19 months between relapses.[668] Following bacteremic episodes, increases in *B. henselae*-specific antibody titers were noted. Serial blood isolates of *B. henselae* from individual bacteremic cats were shown to have different RFLPs over time. The emergence of genetically distinct organisms at various peaks of bacteremia in these animals may contribute to the establishment of persistent bacteremic infections.

The development of polyphasic cultural/molecular approaches for detection of bartonellae has revealed that these organisms have an impact on canine health that was not appreciated before. The involvement of *B. vinsonii* subsp. *berkhoffii* as a cause of endocarditis in dogs was established in the mid-1990s, and other *Bartonella* species have been detected in related species, such as coyotes and foxes.[234] The involvement of other *Bartonella* species, including *B. henselae*, in canine disease has now also

been established. During the last 10 years, at least eight *Bartonella* species, including *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, *B. koehlerae*, *B. clarridgeiae*, *B. elizabethae*, *B. washoensis*, *B. quintana*, and *B. rochalimae*, have been implicated in canine infections and disease syndromes.[166,258,378,533] Although thought to infect only cats, *B. henselae* was isolated from a dog in Gabon in 2004.[528] Subsequently, *B. henselae* was detected in lymphoid and liver tissue specimens in canines with generalized granulomatous lymphadenitis, granulomatous hepatitis, and peliosis hepatis using cultural and molecular techniques.[377,378]

Bartonella species are associated with infections in the compromised host, particularly patients with HIV-1 infection. *Bartonella* infections now include several well-described conditions, including classical and urban trench fever, bacillary angiomatosis with cutaneous and/or systemic

involvement, peliosis hepatis, relapsing fever with bacteremia, endocarditis, and CSD.[1271] The type species of the genus *Bartonella*, *B. bacilliformis*, causes Oroya fever, a geographically restricted febrile illness. The unfolding epidemiology of *Bartonella* species, increasing knowledge regarding animal and human disease, and information concerning the variety of insect vectors responsible for zoonotic infection are largely due to elegant laboratory approaches based on both traditional culture methods and advanced molecular-based technologies.[166,673]

Human Infections Associated With Bartonella *Species*

Oroya Fever and Verruga Peruana. These two clinical entities are manifestations of infection with *Bartonella bacilliformis*. These infections are geographically restricted because of the limited habitats of the principal sandfly vectors *Lutzomyia verrucarum* and *Lutzomyia peruensis*. The reservoir of the organism is unknown, although humans with asymptomatic bacteremia may serve as the reservoir. Infections are seen in river valleys of the west Andes Mountains at altitudes between 500 and 3,200 meters above sea level in Peru, Ecuador, and Columbia.[614] This disease is emerging as a public health problem in Peru, and recent reports have documented human *B. bacilliformis* infections over larger geographic areas, including the Columbia, Ecuador, Andean highlands, and the Amazon region east of the Andes Mountains.[726,820,825] In recent years, the highest incidence of disease has occurred in pediatric age groups.[229] This infection is also known as Carrion's disease after Daniel Carrion, a Peruvian medical student who developed the disease after inoculating himself with material from an infectious lesion. Humans are the only known reservoir/host for *B. bacilliformis*. Following the bite of an infected female vector, symptoms of Oroya fever develop

from 3 weeks to 3 months, although asymptomatic infection may also occur. Disease onset may be abrupt or insidious. With an insidious onset, the patient presents with anorexia, headache, malaise, and a slight fever lasting 2 to 7 days or more. When the onset is abrupt, the patient presents with fever, severe headache, chills, abdominal pain, arthralgias, and nausea and vomiting. Pallor and severe anemia ensue because of destruction of red blood cells by the organisms. Jaundice, hepatosplenomegaly, and generalized lymphadenopathy appear, along with severe muscle and joint pain.[824] Common complications include neurologic involvement, meningeal signs (e.g., delirium, seizures, coma), and respiratory symptoms (i.e., dyspnea). About 30% of patients develop opportunistic infections or reactivation of latent infections (e.g., toxoplasmosis, salmonellosis, tuberculosis, histoplasmosis) because of cellular immune compromise. Noninfectious complications include anemia, intracranial hypertension, and multiorgan dysfunction. During this phase, the organisms may be isolated from the blood and can actually be seen on blood smears within red blood cells. Acute infection during pregnancy can result in abortion or maternal and fetal death. Untreated, the fatality rate of Oroya fever ranges from 45% to 90%; with treatment, fatalities are reduced to less than 10%. Most mortality is seen in pediatric age groups. This "critical" stage lasts from 2 to 4 weeks, at which time the organisms disappear from the circulation, the fever normalizes, and the anemia spontaneously resolves.

After resolution of fever, pain in the bones, joints, and muscles may persist to the eruptive stage of verruga development, which is characterized by the development of lesions on exposed parts of the body, on mucous membranes, or in internal organs.[614]These lesions develop over 1 to 2 months and may persist for months to years. Lesions that develop during the eruptive phase may be miliary (i.e., multiple, widely scattered, and small 2 to 3 mm lesions), nodular (i.e., few eruptions, 8 to 10 mm in diameter), single or diffuse, large, deep-seated lesions.[95] When large lesions are located near joints, they may impede mobility. Mucocutaneous lesions may also appear in the mouth, conjunctiva, and nasal passages. Symptoms associated with the eruptive phase include fever, headache, malaise, arthralgias, osteoarticular pain, and bleeding of the lesions.[825] Joint pain and fever usually subside after appearance of the skin lesions. Anemia is usually not present during this stage of the disease. Histopathology of the lesions show dermal dendrocytes interspersed with macrophages, lymphocytes, and plasma cells; these lesions have been confused with malignant neoplasms, lymphomas, or sarcomas.[56]

B. bacilliformis can be seen in the tissues both intra- and extracellularly with the Warthin–Starry or Giemsa stain and can be cultured from cutaneous lesions and from blood and bone marrow. On Giemsa-stained blood films, the organisms appear as blue-to-purple extraerythrocytic and intraerythrocytic bacilli and coccobacilli. Susceptibility testing of human isolates of *B. bacilliformis* has shown that these bacteria are susceptible to β-lactams, chloramphenicol, tetracyclines, macrolides,

rifampin, the aminoglycosides, and the fluoroquinolones.[1203] Oroya fever has been treated with a variety of agents, including penicillin, tetracycline, and streptomycin, but chloramphenicol is considered the drug of choice. During the last decade, however, resistance to chloramphenicol has emerged in some isolates of *B. bacilliformis*.[1203] Acute-phase infections should be treated with ciprofloxacin for 10 days, with dosages adjusted depending on age.[614] If complications develop, ciprofloxacin is given with ceftriaxone or ceftazidime for 10 days.[1100] Amoxicillin clavulanate has been used for uncomplicated Arroyo fever in pediatric patients with good results. Adjunctive therapies (e.g., packed red blood cell transfusions) may be needed in patients with severe anemia. Complicated infections in pregnant women are treated with chloramphenicol in conjunction with penicillin G for 14 days. For the eruptive phase of the disease, chloramphenicol and penicillins are not effective. Rifampin may be given to both children and adults for 7 to 14 days and should be given along with another active agent, as rifampin resistance may emerge rapidly. Ciprofloxacin, erythromycin, and azithromycin are effective oral agents and should be taken for 7 to 14 days.

"Classical" and "Urban" Trench Fever. *B. quintana* is the agent of trench fever, a louse-borne, debilitating febrile illness that affected almost 1 million people, including German and allied military personnel during World War I.

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After the war, trench fever continued to occur in Spain, France, Italy, parts of Eastern Europe, North Africa, and China. *B. quintana* has also been detected in lice obtained from regions of Peru and South America where trench fever has not been documented previously. Serologic tests indicate the presence of the organism in Bolivia and Mexico as well. The organism is transmitted by the human body louse, *Pediculus humanus*, which has increased in prevalence worldwide during the last 10 to 15 years, particularly in resource-limited countries and in areas of great social, political, and economic turmoil, such as the former Soviet Union and regions of Eastern Europe. The bacterium infects the body louse when it bites an infected human, as humans are the only known reservoir of these agents. The body louse transmits the infection when it subsequently bites another human and sheds organism-laden feces that contaminate the bite wound.[95] After an incubation period of 5 to 20 days, the disease may have a long clinical course that may last 4 to 8 weeks, with relapsing fevers, malaise, shaking chills, drenching sweats, and severe neck, back, and leg pain. Conjunctivitis, retro-orbital pain, and tibial (i.e., shin-bone) pain are characteristic. Splenomegaly and macular rashes have been observed in some patients. Fevers may last 4 to 5 days during each relapsing cycle or may persist unabated for weeks. Macular or papular

rashes may also appear in crops on the truck and extremities. The disease is usually not severe and is self-limited. Patients have a rapid response to treatment with tetracyclines and/or chloramphenicol.

Persistent *B. quintana* bacteremia was a recognized symptom of classical trench fever and is a common manifestation of reemergent or "urban trench fever" among the homeless and alcoholic residents of urban envoironments.[186,1215] This infection is associated with fever, night sweats, prolonged or intermittent bacteremia, louse infestation, and high antibody titers against *B. quintana*. The appearance of *B. quintana* infection among homeless individuals in urban areas has prompted investigations of emergent trench fever and other louse-borne illnesses among indigent people in several cities. Fever and bacteremia caused by *B. quintana* was first documented in 10 homeless, inner-city patients in Seattle who were HIV antibody-negative but had chronic alcoholism as an underlying disease.[1215] All of these individuals presented with fever, two had splenomegaly, two reported a recent cat scratch, and one developed endocarditis (see below).[1214] Brouqui and colleagues documented *B. quintana* bacteremia in 10 (14%) of 71 homeless patients in France.[186] Five of these patients were chronically bacteremic as evidenced by multiple positive blood cultures over several weeks, and eight patients with bacteremia were afebrile. In addition, 30% of the patients had high titers of anti-*B. quintana* antibodies, and recent infections (i.e., bacteremia or seroconversion) were detected in 17 patients (24%). In this study, homeless patients with *B. quintana* bacteremia were more likely to have had exposure to lice, and to have headaches, leg pain, and thrombocytopenia (i.e., low platelet counts), compared with those who did not have bacteremia or were seronegative for *B. quintana* antibodies.[185,186] *B. quintana* DNA was detected by PCR in lice collected from 3 of 15 patients from whom they were collected. Two of these patients were bacteremic, and one was seropositive. Serologic studies have also demonstrated a high prevalence of *Bartonella* infections among inner-city IV drug users. Comer and colleagues reported that 37.5% of serum specimens collected from 630 IV drug users in Baltimore reacted with at least one *Bartonella* antigen (i.e., *B. henselae*, *B. quintana*, or *B. elizabethae*) by indirect FA (IFA).[297] The role of body lice in the transmission of urban trench fever has been confirmed by several epidemiologic and laboratory investigations. In one study, lice were collected from 268 persons at the Moscow Municipal Disinfection Center and subjected to PCR analysis for detection of the *B. quintana gltA* gene sequence. *B. quintana* DNA was detected in 12.3% of the lice samples studied.[1125] In a study of 139 homeless persons seen by San Francisco Project Homeless, 33 (23.9%) had body lice and 12 (8.7%) had head lice infestations. In this survey, 33.3% of those with body lice and 25% of those with head lice had lice pools infected with *B. quintana*.[149] These studies have confirmed the pivotal role of *P. humanus* in the epidemiology of reemergent, 21st century trench

fever.

Bacillary Angiomatosis. Bacillary angiomatosis (BA) is a bacterial infection caused by *B. henselae* and *B. quintana*, which results in an unusual widespread vascular proliferation; this is seen most commonly in patients with advanced HIV disease.[1031,1035,1142] BA has also been reported in immunosuppressed transplant patients, individuals receiving cancer chemotherapy, and patients undergoing long-term corticosteroid treatment; it may also develop in immunocompetent adults and children.[121,600,1412] Patients with AIDS and BA usually have CD4 counts of less than 200 cells/μL when lesions appear. The disease may manifest as cutaneous lesions or may occur as a disseminated infection. Cutaneous lesions manifest as maculopapular lesions, pedunculated lesions, hyperkeratotic or indurated plaques, and nodular lesions with or without subsequent ulceration.[1159,1412] Usually BA develops as discrete lesions that enlarge and coalesce to form larger nodules. Lesions of Kaposi's sarcoma (KS) are usually light pink to brown or black and are plaque like rather than nodular, although on some body surfaces, BA lesions may initially develop as flat, hyperpigmented, indurated plaques. BA formation in part is due to ability of bartonellae to induce angiogenesis and tumorous vasculoproliferative lesions.[1035] Lesions may be located deep in the subcutaneous tissues (e.g., liver, spleen) and may involve bone, forming large osteolytic lesions.[1109] Bone involvement with BA is painful, usually affects the radius, fibula, and tibia, and is associated primarily with *B. quintana* infection. Visceral involvement may occur as disseminated vascular lesions or as bacillary peliosis hepatis when the liver is involved. Extracutaneous BA has been documented in upper and lower respiratory and gastrointestinal tracts, the conjunctivae and orbit, the heart, diaphragm, biliary tract, muscles, liver, spleen, lymph nodes, the genital tract, and the CNS. Internal lesions may cause compression or obstruction of internal organs, with constitutional signs and symptoms of fever, anorexia, vomiting, and weight loss. Histopathologic diagnosis of BA requires punch or excisional biopsies for cutaneous and subcutaneous lesions. Excision is usually used

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for deep-seated lesions. Biopsies may also be submitted for culture and analysis by electron microscopy and molecular methods.

Histologically, cutaneous BA lesions are composed of lobular proliferations of blood vessels composed of plump "epithelioid" endothelial cells within an edematous to fibrotic stroma. Usually, there is an abundant neutrophilic infiltration and aggregates of purplish granular material, representing clusters of bacteria. When biopsied tissue sections are stained with the Warthin–Starry silver stain, blue-black clumps and tangled masses of interstitial bacteria are seen.[475,911] Similar histologic patterns are seen in

other affected tissues, including mucocutaneous lesions, lymph nodes, liver, spleen, bone marrow, gastrointestinal tract, and peritoneum. The differential diagnosis of BA includes CSD, angiosarcoma, KS, pyogenic granuloma, hemangioma, glomangioma, angiokeratoma, and verruga peruana. BA lesions can be histologically differentiated from KS lesions in that there are no bizarre, jagged blood vessels as seen in KS and the inflammatory infiltrate consists of lymphocytes, histiocytes, and neutrophils as opposed to plasma cells.[1368] While BA and pyogenic granulomas are both vascular proliferations with endothelial cell swelling and stromal edema, the latter condition has less of a neutrophilic infiltrate and does not contain the amorphous aggregates of bacteria seen with the Warthin–Starry stain. KS differs histologically from BA in that KS displays slit-like and irregular vascular spaces and spindle-shaped endothelial cells that do not protrude into the lumen. Although there are morphologic characteristics useful in differentiating BA from KS, histochemical and immunohistochemical stains are commonly used to finalize the diagnosis. KS lesions will also stain positively for human herpes virus type 8 using immunoperoxidase staining and will be negative with Warthin–Starry staining. The infiltrating growth pattern, nuclear pleomorphism, and absence of Warthin–Starry bacillary staining help differentiate angiosarcoma from BA. *B. henselae* and, in some cases, *B. quintana* have been isolated from the blood, skin lesions, bone, visceral organs, and brains of patients with BA.[475,600] In a 1997 case–control study of 49 patients with BA in San Francisco, 53% were infected with *B. henselae* and 47% were infected with *B. quintana*. *B. henselae* infection was linked with exposure to cats and cat fleas, while infection with *B. quintana* was associated with low income, homelessness, and lice exposure.[716] BA may be a manifestation of CSD peculiar to the compromised host. *Bartonella* organisms are able to stimulate angiogenesis, the physiologic process that results in the formation of new blood vessels, and to affect the migration and proliferation of endothelial cells.

Cutaneous BA usually responds to treatment with erythromycin, but antimicrobial therapy must be continued for 4 to 12 weeks.[911,1100] Doxycycline may also be used in those who cannot tolerate erythromycin. Longer courses of antimicrobial agents may be necessary since lesions often recur after the cessation of therapy. BA lesions that are confined to the skin may be excised surgically. Good clinical responses to other antibiotics (ceftriaxone, ciprofloxacin, other macrolides, chloramphenicol, SXT, and ciprofloxacin with gentamicin) have also been reported, but relapse is common following cessation of therapy. Drugs that inhibit cell wall biosynthesis (e.g., penicillins and cephalosporins) are not useful in the treatment of BA.

Peliosis. Peliosis hepatis is characterized by the presence of cystic, blood-filled lesions that are scattered throughout the parenchyma of the liver.[1368] Cases of peliosis involving the liver and spleen (splenic peliosis) have been reported in association with HIV infection.[1022] Most patients with hepatic or splenic peliosis also have BA, and

bacteria similar to those seen on Warthin–Starry stained biopsies of BA are also seen within the blood-filled cysts that are present in the liver and spleen. Most patients with bacillary peliosis hepatis present with weight loss, abdominal pain, intractable nausea, anemia, diarrhea, fever, hepatosplenomegaly, and lymphadenopathy. Liver enzymes are usually elevated, with alkaline phosphatase levels being as high as 5- to 10-fold above the normal value. Parenchymal bacillary peliosis of other internal organs (i.e., heart, larynx, lungs, adrenals, cervix, ovaries, pineal gland, and choroid plexus) has been reported as well. Peliosis hepatis appears to be associated exclusively with *B. henselae* infection and is believed by some to be another manifestation of BA.

Fever and Bacteremia. *B. henselae* bacteremia has been described in HIV-infected individuals, patients with AIDS, pharmacologically immunosuppressed recipients of allogeneic transplants, and occasionally immunocompetent hosts with no known risk factors for immunosuppressive disease.[1071,1197,1335] Immunocompromised patients present with relapsing fevers, weight loss, malaise, and fatigue. *B. henselae* bacteremia has been detected in those with and without focal BA or peliosis. In HIV-infected patients, *B. henselae* may also present as a generalized, inflammatory disease of the RES; organisms may be detected histopathologically or by molecular techniques in necrotic, inflammatory lesions in the spleen, liver, heart, bone marrow, and lymph nodes.[1196] In immunocompetent, HIV-negative individuals, *B. henselae* bacteremia presents with an abrupt onset of fever with accompanying joint and muscle pain, and some patients may manifest signs and symptoms of CNS involvement (i.e., headache, photophobia, and meningismus). *B. henselae* has been documented serologically as the cause of "fever of unknown origin" in both children and adults.[635,823,1268] These patients present with fever, anorexia, headache, and other constitutional symptoms. Bacteremia with *B. henselae* and *B. vinsonii* subsp. *berkhoffii* has also been associated with neurologic and neurocognitive dysfunction and neurologic disease.[167,169]

As mentioned earlier persistent fevers and bacteremia are hallmarks of both classical and urban Trench fevers as well.

Immunocompetent patients usually have a rapid response to a short (10 days or less) course of therapy, and generally do not relapse; in some cases, infections may resolve without antimicrobial therapy. However, Lucey and colleagues reported on two immunocompetent patients who had relapsing illness with positive blood cultures for *B. henselae*.[810] Interestingly, both of these patients had experienced tick bites before becoming ill. These two cases were the first in

which transmission of *B. henselae* by insects was epidemiologically suggested.

Drancourt, Raoult, and others reported two patients with bacteremia and chronic granulomatous mediastinal adenopathy from whom *B. quintana* was recovered from blood and bone marrow cultures.[370,1062] These patients were middle-aged women who were seronegative for HIV, had underlying diseases (i.e., corticosteroid treatment, chronic renal failure requiring hemodialysis), had exposure to cats, kittens, and cat fleas, and were successfully treated with parenteral aminoglycosides. Interestingly, neither patient developed a detectable serologic response against *B. quintana*.

Endocarditis. *Bartonella* species, and *B. quintana* in particular, have been isolated from several patients with "culture-negative" endocarditis and myocarditis.[371,385,442,540, 639,905,1393] Endocarditis due to *B. quintana* was first reported in 1993 in a 50-year-old, HIV-1 infected homosexual man who presented with night sweats, weight loss, and fatigue.[1213] On physical examination, mild renal insufficiency, splenomegaly, anemia, and a holosystolic heart murmur were noted. Echocardiography revealed mitral and aortic valve vegetations. Subsequently, *B. quintana* was documented as a cause of bacteremia in 10 febrile, homeless, alcoholic patients in Seattle; one of these patients developed aortic valve endocarditis.[1214] *B. quintana* was also recovered as the cause of endocarditis in three homeless, alcoholic men in France.[369] All four of these patients required aortic and/or mitral valve replacement. In 1993, Hadfield and coworkers described the first case of endocarditis caused by *B. henselae* in a 59-year-old HIV-negative man with a history of alcohol abuse; another instance of *B. henselae* endocarditis was subsequently reported in 1995 in a previously healthy 41-year-old man.[542,599] Both of these patients required valve replacement surgery. Since then, *B. henselae* has been identified as the cause of both native and prosthetic valve endocarditis in immunocompromised and immunocompetent patients.[620,684,1393] *B. elizabethae* was first reported as a cause of endocarditis in an immunocompetent 31-year-old man.[324] This infection resolved with valve replacement and treatment with vancomycin and imipenem. In 1996, Raoult and colleagues reported on 22 additional French patients with *Bartonella* endocarditis who were diagnosed by serology, culture, and molecular techniques.[1063] Of the 22 patients, 11 were alcoholics, 9 were homeless, 13 had preexisting valvular injuries, and 4 had histories of cat exposure. Most patients had aortic valve vegetations, and many developed embolic complications, with all but two patients requiring valvular surgery. Implicated organisms included *B. quintana* (5 patients), *B. henselae* (4 patients), and an undetermined *Bartonella* species (13 patients). The last 13 patients were diagnosed by serologic tests that could not distinguish between *B. quintana* and *B. henselae*. In this study, *B. quintana* endocarditis was associated with homelessness and chronic alcoholism without previously existing valvular damage, while *B. henselae* endocarditis occurred mostly in patients with preexisting valvular pathology and a history of contact

with cats. *Bartonella* endocarditis has been successfully treated with combinations of parenteral amoxicillin and gentamicin, parenteral vancomycin with ofloxacin and netilmicin followed by oral ofloxacin, rifampin, and pristinamycin, and with parenteral ceftriaxone followed by a prolonged course of oral erythromycin.[369,1063,1214] Two patients with endocarditis caused by *B. vinsonii* subsp. *berkhoffii* have also been reported.[972,1116]

Bartonella species also cause endocarditis and other cardiac disease in dogs.[263] Breitschwerdt and colleagues in North Carolina described a case of endocarditis involving the aortic and mitral valves in a 3-year-old spayed Labrador retriever.[165] A fastidious gram-negative bacillus was isolated from the dog's blood using the lysis-centrifugation method, and DNA extracted from the involved heart valves was amplified by PCR and compared with other *Bartonella* species. DNA hybridization, 16S rRNA sequencing, cellular fatty acid analysis, and phenotypic testing of the isolate characterized the organism as a new *Bartonella* species that most closely resembled *B. vinsonii*. The name *B. vinsonii* subsp. *berkhoffii* was proposed for this new organism.[723] This agent represented a new veterinary pathogen causing cardiac arrhythmia, myocarditis, and endocarditis in dogs; it has also been reported as the agent of endocarditis in a human.[164,1116] *B. henselae*, *B. elizabethae*, and *B. clarridgeiae* have been detected by molecular techniques in blood specimens from dogs.[263] Wild and domesticated canines may be reservoirs for all of these agents.

Cat Scratch Disease. CSD is a common cause of lymphadenopathy, particularly in children and adolescents. Most infections occur between September and March in warm climates; the peak incidence in July and August. About 90% of patients have a history of exposure to cats, with a cat scratch or a cat bite having occurred in about 75% to 80% of these persons. Kittens seem to be more frequently implicated than adult cats, and transmission appears to be by direct contact, since the disease generally follows a bite, scratch, or lick from a young cat. About 3 to 10 days after the scratch or bite, a primary erythematous papule or pustule forms. This lesion persists for about 2 to 3 weeks. During this time, low-grade fever may be present in one-third of patients, with both malaise and fever being noted in about one-fourth of patients. About 10% will have headache and muscle aches with a sore throat and a faint rash that lasts for 1 to 2 weeks. Over 90% of patients develop regional lymphadenopathy, the location of which is dependent on the site of the cat bite or scratch. Since most lesions are on the upper extremities, the adenopathy usually develops in axillary, cervical, or submandibular lymph nodes. Noncontiguous or generalized lymphadenopathy and hepatosplenomegaly are uncommon, but when present may be confused with malignancy (i.e., lymphoma).[485,694] Affected lymph nodes usually exhibit granulomas, suppurative granulomas, stellate microabscesses, and follicular

hyperplasia. Clusters of bacillary organisms may be seen in lymph node biopsies stained with the Warthin–Starry silver impregnation stain. Enlarged lymph nodes usually regress spontaneously over several months, although up to 20% of patients may show lymphadenopathy for 12 to 24 months. Disease may be more severe, complicated, or disseminated in immunocompromised hosts; *Bartonella* infections have been transmitted by solid organ transplantation (e.g., liver transplantation).[146,1082,1163]

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Patients with CSD may have atypical presentations and complications may develop; so clinical acumen and a thorough patient history are important. CSD was reported in a 32-year-old veterinarian who initially sought medical attention for fever of unknown origin and persistent back pain over a month duration. This began after an accidental needlestick injury. Infection was confirmed by serologic and molecular testing.[790] Some patients, particularly children, may require surgical drainage of abscesses and/or removal of infected lymph nodes.[917] Complications include oculoglandular syndrome of Parinaud, which presents as an ocular granuloma or conjunctivitis with preauricular lymphadenopathy, osteomyelitis, epitrochlear mass mimicking rhabdomyosarcoma, hepatic and splenic microabscesses, hepatitis, and granulomas.[59,510,1098,1290] The oculoglandular syndrome results from a cat scratch, lick, or bite on the eyelid or conjunctivae, although autoinoculation from a primary lesion into the eye may also occur. CSD encephalopathy and aseptic meningitis are rare complications that occur 1 to 6 weeks after regional lymphadenopathy becomes apparent; these may be characterized by encephalitis, seizures, myelitis, neuropathy, and retinitis.[483,949,1367] The patient may exhibit headache, mental status changes, seizures, convulsions, combative behavior, and/or coma. Neuroretinitis associated with CSD has also been reported, including a patient with *B. henselae* bacteremia that resulted in acute loss of vision.[318,800] Systemic CSD may cause multifocal osteomyelitis at sites remote from the initial scratch or bite. Pulmonary manifestations of CSD are extremely rare.[845]

CSD is usually a self-limited illness that does not generally respond to targeted antimicrobial therapy. Despite in vitro susceptibility of *B. henselae* to many agents, their use for treatment of CSD has not resulted in clear-cut benefits for the patient. Only the aminoglycosides, and gentamicin in particular, have demonstrated effectiveness in the treatment of suppurative complications of CSD.[924] Reports of successful treatment of CSD with azithromycin may be related not only to the susceptibility of *Bartonella* species to this agent, but also to penetration and intracellular accumulation of azithromycin within neutrophils and macrophages, resulting in ratios of 40:1 for intracellular:extracellular drug concentrations.[252,872]

Ciprofloxacin has also been used successfully for the treatment of CSD, again presumably because of its intracellular accumulation.[597]

Microbiologic and serologic studies conducted over the last several years have established that *B. henselae* is the principal bacterial agent responsible for CSD. In 1992, Regnery and colleagues isolated *B. henselae* from the blood of an asymptomatic cat on two occasions and demonstrated that 88% of patients with CSD had high antibody titers to *B. henselae* antigens by IFA.[1073,1074] Molecular techniques were used subsequently by several research groups to demonstrate the presence of *B. henselae* DNA in purulent material from the suppurative lymph nodes of patients with CSD.[36,38,503] Using IFA, Zangwill and his collaborators found that 84% of 45 patients with CSD had anti-*B. henselae* antibodies compared with 3% of 112 control patients.[1411] Furthermore, 81% of cats belonging to CSD patients were also seropositive compared with 38% of 29 control animals. This study also reported that the risk of CSD was greater for those individuals with kittens than for those with adult cats, that individuals with bites and scratches from kittens were at higher risk for CSD than those with wounds from adult cats, and that risk of CSD was also greater if the kitten had fleas. Bacilli observed in biopsies from suppurative lymph nodes have also been identified as *B. henselae* by immunocytochemical staining with antibodies against *B. henselae*.[892] The etiologic role of *B. henselae* in CSD has been further strengthened by the large number of epidemiologic studies on *B. henselae* infection in domestic cats.

In 1997, a second *Bartonella* species, *B. clarridgeiae*, was also described in association with a case of CSD.[721] In this case, a veterinarian developed CSD after a bite wound from a 6-week-old cat. Blood cultures collected from the patient and serologic tests (IFA) for *B. henselae*, *B. quintana*, and *B. elizabethae* antibodies were negative. However, the serum of the patient reacted in an IFA test with a *Bartonella* isolate recovered from blood cultures obtained from the cat. Serologic evidence for *B. clarridgeiae* infection was also obtained from a CSD patient with constitutional symptoms, a history of exposure to cats and cat scratches, and a large chest wall abscess.[845] While cultures from the patient remained negative, *B. clarridgeiae* was isolated from the blood of one of the patient's cats.

Miscellaneous Infections. Since the discovery of the association of *Bartonella* species with CSD, BA, and peliosis hepatis, several other manifestations of *B. henselae* infection have been reported in both immunocompromised and immunocompetent hosts. In most of these infections, an association with young cats has been documented, lending further credence to the possibility that uncommon clinical manifestations of *B. henselae* infection represent part of a continuum in the clinical presentation of CSD. These manifestations have included neuroretinitis without

the oculoglandular syndrome of Parinaud, isolated unilateral and diffuse lymphadenitis, severe myocarditis, and neurologic disease associated with rapidly progressive dementia.[871,1161,1367] Meininger and colleagues described a healthy man who developed chronic active myocarditis due to *B. henselae* that resulted in heart failure and the need for heart transplantation.[879] The association of *Bartonella* with AIDS-related dementia was examined by a serologic study conducted in Los Angeles by Schwartzman and colleagues in which both cat ownership and neuropsychological decline and dementia were associated significantly with the presence of IgM antibodies to *B. henselae*.[1160] Caniza and coworkers described a case of opportunistic pulmonary infection due to *B. henselae* in a 19-year-old woman who was a kidney transplant recipient.[197] Tissue from pulmonary nodules of this patient was negative on culture but contained 16S rRNA specific for *B. henselae* by PCR. All eight of the domestic cats that lived with the patient had positive blood cultures for *B. henselae*; PCR analysis of these isolates established that the feline isolates were identical to that found in the lung tissue of the patient. *B. henselae* infection has also been responsible for intraocular and retinal inflammation and optic disk edema resulting in substantial loss of visual acuity.[1309] Golnick et al. reported four patients who presented with vision loss resulting from intraocular inflammation, swelling of the optic nerve, and retinal detachment.[496] Patients had elevated titers against *B. henselae* and responded to treatment

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with resulting improvements in vision. Neuroretinitis has also been diagnosed in a patient with serologic evidence of infection with *B. elizabethae*.[968] In an unusual presentation of infection, bilateral neuroretinitis accompanied by severe headache and behavioral changes was diagnosed in a 55-year-old infected woman, who also had insulin-dependent diabetes mellitus and hypothyroidism. PCR analysis of DNA extracted from intraocular fluids revealed the presence of a 16S rRNA gene sequence that showed 100% homology with *B. grahamii*, a species normally found in rodents in Europe and North America.[695] The patient owned a dog but had no history of exposure to cats, rodents, or other animals. The patient also had serologic evidence of *B. henselae* infection by EIA, which was likely due to cross-reactivity with *B. grahamii*. She responded to therapy with daily oral doxycycline and rifampin for 4 weeks. Intraorbital and retinal infections with *B. henselae* have also been diagnosed by detection of DNA in intraocular specimens.[496,1327] Renal microabscesses and vertebral osteomyelitis due to disseminated *B. henselae* infection have also been reported.[512,1132]

Emerging Pathogens in the Genus Bartonella

Bartonella species originally described in animals are increasingly being detected in humans, often in association with febrile illnesses. In 2006, Raoult and coworkers described a 74-year-old man who developed endocarditis. Excised valvular tissues and blood samples were inoculated into human endothelial shell vials, and growth of *Bartonella* species in the shell vials was demonstrated by immunofluorescence after 2 months. Extraction of DNA from the cultures, blood, and valve tissue, followed by amplification and sequencing, revealed that the isolate was *B. alsatica*, a species previously described in wild rabbits.[574,1064] In 2008, Angelakis and colleagues described a 79-year-old woman from France with a large axillary mass that developed 1 month after she had scratched her finger while butchering a wild rabbit. A fragment of her excised lymph node and some rabbit tissues was subjected to nucleic acid extraction, and the extracted DNA was used as a template in three PCR assays specific for the *B. alsatica* 16S-23S intergenic spacer region, the *ftsZ* gene, and 16S rDNA.[43] All results on the lymph node indicated infection with *B. alsatica.* In 2009, another patient in France was diagnosed with endocarditis due to *B. alsatica*.[645] *B. rochalimae*, an agent of infective endocarditis in dogs, has been isolated from rodents, gray foxes, and from humans.[399,579,791] *B. washowensis*, a species found in ground squirrels, was isolated from a 70-year-old man with fever and myocarditis; molecular studies documented that the source of the isolate was probably exposure to rodents or arthropod vectors carrying the agent.[728] *B. tamiae*, a relatively new species described in 2008, was isolated from blood cultures of three patients in Thailand.[727] Candidatus *B. melophagi*, a proposed but as yet unvalidated new *Bartonella* species that is associated with sheep, was isolated from blood cultures of two women, both of whom developed acute febrile illnesses.[107,822] The identification was confirmed by molecular analyses of 16S rRNA, the citrate synthase, and RNA polymerase gene sequences and assessment of the 16S-23S intergenic spacer region. Both women lived and worked with and around animals, but only one had any exposure to sheep. In 2010, a new *Bartonella* candidate species, candidatus *B. mayotomonensis,* was isolated from native aortic valve tissue of a 59-year-old male with endocarditis.[788] The unique identity of this isolate was determined by amplification and sequencing of the 16S rRNA, *ftsz*, *rpoB*, and *gltA* genes and assessment of the ITS region.

Detection, Isolation, and Identification of Bartonella *Species*

Specimen Types. Specimens that may be submitted for recovery of *Bartonella* species include blood, biopsy specimens of suspected cutaneous or systemic BA lesions, lymph node biopsies, and aspirates. Specimens collected early in the clinical course are preferable, particularly lymph node biopsy specimens from patients with

suspected CSD, and should be collected prior to the administration of antimicrobial agents. Late-stage, suppurative lymph nodes may not yield organisms because of the intense local cellular immune response. Lysis-centrifugation methods using the Isolator blood lysis method (Wampole, Cranbury, NJ) have been reported to enhance recovery of these fastidious bacteria from blood specimens. Blood may also be submitted in sterile plastic ethylenediaminetetraacetic acid (EDTA) or sodium citrate tubes. With EDTA blood specimens, freezing the blood and then thawing it at room temperature prior to direct plating may also enhance recovery. Tissue specimens should be homogenized prior to culture; the homogenate can be used for direct culture and for DNA extraction prior to molecular amplification/detection. Since the organisms produce little or no CO_2 during growth, detection of *Bartonella* species in automated blood culture systems (e.g., BACTEC; BacT/Alert, etc.) may be problematic. Isolates have been recovered from BACTEC high-volume aerobic resin PLUS 26 bottles, although the organisms did not register growth indices above the positive threshold. Therefore, staining smears prepared from inoculated bottles with the acridine orange fluorescent stain intermittently during incubation may be helpful for detection.[5]

Microscopic Examination of Clinical Specimens. The Warthin–Starry silver stain has been used to visualize bartonellae in tissue. This stain is nonspecific, and other microorganisms may also exhibit staining. Lymph node specimens from patients with suspected CSD may or may not exhibit positive staining if the biopsy is not submitted early in the clinical course. Biopsies from BA lesions will usually show large numbers of organisms in Warthin–Starry stained preparations.

Culture. *Bartonella* species may be isolated by inoculation of the appropriate agar media or by cocultivation in cell culture. Blood submitted in either Isolator or EDTA tubes and homogenized tissue specimens can be inoculated directly onto freshly prepared chocolate agar or brain–heart infusion (BHI) agar containing 5% fresh rabbit or horse blood. Blood-supplemented BHI, tryptic soy, and Columbia agars and enriched chocolate agar may also

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support growth. Selective media are not available, and media containing antimicrobial agents should not be used. Inoculated plates are incubated in a humid atmosphere at 35°C to 37°C in 5% to 7% CO_2 for at least 28 days. Growth is usually noted within 5 to 15 days, although some *Bartonella* strains may require up to 45 days incubation before growth is apparent.[760,1269] Subcultures from slow-growing primary cultures may require 15 to 20 days' incubation before good growth is obtained. Most bartonellae do not grow under anaerobic conditions, at temperatures of 25°C or 42°C,

or in the absence of hemin and CO_2. *B. bacilliformis* is exceptional, in that this species favors a lower growth temperature (i.e., 25°C to 28°C) and does not require supplemental CO_2 for growth. Some *Bartonella* species (e.g., *B. koehlerae*) may not grow on fresh rabbit blood agar and requires the use of freshly prepared chocolate agar for optimal recovery.

Isolation of *Bartonella* species has also been accomplished using cell culture methods, which are performed with shell vials containing a human endothelial cell monolayer (e.g., ECV 304 cell line), the T24 bladder carcinoma cell line, or other cell types (e.g., Vero cells, HeLa cells, L292 cells).[42,44,369,715,1062,1413] Specimens processed by this technique have included biopsy specimens from cutaneous and osseus BA lesions and heparinized blood. Koehler and colleagues used a bovine endothelial cell line (i.e., CPA cells, ATTC cell line #207) to isolate both *B. henselae* and *B. quintana* from minced tissue biopsies of BA lesions. After 9 to 36 days of incubation, the turbid culture supernatants were subcultured to agar media for subsequent growth and identification.[715] Drancourt and colleagues used ECV 304 cells (a continuous human endothelial cell line) to recover *B. quintana* from blood specimens of homeless patients with endocarditis.[369] With this technique, organisms can be detected in the shell vial by immunofluorescence and identified by PCR or other molecular techniques.[42,44] Broth-enrichment recovery of *Bartonella* species has also been performed with a defined medium containing RPMI 1640 (a tissue culture-type liquid medium) supplemented with hemin, amino acids, and pyruvate.[1362] This medium was used to successfully recover *B. henselae* from human and cat blood and from lymph node biopsies of CSD patients.

One of the most helpful developments in the cultural diagnosis of *Bartonella* infections is the use of preenrichment for subsequent molecular and cultural organism detection as part of a three-specimen source polyphasic cultural/molecular approach. Preenrichment is performed in *Bartonella* α-*Proteobacteria* growth medium (BAPGM), an optimized insect cell culture-based growth medium that is able to support growth of seven *Bartonella* species.[166,821] BAPGM uses a basal medium formulated for growth of insect cells in culture.[1086] This basal medium is supplemented with NAD, ATP, pyruvate, and several amino acids. After adjustment to a pH of 7.4, the medium is filter-sterilized and supplemented with defibrinated sheep blood at a final concentration of 5%. The polyphasic approach involves DNA extraction of the patient specimen for direct detection by PCR (first sample), a second PCR after enrichment culture in BAPGM for 4 weeks (second sample), and another PCR performed on any visible growth resulting after 4 weeks from a blood agar subculture of BAPGM after the 4-week incubation period.[359] BAPGM is commercially available (Galaxy Diagnostics, http://www.galaxydx.com). This polyphasic approach has been used for detection of *Bartonella* species in several

veterinary specimens from dogs and cattle (i.e., blood, tissue biopsies, CSF, abdominal, pericardial, seroma, and aqueous fluids, joint fluid).[82,249,359,377,907] Polyphasic detection using BAPGM has resulted in the recovery of various *Bartonella* species (i.e., *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, *B. bovis*, *B. koehlerae*, *B. volans*-like) from canines and the isolation of *B. tamiae* from febrile human patients in Thailand.[727,1019] Riess and colleagues described another enrichment growth medium that used Schneider's *Drosophila* powder medium (Serva, Heidelberg, Germany) supplemented with 10% fetal calf serum and glutamine.[1086] This medium grew *B. henselae* more efficiently than BAPGM. The Schneider's medium/fetal calf serum formulation does not contain whole blood or hemin; so the growth of *B. henselae* could be detected visually. The addition of 5% sucrose to this medium resulted in even faster growth of this fastidious organism, allowing these workers to actually calculate a generation time of about 5.6 hours for the *B. henselae* Marseille strain.[1086] Supplemented Schneider's medium also supported the growth of *B. quintana* and *B. vinsonii*. Lynch and colleagues compared several culture methods using strains of *B. henselae*, *B. quintana*, *B. elizabethae*, and *B. tamiae* and found that optimal growth of these organisms occurred using combined mammalian cell and insect cell culture medium with supplementation (i.e., 10% fetal calf serum, pyruvate, sucrose, HEPES buffer).[817] This combination medium also supported the growth of Vero E6 cells (primate kidney epithelial cells, ATCC CRL-1586). Although the cell-free medium supported adequate growth of laboratory-adapted *Bartonella* strains, coculture with Vero E6 cells may still be more sensitive for primary isolation of *Bartonella* species from clinical specimens of veterinary or human origin.

Gram's Stain and Colony Morphology. On primary isolation, *Bartonella* species appear initially as white, small adherent colonies that vary in size and shape. Some strains of *B. henselae*, *B. quintana*, and *B. elizabethae* may pit the agar surface during growth.[1269] Characteristically, *B. henselae* colonies are white or tan, dry, adherent, "cauliflower-like," embedded in the agar, and morphologically heterogeneous. Colonies of *B. quintana* are glistening, flat, smooth, and nonpitting on agar media. *B. elizabethae* colonies resemble *B. henselae* except that weak or partial hemolysis may be noted around colonies growing on heart infusion agar with 5% rabbit blood. Isolates of *B. clarridgeiae* form raised, white, adherent colonies on agar. With multiple passages, the colonies of most *Bartonella* species colonies become less dry, less adherent, and larger, and tend to grow faster. Colonies of *B. bacilliformis* differ from the others species in that they are initially small, smooth, and translucent and remain so upon serial subculture. On Gram's stain, *Bartonella* organisms appear as small, slightly curved, pleomorphic, gram-negative bacilli that measure about 1 µm to 2.5 µm in length by 0.5 µm to 0.6 µm. Strains of *B. henselae*, *B. quintana*, and several other species may show "twitching" motility when mounted in saline. Bartonellae may be

better visualized using the

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Gimenez stain, since counterstaining using either safranin or carbol fuchsin may be weak.

Identification Methods. In general, *Bartonella* species are biochemically nonreactive in routine phenotypic biochemical identification tests, including oxidase, catalase, indole, urease, decarboxylase, and nitrate reduction tests. A general presumptive identification may be based on the slow growth (usually greater than 7 to 10 days), characteristic colony morphology on agar-based medium, and negative oxidase and catalase tests. This presumptive identification must also consider the specimen type and the clinical presentation of the patient. Presumptive identification of certain *Bartonella* species, including *B. henselae*, *B. quintana*, and *B. vinsonii*, may be determined with commercial identification systems that use chromogenic enzyme substrates to detect preformed bacterial enzymes (e.g., Microscan Rapid Anaerobe panel, RapID ANA II, Rapid ID32A).[1334] These systems produced biochemical reaction patterns that were unique within their own data bases, but only the reactions on the Microscan Rapid Anaerobe Panel were able to separate *B. henselae* and *B. quintana* at the species level. This panel distinguished all species tested, generating unique biotype codes for each species (i.e., code 10077640 for *B. henselae*, code 10073640 for *B. quintana*, and code 10077240 for *B. bacilliformis*) (Table 9-15).

Bartonella species may be identified by gas-liquid chromatographic analysis of cellular fatty acids by mass spectrometry.[1335] All *Bartonella* species contain greater than 50% C18:1, 16% to 25% C18:0, and 16% to 22% C16:0, with minor amounts of C13:1 and C17:03 fatty acids. *B. henselae* isolates lack cellular fatty acids C15:0 and C12:0, which are present in isolates of *B. vinsonii* and *B. bacilliformis*, respectively. *B. quintana* could be differentiated from most *B. henselae* isolates by the presence of less than 20% C18:0 in *B. quintana* and greater than 20% C18:0 in *B. henselae*. The cellular fatty acid composition of *B. elizabethae* is most similar to that of *B. vinsonii* and includes C15:0 (not found in *B. henselae* or *B. quintana*) and larger amounts (21%) of C17:0 than *B. henselae* (3%), *B. quintana* (1%), and *B. vinsonii* (9%). *B. elizabethae* also contains smaller amounts of cellular fatty acid C16:0 (13%) than the other species, which contain from 17% to 20% C16:0 cellular fatty acids. MALDI-TOF mass spectrometry has also been used to identify *Bartonella* species. Reference strains representing 17 recognized *Bartonella* species were analyzed, and the consensus spectrum obtained for each of the species was unique among the various spectra for 2,843 bacterial organisms included in the Brucker MALDI-TOF database, including 109 other α-*Proteobacteria*. In addition, 39 blinded *Bartonella* strains were

correctly identified to the species level.[441]

Definitive identification of *Bartonella* species is best accomplished by amplification-based molecular methods. These methods can be used for confirmation of isolates recovered in culture, or may be performed directly on tissue, pus, or biopsies. PCR amplification of various genes, including the citrate synthase gene (*gltA*), heat-shock proteins, riboflavin synthesis genes, cell division genes, or 16S-23S rRNA intergenic spacer regions, and restriction endonuclease analysis of the amplicons have been used successfully for this purpose.[76,650,848,851,1416] Matar and colleagues used PCR along with primers in the 16S rRNA and 23S rRNA genes to amplify the spacer region between the 16S rRNA gene and a part of the the 23S rRNA gene, followed by restriction enzyme cleavage.[851] This approach resulted in characteristic restriction profiles for *B. bacilliformis*, *B. vinsonii*, and *B. quintana* and two profiles for *B. henselae*, suggesting that this method may be useful both for identifying *Bartonella* species and for subtyping isolates of *B. henselae*. Jensen et al. described a single-step PCR-mediated amplification of the 16S-23S rRNA intergenic spacer region that resulted in amplicons of unique size for each *Bartonella* species.[648] Joblet and colleagues found that *gltA* gene amplification followed by digestion with two different restriction enzymes allowed differentiation of *Bartonella* species.[650] Sequence analysis of the 940 base pair fragment of the citrate synthase-encoding gene (*gtlA*) and the 60-kDa heat-shock protein gene (*groEL*) could also be used to construct phylogenetic trees for determining relationships between validly described *Bartonella* species and uncharacterized *Bartonella* strains.[130,848] Primer/probe oligonucleotides have been designed based on localized sequence differences within the riboflavin synthesis gene (*ribC*), and these sequences have been used to develop species-specific PCR assays for identification of *B. henselae*, *B. quintana*, *B. bacilliformis*, and *B. clarridgeiae*.[111] Rodriquez-Barradas and coworkers developed a similar "repetitive element" PCR identification method using primers derived from either repetitive extragenic or intergenic consensus sequences. Because these sequences already existed in multiple, discontinuous copies that result in multiple, discontinuous amplicons following PCR, treatment with restriction endonucleases prior to PAGE was not necessary.[1097] Handley and Regnery at the CDC reported a PCR-dependent identification method based on amplification of restriction endonuclease-cleaved segments. The enzymes used in this procedure resulted in large fragments that could be annealed with primers corresponding to base sequences adjacent to endonuclease restriction sites for subsequent PCR and resolution by PAGE.[554] Molecular approaches have also been used for direct detection of *Bartonella* species in clinical specimens. Matar and colleagues reported a PCR-RFLP method for simultaneous detection and identification of bartonellae directly in clinical specimens, including lymph node biopsies and aspirates, skin, subcutaneous nodules, and other tissues.[851]

This method involved the use of PCR primers from conserved regions of the 16S rDNA gene, followed by restriction endonuclease digestion and amplicon analysis. Sander and Penno used PCR to generate biotinylated amplicons of the 16S rRNA gene region and immobilized the amplicons in streptavidin-coated microtiter wells.[1136] Hybridization of these amplicons with species-specific digoxigenin-labeled 16S rDNA oligonucleotide probes and subsequent addition of antididigoxigenin peroxidase in a modified EIA format provided rapid identification of both *B. henselae* and *B. quintana*. This method could both detect and quantify *B. henselae* and *B. quintana* directly in clinical specimens in concentrations as low as 10^3 cfu/mL. Avidor and colleagues evaluated PCR-mediated 16S rRNA gene amplification followed by *B. henselae* probe hybridization with PCR-based amplification of either *gltA* or *htrA* (heat-shock

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TABLE 9-15 Phenotypic Characteristics for Identification of *Bartonella* Species

Characteristic	*B. bacilliformis*	*B. quintana*	*B. henselae*	*B. elizabethae*	*B. clarridgeiae*	*B. grahamii*	*B. vinsonii* subsp. *vinsonii*	*B. vinsonii* subsp. *berkhoffii*	*B. vinsonii* subsp. *arupensis*
Optimal growth temperature	25°C–30°C	35°C–37°C	35°C–37°C	35°C–37°C	35°C–37°C	35°C–37°C	35°C–37°C	35°C–37°C	35°C–37°C
Hemolysis	–	–	–	+[w]	–	–	–	–	–
Oxidase	–	V	–	–	–	–	V	–	–
Catalase	+	V	–	–	–	–	V	V	–
Nitrate reduction	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–
Acetoin	–	–	–	–	–	+	–	NA	NA
O/F Glucose[a]	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
Flagella	+	–	–	–	+	–	–	–	–
Twitching motility	–	+	+	–	–	–	–	–	–
Major cell fatty acids	$C_{18:1\omega7C}$, $C_{16:0}$, $C_{16:1\omega7C}$	$C_{18:1\omega7C}$, $C_{16:0}$, $C_{18:0}$	$C_{18:1\omega7C}$, $C_{18:0}$, $C_{16:0}$	$C_{18:1\omega7C}$, $C_{17:0}$, $C_{16:0}$	$C_{18:1\omega7C}$, $C_{16:0}$, $C_{18:0}$	NA	$C_{18:1\omega7C}$, $C_{18:0}$ $C_{17:0}$, $C_{16:0}$	$C_{18:1\omega7C}$, $C_{18:0}$, $C_{16:0}$	$C_{18:1\omega7C}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$
Bis-p-Nitrophenylphosphate	+	V	+	+	NA	NA	+	+	+
L-Arginine-β-naphthylamidase	+	+	+	+	+	NA	+	+	+
Glycine-β-naphthylamidase	+	+	+	+	+	NA	+	+	+
Glycylglycine-β-naphthylamidase	+	+	+	+	NA	NA	+	+	+
L-Leucine-β-naphthylamidase	+	+	+	+	NA	+	+	+	+
L-Lysine-β-naphthylamidase (acidic)	+	–	+	+[w]	NA	NA	–	+	–
L-Lysine-β-naphthylamidase (basic)	+	+	+	+	NA	NA	+	+	+
DL-Methionine-β-naphthylamidase	+	+	+	+	NA	NA	+	+	+[w]
L-Proline-β-naphthylamidase	–	+	+	–	+	V	V	+	–
L-Pyrrolidonyl-β-naphthylamidase	–	–	–	–	NA	–	–	–	–
L-Tryptophan-β-naphthylamidase	+	+	+	+	NA	NA	+	+	+

[a] Oxidation/fermentation of glucose.
+, positive reaction; –, negative reaction; V, variable reaction; +w, weak positive reaction; NA, data not available.

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protein genes) followed by RFLP analysis of amplicons for direct detection of *B. henselae* in clinical specimens.[76] *B. henselae* DNA was detected in 100% of 32 pus and lymph node specimens by the 16S rRNA PCR/probe identification method, and in 94% and 69% by the *gltA*/RFLP and *htrA*/RFLP methods, respectively. These investigators suggested that direct testing by *gltA*/RFLP was preferable and easier since species-specific hybridization (via probe) was not necessary with this method.[76] The single-step PCR assay using the 16S-23S rRNA intergenic spacer region target developed by Jensen et al. was able to detect *B. henselae* DNA in blood specimens from infected cats.[648] DNA was amplified from 100% of infected cat blood specimens containing >50 cfu of *B. henselae* per mL and from 80% of blood specimens containing 10 to 30 cfu of *B. henselae* per mL of blood. More recent evidence indicates that some gene sequences found in bartonellae may not be as specific as once thought. For example, the citrate synthase gene (*gltA*) found in *Bartonella* species has homologies with gene sequences found in some host genomes, including mice, rats, humans, and other human pathogens.[291]

Real-time PCR assays have also been described for the direct detection and differentiation of *Bartonella* species and genotypes. Colborn et al. performed whole-genome scans on isolates of *B. henselae*, *B. quintana*, and *B. bacilliformis*, and compared nucleotide sequences with those for other *Bartonella* species published in Gene Bank.[291] They also searched for similar sequences in other bacteria capable of infecting human blood and tissues and in potential mammalian or arthropod vectors for bartonellae. Using this approach, a real-time PCR primer set flanking the NADH dehydrogenase gamma subunit (*nuoA*) gene was identified. Detection of the *nuoA* gene was compared with detection of the citrate synthase gene (*gltA*) and the *fitZ* gene using the requisite primer pairs. These three assays were compared for their ability to amplify reference DNAs from several *Bartonella* species. The *nuoG* primer set successfully amplified all 11 *Bartonella* species tested. When DNA from field-collected specimens, including specimens from rat livers and from ticks, was examined, the *nuoG* primer set demonstrated significantly higher sensitivity and specificity for *Bartonella* than the other primer sets by consistently yielding more sequence-confirmed PCR-positive results. For 61 ticks sampled, the *nuoG* primer set yielded 7 *Bartonella*-positive samples, compared to 1 and 0 for the *ftsZ* and *gltA* sets, respectively. Of 24 liver samples tested, 18 were found to be *Bartonella* positive by the *nuoG* primer set, compared to 10 and 2 for the *ftsZ* and *gltA* sets, respectively.[291] Diaz and colleagues developed a genus-specific real-time PCR assay targeting a 301 base pair region of the *ssrA* gene, which encodes an RNA species also known as transfer-messenger RNA (tmRNA).[355] This is a single copy molecule involved in processing of nascent peptides during translation. Unlike some targets used for detection and identification of *Bartonella* species (e.g., *gltA*), gene sequences that are

homologous with the *ssrA* target sequence have not been detected in any eucaryotic organisms to date.[291] Using this target gene sequence, over 30 unique species, subspecies, and strains within the genus *Bartonella* were detected in blood specimens from elk and cattle. Species identification was achieved by sequencing of the *ssrA* amplicon.

Real-time PCR methods are increasingly being used for direct diagnosis of *Bartonella*-associated diseases. A real-time RT-PCR assay specific for the *Bartonella* 16S-23S intergenic spacer region and the *pap31* gene of *B. henselae* has been used for direct detection of *B. henselae* in skin biopsy specimens, lymph nodes, and swab specimens from CSD patients.[42,44] Broad-range, real-time PCR for 16S and 18S rDNA has been used to detect *Bartonella* species in EDTA blood specimens and in valve specimens.[444]

Serologic Diagnosis

The laboratory diagnosis of CSD is mainly based on molecular methods and serologic testing by either indirect immunofluorescence (IFA) or EIA. IFA is the most widely used technique although it is much more time-consuming than EIA methods. Serologic methods for the diagnosis of *Bartonella* infections have been used in both clinical and veterinary settings. Evaluations of serologic tests report a range of sensitivity and specificity depending on the study population, case definitions of CSD, and materials and methods employed in the testing procedure. Variability in the sensitivity of *B. henselae* serology, particularly IgM testing, may also be due to regional variability in the distribution of different *B. henselae* genotypes (see below). For example, in the Netherlands, *B. henselae* genotype I (serotype Houston I) and genotype II (serotype Marseille) have been found in CSD patients. Some serologic tests include only genotype I *B. henselae*; so test sensitivity may be improved by inclusion of genotype II strains as antigen sources.[115,117]

IFA methods for detection of *Bartonella* antibodies are not highly standardized and vary principally in the source of the bacterial antigens that are fixed to the IFA slides.[1414] Some methods use bacteria grown on agar media, while others use *Bartonella* isolates cocultivated with tissue culture cells as the fixed antigen on the IFA slides. Bergmans and coworkers in the Netherlands evaluated the detection of anti-*B. henselae* IgM and IgG using both IFA and EIA and found significant variability depending on the source of the bacterial antigens for IFA slide preparation.[116] Sera from 21 patients with "possible CSD" (patients fulfilling only 1 of 4 clinical criteria) and 22 with "probable" CSD (patients fulfilling 2 of 4 clinical criteria) were examined. When *B. henselae* cells cocultivated in Vero cells were used as the IFA antigen, the sensitivity of IgG detection was only 31.8% for sera from patients with "probable CSD" and 33.3% for patients with "possible CSD." When *B. henselae* grown on agar

media was used as the IFA antigen, the corresponding sensitivities of the assay for the two patient groups were 40.9% and 14.3%, respectively. Sensitivities of less than 50% were also seen for IgM determinations made by IFA in both groups of patients, regardless of antigen source. Specificities of both the IgG and IgM assays were assessed using sera from healthy blood donors and ranged from 95% to 100%.[116] Vermeulen and colleagues evaluated an in-house IFA test that used agar-grown genotype I *B. henselae* as the antigen.[1302,1303] This assay was challenged with specimens from 61 patients with CSD, based on clinical data and a positive PCR assay for *B. henselae*, and 56 specimens from a group of patients with different clinical diagnoses and a negative *B. henselae* PCR assay. The sensitivity and specificity of the IFA for IgM

detection were 53% and 93%, respectively, while the sensitivity and specificity for IgG detection were 67% and 82%, respectively.

IFA assays have also been developed that use *Bartonella* species that have been cocultivated in Vero cells, ECV endothelial cells, or other cultured cell monolayers. In 1995, Dalton and colleagues at the CDC cocultured *B. henselae*, *B. quintana*, and *B. elizabethae* in Vero cell monolayers and used these organisms to prepare slides for IFA testing. Of 91 patients whose illness met a strict clinical definition of CSD, 95% had IFA titers of ≥64 to either *B. henselae* or *B. quintana*.[323] Since *B. quintana* has not been associated with CSD, it was believed that positive titers against *B. quintana* represented serologic cross-reactions between the two species. Cross-reactions between *B. elizabethae* and the other two *Bartonella* species were observed only with specimens having extremely high titers against *B. henselae* or *B. quintana*. Vero cell–cocultivated *B. bacilliformis* organisms were also used to develop an IFA procedure for diagnosis of bartonellosis in endemic regions of South America. The IFA was found to be 82% sensitive in detecting antibodies in acute-phase sera of 106 patients with slide-positive (i.e., organisms observed in erythrocytes on a Giemsa-stained thin smear), PCR-positive, or culture-confirmed bartonellosis.[228] Based on the high point prevalence of 45% observed prior and during the study period, the IFA test had a positive predictive value of 89%. In their report of *B. quintana* endocarditis in three homeless men, Drancourt and associates found that all three men had high IgG titers against *B. quintana* by a microimmunofluorescence technique.[369] Titers were higher when the *B. quintana* cells used for the procedure were cocultivated in the ECV endothelial cell line (titers of 6,400 to 12,800) than when the cells were from cultures grown on 5% sheep blood agar (titers of 400 to 800).

IFA slides prepared with either blood agar-derived or cell-associated *B. henselae* or *B. henselae/B. quintana* as the antigen are available commercially and include MRL-

BA and MRL-Vero (MRL Diagnostics, USA; Virion, Institut Virion, Switzerland),*Bartonella* IFA (BION Enterprises, USA), *Bartonella* IFA IgG (Focus Diagnostics, Cypress, CA), *B. henselae IgM/IgG*, *B. quintana IgM/IgG*, *B. henselae/B. quintana* IgM/IgG (EUROIMMUN Schweiz AG, Hirschmattstrasse, CH-6003 Luzern), and *B. henselae*/*B. quintana* IFA (Fuller Laboratories, Fullerton, CA). Zbinden and associates studied two commercial IFA kits that use blood agar-derived organisms (MRL-BA, MRL Diagnostics, USA; Virion, Institut Virion, Switzerland) and cell-associated organisms (MRL-Vero/*Bartonella* IgG Substrate slides, MRL Diagnostics, USA; *B. henselae* slides, Bios, Germany).[1413] These commercial slides were compared with their in-house IFA that used Vero cell–cocultivated *B. henselae* as the antigen. In general, kits that used agar-grown *B. henselae* antigen showed higher titers (less specificity) than kits using cell-associated organisms. The MRL-Vero *B. henselae* kit alone was then compared with the in-house system. Using a cutoff titer of 256 and sera from 26 patients with CSD and 240 controls, the MRL-Vero IFA kit showed a sensitivity of 84.5% and a specificity of 93.4%. Similar results were found in a second evaluation of the MRL IFA test.[862,1413,1414]

Focus Diagnostics (Cypress, CA) markets an IFA kit for detection of IgG against *B. henselae* and *B. quintana*. Each well of individual eight-well slides contains two antigen spots consisting of Vero cells infected with either *B. henselae* (Houston strain) or *B. quintana*. Sera are screened at a dilution of 1:64, and positive results at this dilution are titered further. According to the package insert, titers of <64 are considered negative for current infection. Titers of ≥64 and <256 are considered to be evidential of infection at an undetermined time, with the suggestion that a second specimen be drawn 10 to 21 days after the first specimen, and that the first and second specimens be tested together in order to detect a fourfold or greater increase in titer. IFA titers of ≥256 are considered as presumptive evidence of recent infection. This assay was evaluated with 154 sera from normal blood donors (58 from rural Switzerland and 96 from the United States) and with 60 sera from patients with a clinical diagnosis of CSD (including lymphadenopathy and cat exposure).[427] The overall specificity of the IFA test was 89.5% (75.4% for Swiss blood donors and 97.9% for US blood donors), while the test sensitivity was 96.7%. An evaluation using sera from 50 CSD patients and 55 controls without CSD showed an overall sensitivity of 98% and a specificity of 69% at the 1:64 cutoff recommended by the manufacturer.[1303] The Focus Diagnostics *Bartonella* IFA IgG test is for export only and is not for distribution in the United States. A similar IFA product is available from Fuller Laboratories (Fullerton, CA).

The BION Enterprises assay utilizes human larynx carcinoma cells that have been infected with *Bartonella henselae* Houston-1(ATCC 49882). In a study of this assay, 63% of 19 children with CSD had antibody titers (≥512) on hospital admission; titer increases in a second serum sample obtained 1 to 3 weeks later were detected in four

additional patients. Using this assay, 13% of 116 children without CSD had IgG titers of ≥64.[1135] EUROIMMUN markets three *Bartonella henselae* assays in Europe. Two are IgM IFAs that use either a mammalian cell–cocultivated *B. henselae* Houston strain or a cocultivated *B. henselae* Marseille strain. An IgG IFA assay is also available, which uses the cocultivated Houston strain. The IgM IFAs are screened at a cutoff of 1:100, while the IgG assay is screened at a cutoff of 1:320. In a study of 50 CSD patients and 55 controls without CSD, the Houston and Marseille strain assays showed sensitivities of 54% and 50%, respectively, while the corresponding specificities of the assays were 96% and 87%, respectively. Screening of the same patients with the Houston IgG assay showed a sensitivity of 88% and a specificity of 89%.[1303]

EIA has also been evaluated as a serologic method for diagnosis of CSD. As with IFA procedures, EIAs have also used agar-grown or cocultivated organisms as the antigen. Barka and colleagues at what was then Specialty Laboratories (Santa Monica, CA) developed an EIA for detection of specific IgG, IgM, and IgA using *B. henselae* cells grown on agar as the solid-phase antigen.[91] Of serum specimens from 40 confirmed cases of CSD (by clinical history, culture, and/or histopathology), 38 (95%) had elevated levels of antibodies against *B. henselae*. Although none of the 40 CSD patients in this study had *B. henselae*-specific IgM or IgA in the absence of IgG, the authors stated that they had observed *B. henselae*-specific IgM as the only marker in some patients and had also seen IgG seroconversion in the presence of IgM occur over a 2- to 3-week period. Positive EIA results were not observed in any of 92 specimens from patients with documented high antibody titers for other agents, including

Afipia felis, *Rickettsia typhi*, *Rickettsia rickettsii*, *Borrelia burgdorferi*, *Yersinia pestis*, *Chlamydia trachomatis*, rubella virus, and cytomegalovirus (CMV). Using the same EIA, Bergmans and colleagues compared serologic methods with PCR for diagnosis and found that test concordance for diagnosis was highest between the IgM EIA and PCR.[116] These workers suggested that the most appropriate strategy for diagnosis of CSD was to perform an IgM EIA first. If the IgM serology by EIA was positive, the patient had CSD. If the IgM EIA was negative, *Bartonella* PCR from pus or tissue should be performed.

In 2001, Giladi and colleagues described an EIA for IgG and IgM that used and an *N*-lauroyl-sarcosine-insoluble outer membrane antigens from agar-grown *B. henselae* as the antigen.[486] This assay was evaluated in a group of 84 patients with definite CSD by history and at least one confirmatory test (PCR, culture, or skin test). This group was compared with a control group of 34 patients without CSD. Anti-*B.*

henselae IgG was detected in 63 and IgM in 40 of the 84 patients with CSD. Both antibodies were found in 32 patients, IgG alone was found in 31 patients, and IgM alone was found in 8 patients. The sensitivity of the EIA was calculated to be 75% for IgG and 48% for IgM, respectively. When either or both results were used for diagnosis, sensitivity increased to 85%.[486] This same assay was subsequently evaluated with specimens from 98 patients with confirmed CSD.[885] Of these, 53% tested positive for anti-*B. henselae* IgM; 88% of these patients had an initial sample that tested positive, while the remaining 12% of patients seroconverted within 1 to 8 weeks after the first serum was collected. All patients with CSD and a high IgG titer experienced disease onset within the prior 12 months. Biopsy specimens for PCR analysis were obtained from 26 of the 98 patients, and of the 24 patients who were PCR-positive, 83% and 58% were IgG- and IgM-positive, respectively. In this study, IgM seropositivity lasted for approximately 3 months, with only 4% of the study population remaining positive for IgM for more than that time. These workers concluded that IgM seropositivity in a patient with CSD is indicative of acute disease. IgG titers decreased over a longer period of time. Vermeulen and coworkers also evaluated a laboratory-developed EIA for diagnosis of CSD using a whole-cell sonicate of *B. henselae* grown on Columbia sheep blood agar.[1303] This assay was compared with an IFA test using organisms grown in the same fashion, but without the sonication step. EIA for IgM detection had a sensitivity and specificity of 65% and 91%, respectively, compared to IFA, which had a sensitivity of 53% and a specificity of 93%. For detection of anti-*B. henselae* IgG, the sensitivities of IFA and EIA were only 67% and 28%, respectively. From these studies, it is clear that serologic tests for diagnosing CSD need to be improved and that no ideal serologic test has been described. Detection of IgM antibodies against *B. henselae* by either IFA or EIA is highly suggestive for diagnosis in patients suspected of having CSD, with positive predictive values of about 87%, and that tests for IgG appear to be of limited value.[1302,1303] These workers concluded that, given the low sensitivity of serologic assays, PCR analysis for *B. henselae* should be performed for patients with suspected CSD who have negative serologic tests.

Serologic cross-reactions have also been noted between *Bartonella* species and other organisms, including *Coxiella burnetii* (the causative agent of Q fever) and *Chlamydophila* species.[369,758,858] In patients with *B. quintana* endocarditis, titers of >256 were observed against *C. pneumoniae* and titers of >64 were observed against both *C. psittaci* and *C. trachomatis*.[369,370] While absorption of the serum specimens with *C. pneumoniae* did not reduce the titers against *B. quintana*, absorption with *B. quintana* successfully quenched reactivity with *C. pneumoniae*. In another study, more than 50% of patients with chronic Q fever had significant antibody titers to *B. henselae*. Cross-absorption and immunoblot studies established that reactivity with *B. henselae* resulted from cross-reactions with *C. burnetii* antibodies; absorption of

serum with *C. burnetii* removed reactivity with *B. henselae* antigen, while adsorption with *B. henselae* removed only the reactivity against *B. henselae*.[758] Absorption of sera with *C. pneumoniae* or *B. quintana* removed anti-*C. pneumoniae* antibodies, while adsorption with *C. pneumoniae* did not change titers against *B. quintana*.[858] In the case of *Bartonella* endocarditis, specific titers may be high, with the titers against these other agents being significantly lower, suggesting that misdiagnoses should not occur provided that antibodies against all of these agents are sought simultaneously. As mentioned, sera with high anti-*B. henselae* titers may cross-react with other *Bartonella* species as well. Conversely, patients with chronic Q fever endocarditis may develop an array of antibodies that cross-react with *B. henselae*, *B. quintana*, and several other agents, including *Rickettsia rickettsii*, *Ehrlichia chaffeensis*, *Legionella* species,*Chlamydia* species, and *Anaplasma phagocytophilum*.[509] Zbinden and coworkers reported significant *B. henselae* IgM IFA cross-reactivity in 20 patients infected with Epstein–Barr virus (EBV); 45% were seropositive with agar-derived *B. henselae* antigen, and 95% were seropositive with cocultivated *B. henselae* antigen by IFA.[1415] Cross-reactivity with *C. burnetii*, *C. pneumoniae*, EBV, CMV, and *T. gondii* has been reported for several of the commercially available IFA assays as well.[1303]

The ability of serology to provide a diagnosis of *Bartonella* infections is impacted by several factors, not all of which are clear at the present time. In HIV-infected patients with manifestations of *Bartonella* infection, a significant antibody response may not be observed because of HIV-related immunosuppression. Failure to mount an antibody response has also been observed in immunocompetent patients, as evidenced by the absence of an antibody response in a host with chronic lymphadenopathy and positive blood cultures for *B. quintana*.[1062] Failure to detect antibodies may also be due to antigenic heterogeneity of the organisms themselves. Antigenically different strains of *B. henselae* have been recovered from patients with endocarditis and from cats; these strains were not only serologically different, but were also noted to be different by protein profiles and 16S rDNA sequences.[117,369] Using recombinant DNA technology, a 17-kDa protein has been isolated from *B. henselae* that is specifically reactive with sera from patients with CSD and may have value as a diagnostic serologic reagent.[37] Using an immunoproteomic approach, along with immunoblotting and MALDI-TOF, several candidate proteins of *B. henselae* have been detected that may be useful for serodiagnosis of CSD and other *Bartonella* infections in the future.[1128] At the present time, IFA is the most frequently used serologic test for CSD. Using this technique, IgG antibodies in CSD

patients are usually high (e.g., ≥512), while lower IgG titers may be found in both

CSD patients and healthy controls. In specimens with low titers, a second serum sample collected 10 to 14 days later may help to confirm the diagnosis. Seroconversion, a single high titer, or a fourfold titer increase in a second serum specimen is highly suggestive of CSD.[1135]

Products for *Bartonella* serologic testing are available from commercial vendors, and many reference laboratories also offer testing for these agents. Focus Diagnostics (Cypress, CA) markets an IFA kit for detection of IgG against *B. henselae* and *B. quintana*. Each well of individual eight-well slides contains two antigen spots consisting of Vero cells infected with either *B. henselae* or *B. quintana*. Sera are screened at a dilution of 1:64, and positive results at this dilution are titered further. According to the package insert, titers of <64 are considered negative for current infection. Titers of ≥64 and <256 are considered to be evidential of infection at an undetermined time, with the suggestion that a second specimen be drawn 10 to 21 days after the first specimen, and that the first and second specimen be tested together in order to detect a fourfold or greater increase in titer. IFA titers of ≥256 are considered as presumptive evidence of recent infection. This assay was evaluated with 154 sera from normal blood donors (58 from rural Switzerland and 96 from the United States) and with 60 sera from patients with a clinical diagnosis of CSD (including lymphadenopathy and cat exposure).[427] The overall specificity of the IFA test was 89.5% (75.4% for Swiss blood donors and 97.9% for US blood donors), while the test sensitivity was 96.7%. The Focus Diagnostics *Bartonella* IFA IgG test is for export only and is not for distribution in the United States. A similar IFA product is available from Fuller Laboratories (Fullerton, CA).

IFA serology for detection of *B. henselae* and *B. quintana* IgG and IgM and molecular tests for detection of these agents in whole blood, serum, plasma, tissue, and CSF specimens are available from several commercial reference laboratories (Associated Regional University Pathologists [ARUP], Salt Lake City, UT; Quest Diagnostics, Santa Monica, CA; Microbiology Reference Laboratory [MRL], Cypress, CA).

In Vitro Antimicrobial Susceptibility

Using agar dilution, *B. henselae*, *B. quintana*, and *B. vinsonii* are susceptible to a variety of antimicrobial agents, including ampicillin, third-generation cephalosporins, tetracyclines, macrolides, rifampin, SXT, and aminoglycosides.[861] MICs for oxacillin, cephalothin, clindamycin, chloramphenicol, and the fluoroquinolones were near the maximum concentrations of drug available in the serum. A subsequent study used agar dilution with blood-supplemented Columbia agar to determine the in vitro susceptibilities of 9 *B. quintana*, 3 *B. henselae*, and single isolates of *B. elizabethae* and *B. bacilliformis*.[859] All isolates were susceptible to β-lactams, macrolides,

doxycycline, aminoglycosides, and rifampin. The isolates appeared less susceptible to the semisynthetic penicillins, first-generation cephalosporins, and clindamycin. Susceptibilities of these 14 isolates to the fluoroquinolones were variable. Antimicrobial susceptibility testing of *Bartonella* species has also been performed using Etest methods.[135,365,1017,1270] In general, MIC results with the Etest have correlated well with agar dilution results for all classes of agents tested. Tsuneoka and coworkers used Etest methodology to test 32 *B. henselae* isolates from cats and a single strain from a human.[1270] All isolates were susceptible to minocycline, the macrolides, and the β-lactams, with clarithromycin being the most active macrolide (MICs ≤0.023 μg/mL). Gentamicin was the least active agent against *B. henselae* in this study.

Other in vitro susceptibility studies on *Bartonella* species have focused on the ability of antimicrobial agents to inhibit organism growth in a cell culture environment. Ives et al. studied the susceptibility of *Bartonella* species to several macrolide antimicrobial agents using a Vero cell monolayer cocultivation method.[630,631] Organism inhibition by different concentrations of antimicrobial agents was assessed by visual enumeration of organisms as determined by specific immunofluorescence in comparison with cultures without antibiotics after 5 days' incubation. Using this method, individual isolates of *B. henselae*, *B. quintana*, and *B. elizabethae* were susceptible to erythromycin, clarithromycin, azithromycin, dirithromycin, and roxithromycin.[630,631] These workers also pointed out that both erythromycin and doxycycline are concentrated in phagocytic cells, reaching levels that exceed the achievable serum concentrations. While the growth of organisms phagocytized by neutrophils and macrophages would likely be inhibited due to the high drug levels attained in these cells, different species or strains of *Bartonella* may behave differently within endothelial cells or other types of cells. Kordick et al. found that all strains of *B. henselae* and *B. clarridgeiae* recovered from naturally and experimentally infected bacteremic cats were susceptible to doxycycline, enrofloxacin, and ciprofloxacin.[721,722] However, successful treatment (defined by failure to detect bacteremia by culture or by PCR) occurred in only 9 of 14 cats that received enrofloxacin and in 2 of 8 cats that were treated with doxycycline. These workers concluded that standard agar dilution susceptibility tests on *Bartonella* species do not provide any clear-cut information on the therapeutic efficacy of the agents, since there is very little information on the cellular tropisms of *B. henselae* and *B. clarridgeiae* in cats. It was postulated that feline cells infected with *Bartonella* may be functionally impaired to the extent that antimicrobials are not taken up or are compartmentalized away from the organisms themselves.[721]

Bordetella Species

Background and Taxonomy of Bordetella *Species*

The genus *Bordetella* contains nine species: *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. hinzii*, *B. holmesii*, *B. trematum, B. petrii*, and *B. ansorpii*.[518] Genetic studies have shown that these organisms are very closely related to each other. In fact, DNA hybridization techniques indicate that these organisms may not be sufficiently different from one another to justify the assignment of individual species, although definite genetic, phenotypic, and immunologic differences exist among them. Sequence analyses of 16S rRNA of the described *Bordetella* species have demonstrated

common phylogeny with the genus *Alcaligenes*, and the genus *Bordetella* now resides in the β-2 subdivision of the *Proteobacteria* in the family *Alcaligenaceae*, order *Burkholderiales,* along with the genera *Achromobacter*, *Alcaligenes*, *Pelistega*, *Sutterella*, *Oligella*, and *Taylorella*. The genetic relatedness of *Bordetella* species to the genus *Alcaligenes* is also supported by the finding that both *B. pertussis* and *B. bronchiseptica* produce a siderophore called alcaligin that is almost identical to that produced by *Alcaligenes denitrificans*.[906]Members of the genus *Bordetella* are small, gram-negative coccobacilli on primary isolation. On subculture, they tend to become more pleomorphic. They are obligately aerobic, grow optimally at 35°C to 37°C, do not utilize carbohydrates, and are inactive in most biochemical tests. *B. pertussis*, *B. parapertussis*, *B. holmesii*, and *B. petrii* are nonmotile, while *B. bronchiseptica*, *B. avium*, *B. hinzii*, and *B. ansorpii* are motile via peritrichous flagella. These organisms do not require hemin or NAD. However, primary isolation of *B. pertussis*, in particular, requires the addition of charcoal, ion exchange resins, or 15% to 25% blood to neutralize the growth-inhibiting effects of unsaturated fatty acids, sulfides, peroxides, and heavy metals. *B. parapertussis* is somewhat less exacting in its growth requirements, but isolation still requires the use of the same special media used for *B. pertussis*. The remaining species are less fastidious and will grow on routinely used agar media, including blood, chocolate, and MacConkey agars.

The genus *Bordetella* includes species isolated from both man and other animals. Humans are the only recognized host of *B. pertussis*. *B. parapertussis* was also thought to be restricted to human hosts, but in addition to humans it has also been isolated from the respiratory tracts of pneumonic lambs in Scotland and New Zealand.[1041] *B. parapertussis* is also able to cause a pertussis-like illness in humans. *B. bronchiseptica* is found in a variety of animals (e.g., nonhuman primates, dogs,

cats, rabbits, horses, turkeys, swine, foxes opossums) and is occasionally found in humans, primarily as an opportunistic agent.[1339] As the name suggests, *B. avium* is found in birds and causes rhinotracheitis in turkeys.[696] *B. hinzii* was formerly called "*B. avium*-like bacterium," turkey coryza bacterium type II, *Alcaligenes faecalis* type II, and *Alcaligenes* species strain C2T2; this organism was formally assigned to the genus *Bordetella* in 1995.[1288] *B. hinzii* was originally described as a commensal organism in poultry, and has since been isolated from a variety of human clinical specimens. *B. holmesii* was formerly called CDC nonoxidizer group 2 (NO-2).[1343] Characterization of these isolates by DNA–DNA hybridization, 16S rRNA sequencing, and cellular fatty acid/ubiquinone analysis established their relationship to genus *Bordetella*. Since 1995, *Bordetella trematum* has been described as a new species that is found in human wounds and ear infections, and novel "*Bordetella holmesii*-like" strains have been isolated from patients with bacteremia, endocarditis, and respiratory tract infections.[1245,1287] *B. petrii* was first described as an environmental organism that was initially isolated from an anaerobic bioreactor culture enriched from river sediment.[1307] Since its initial description, *B. petrii* has also been isolated from several human clinical sources, including blood and sputum. *B. ansorpii* was first described in 2005, when it was isolated from a purulent epidermal cyst on the neck of a patient with rhabdomyosarcoma.[711]

Epidemiology of Pertussis

Pertussis continues to be an endemic disease of worldwide importance and remains an important cause of death in children less than 1 year old worldwide, with an estimated 10 million cases and as many as 400,000 deaths annually. Humans are the only known host of *B. pertussis*, and transmission occurs via direct contact with aerosolized droplets from infected, coughing individuals. The attack rate is very high, with greater than 90% of susceptible individuals becoming infected after significant exposures. For unknown reasons, disease among children occurs more often in females than in males and tends to occur in epidemic cycles of 3 to 5 years. Prior to the availability of the whole-cell killed vaccine in 1947, about 200,000 cases of pertussis were reported in the United States each year.[1346] At that time, most cases of pertussis occurred among children from 1 to 5 years of age. After 1947, when the whole-cell DPT vaccine was licensed and recommended by the American Academy of Pediatrics, reported cases of pertussis began to decline until 1976, when only 1,010 cases were reported.[1346] Subsequently, the annual number of reported cases has increased steadily, and in 2004, the incidence reached 8.9 cases per 100,000, with nearly 19,000 cases being reported that year.[247] Despite sustained high coverage for childhood pertussis vaccination, a total of 16,858 cases of pertussis and 12 infant deaths were reported in

2009.[223] Increasing incidences of pertussis despite high vaccination coverage is also being seen in several other countries, including Canada, Italy, Japan, Switzerland, and the Netherlands.[1242] Because the whole-cell vaccine provides protection for a limited duration, fully immunized individuals were protected, but adults had little or no immunity for passive transfer to infants. Consequently, during the 1980s and throughout the 1990s, the highest incidence of pertussis during this era of vaccination has progressively shifted to younger and younger infants. In the United States, during the early 1990s, between 40% and 50% of reported pertussis cases occurred in children who were less than 1 year old.[213,214] Similar incidence rates have been reported in other countries that have maintained vaccination protocols. For example, during the 15-month period from July 1993 through October 1994, 65% of index cases of pertussis in a French medical center occurred in children less than 1 year of age.[96] In resource-rich (i.e., developed countries) with well-established immunization protocols, pertussis has now emerged as an infectious problem in two age groups: individuals greater than 10 years of age and those under the age of 5 months. In the United States, during the 1980s, the mean annual incidence of pertussis in infants under 4 months increased from 63.4 cases per 100,000 to 88.7 cases per 100,000 in the 1990s.[1243] For US data from 2001 through 2003, the highest incidence was in infants under 6 months of age (98.2 cases per 100,000) compared with 12.3 cases per 100,000 in infants aged 6 through 11 months.[219] The greatest amount of pertussis-related mortality also occurs primarily among infants; surveillance data collected from 1992 through 1993 showed that infants less than 1 year of age accounted for 42% of the reported cases and 87% of the reported deaths. In countries where vaccination is not commonly practiced, the 1- to 5-year age group remains at highest risk.[214]

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Pertussis has also become a significant problem in older children, adolescents, and adults. In Europe, incidence rates between 1998 and 2002 increased 115% in children older than 14 years.[211] In the United States, incidence rates in adolescents aged 10 to 19 years increased from 5.5 cases per 100,000 in 2001 to 10.9 cases per 100,000 in 2003.[219] Several factors have contributed to the change in pertussis epidemiology, leading to increasing disease rates in infants less than 5 months of age and in children greater than 10 years. Waning immunity following childhood vaccination in the absence of booster immunizations is an important contributing factor. The duration of protection following immunization with whole-cell vaccines ranges from 4 to 14 years, while the duration following acellular vaccine administration is about 5 to 6 years.[1337] Adolescents who received childhood acellular pertussis vaccines and adults who receive the whole-cell vaccine are at risk for pertussis in the absence of booster

inoculations. Studies have also shown that at least two vaccine doses are required for complete protection, and according to most immunization schedules, 5-month-old infants are too young to have received 2 vaccine doses and continue to be at risk for pertussis.[973] In a large multinational study of infants hospitalized with pertussis, 75% had received either no vaccine or only one dose of pertussis vaccine.[732] Therefore, continued susceptibility due to incomplete immunization status is another factor contributing to increased rates of disease. Adults and adolescents who acquire the organism due to waning vaccine- or disease-induced immunity act as reservoirs that may transmit the infection to unvaccinated or partially vaccinated infants.[1242] Because of atypical presentations of pertussis in older individuals, the disease is often unrecognized and treatment is not given, is delayed, or is incomplete. These infected individuals are an important source for transmission of *B. pertussis* to others, but especially to infants and young children who are not adequately immunized. In many cases, older relatives, including parents and grandparents, healthcare workers, and classmates are the source of infections in infants and children.[390,1004,1323] Reduced vaccine-induced immunity may also be due to structural polymorphisms of virulence factors between circulating *B. pertussis* strains and vaccine components, including pertactin and pertussis toxins (PTs).[492,568] Finally, improved methods for diagnosis such as PCR have resulted in increased testing volumes and have also contributed to marked increases in detection and a better assessment of disease incidence.

Clinical Significance of Bordetella pertussis

B. pertussis causes the syndrome called pertussis or "whooping cough." The organism is acquired via droplet infection, and is highly contagious, with an attack rate of greater than 90% in nonimmunized individuals. Partial immunization of children against pertussis may alter the classical clinical presentation. Furthermore, the presentation and diagnosis of pertussis in young children (i.e., less than 1 year old) may be complicated by concomitant viral respiratory tract infections, including influenza and respiratory syncytial viruses in particular. Classically, clinical pertussis in unvaccinated children can be divided into three stages. After an incubation period of 7 to 10 days (range 5 to 21 days), the **prodromal** *or catarrhal stage* begins and is characterized by nonspecific "cold" or "flu" symptoms, such as rhinorrhea, conjunctival injection, low-grade fever, and malaise.[582,1263] The disease is highly communicable at this stage since large numbers of organisms are present in the upper respiratory tract, and cultures collected at this time have the greatest likelihood of being positive. A dry, nonproductive cough appears late in this stage and increases in persistence, severity, and frequency. This evolves into the **paroxysmal or spasmodic stage** after several days to a week. This stage is characterized by the "staccato cough," with the prolonged inspiratory "whoop" heard at the end of the coughing spell; this phase may

last for 2 to 6 weeks. Not all children with pertussis have the characteristic cough. During this stage, there is no fever or other systemic signs and symptoms. Inspiratory efforts are futile during the coughing paroxysm, and the "whoop" is caused by the inspiration of air through the swollen and narrowed glottis. The coughing spell is frequently followed by by cyanosis and vomiting. This stage may be so severe that intermittent ventilatory assistance may be required. Complications that may occur during the course of the disease include pneumonia secondary bacterial infections, otitis media, CNS symptoms (i.e., convulsions, high fever), encephalopathy, cerebellar ataxia, inguinal hernia, and rectal prolapse associated with the severe coughing. The pathophysiology of encephalopathy associated with complicated pertussis is not known because of the unavailability of a suitable animal model, but suggested mechanisms include anoxia secondary to the coughing paroxysms, hypoglycemia secondary to the toxic effects of PT, and intracerebral hemorrhage. The **convalescent stage** generally begins within 4 weeks of onset, and during this time, there is a decrease in the frequency and severity of the coughing spells. The virulence factors of *B. pertussis* are discussed in Box 9-10.

The clinical presentation of pertussis is markedly different in infants, adolescents, and adults. Pertussis in infants can have severe presentations, have high mortality, and requires rapid diagnosis and treatment. A 2007 report on infants with pertussis admitted to the intensive care unit of an Australian tertiary-care hospital over a 20-year period described 49 patients, aged 4 to 8 weeks.[933] None of the infants had received a full immunization series, and 94% had not received any pertussis immunizations. Reasons for admission included apnea with and without paroxysms (63%), pneumonia (18%), and seizures (10%). Infants presenting with apnea with or without severe cough survived, but all seven deaths occurred with infants admitted to the ICU with pneumonia. Premortem complications included cardiovascular compromise, severe encephalopathy, and multisystem organ failure.

Atypical pertussis seen in older children, adolescents, and adults is attributed to the fact that most adults were immunized as children and that waning immunologic recall results in a modified, less severe illness.[787] Older children and adults with symptomatic but unrecognized pertussis are often sources of the organism in pediatric cases. Studies of pertussis in adults have focused on individuals with persistent cough and have used culture/molecular methods and serology to demonstrate the presence of the organisms or significant titer increases against unique antigens of *B. pertussis*, respectively. In a 2006 study of children aged 5 to 16 years presenting to

BOX 9-10

Virulence Factors of *Bordetella pertussis*

Factor	Comments
Pertussis toxin	Pertussis toxin (PT) is a major virulence factor of *B. pertussis*. PT is a single protein of 105–117 kDa molecular weight that has a wide spectrum of biologic activity and is produced only by *B. pertussis*.[795,796] Biologic effects of PT include the sensitization of mice to histamine, the production of lymphocytosis, activation of pancreatic islet cells, and stimulation of immune responses. Antibody directed against PT is protective for mice when challenged by either intracerebral or respiratory tract inoculation. The genes for the PT peptides are arranged as an operon; this operon is present in *B. pertussis*, *B. parapertussis,* and *B. bronchiseptica*, but the genes are neither transcribed nor translated in the last two species.[58,1220] Antibody directed against PT is also protective in animal models of pertussis. PT is the only molecule that is represented in every acellular pertussis vaccine formulated thus far.
Filamentous hemagglutinin	Filamentous hemagglutinin (FHA) is a cell surface adhesin of 220-kDa molecular weight that has hemagglutinating activity and, along with PT, mediates adhesion of *B. pertussis* to eukaryotic cells in vitro and to ciliated cells of the upper respiratory tract. FHA also displays immunomodulatory activity in human peripheral blood mononuclear cells (PBMCs).[356,795] FHA has multiple binding activities that enable *B. pertussis* to adhere to and, in some cases, invade different cell types, including macrophages, at different stages of infection and in concert with other adhesins. Binding of FHA to macrophages leads to phagocytosis of the organisms without the oxidative burst associated with phagocytosis of other microorganisms. This unusual circumstance may be critical for intracellular survival of *B. pertussis*. Antibody against FHA provides some immunity against respiratory infections, but not intracerebral challenge in animals, presumably by inhibiting attachment of the organisms.
	Pertactin describes several surface-associated cell membrane proteins found in three *Bordetella* species. Pertactin P.69 of *B. pertussis* has a molecular weight of 69 kDa, and homologous proteins of slightly different molecular weights are found in *B. parapertussis* (pertactin P.70) and *B. bronchiseptica* (pertactin P.68).[795] Pertactins are encoded and produced as slightly larger

Pertactin	proteins that are proteolytically cleaved to yield the biologically active molecules. *B. pertussis* pertactin was originally called P69 or 69K protein; the true molecular weight has been determined to be about 60.5 kDa after it was found that pertactin was derived from post-translational processing of a larger precursor protein. The pertactin of *B.pertussis* functions along with FHA to mediate attachment of the bacterium to tissue culture cells by helping the FHA molecule achieve a conformation that maximizes bacterial cell binding.[57] Pertactin is also required for *Bordetella* species to resist neutrophil-mediated clearance.[624]Antibodies against pertactin P.69 protect mice against infection with *B. pertussis* administered by the aerosol route, but protection against intracerebral challenge requires antibodies against both P.69 and FHA.
Adenylate cyclase-hemolysin	*B. pertussis* produces an adenylate cyclase-hemolysin (AC-H), a bifunctional 177-kDa protein of that is secreted into the medium and possesses both adenylate cyclase and hemolytic activities.[198,795] The protein is able to bind to susceptible cells and is translocated into the cell intact. Inside the target cell, the molecule is proteolytically cleaved. Activation of the adenylate cyclase activity by the eucaryotic protein calmodulin results in intracellular accumulation of cyclic AMP. This accumulation may suppress expression of the local immune response by inhibiting neutrophil chemotaxis and phagocytosis.[581]
Tracheal cytotoxin	Tracheal cytotoxin (TCT) is a small molecule of about 921 Da that is composed of a disaccharide tetrapeptide derived from the cell wall peptidoglycan. The molecule contains glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid in a 1:1:2:1:1 ratio and is released into the culture supernatant during the logarithmic growth phase.[302] TCT specifically damages and kills the ciliated epithelial cells lining the airways where the organism attaches and may contribute to the characteristic cough of clinical pertussis. TCT has also been shown to adversely affect polymorphonuclear cell function at low concentrations, and is toxic to these cells at higher concentrations.[795]TCT also elicits IL-1 production and induces nitric oxide synthase, which is believed to mediate damage to the respiratory tract epithelium. TCT and other similar muramyl peptides have several other biologic activities, including pyrogenicity and adjuvanticity.
	B. pertussis also produces a heat-labile toxin (HLT or dermonecrotizing toxin) that is a single polypeptide of about 140

Heat-labile toxin (dermonecrotizing toxin)	kDa.[795] HLTs produced by *B. pertussis, B. parapertussis,* and *B. bronchiseptica* are similar in their biologic properties and are physicochemically and serologically indistinguishable, whereas HLT produced by *B. avium* is slightly different in these respects. HLT induces contraction of blood vessels due to specific constrictive effects on vascular smooth muscle tissue and causes hemorrhagic necrosis in mice and guinea pigs. Although its role in the pathogenesis of pertussis, if any, is not known, constrictive effects of HLT on the highly vascularized tissues of the respiratory tract could lead to local inflammation and may explain some of the respiratory tract pathology associated with pertussis.

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Lipooligosaccharide	Like other gram-negative bacteria, *B. pertussis* possesses lipopolysaccharide (LPS or endotoxin) in its cell wall outer membrane. Like some other bacterial pathogens, the endotoxin of *B. pertussis* is actually a lipooligosaccharide (LOS). This LOS contains lipid A and an oligosaccharide core with 2-keto-3-deoxyoctulosonic acid, but lacks the long-chain polysaccharide "O" antigens found in LPS molecules. The role of LOS in the pathogenesis of pertussis is not known, although it has the usual properties associated with LOS of many organisms, including pyrogenicity, adjuvanticity, induction of interferon production, mitogenicity, and polyclonal activation of B cells. *B. pertussis* LOS may be at least partially responsible for the reactogenicity associated with the whole-cell pertussis vaccine.
Heat-labile "O" agglutinogens	*Bordetella* species also possess specific antigens termed "O" agglutinogens (AGGs) on the bacterial cell surface. Fourteen heat-labile AGGs have been described for *Bordetella* species. These agglutinogens are associated with fimbriae and apparently are also involved in the mediation of attachment to target cells. AGGs 1 through 6 are found in *B. pertussis*; AGG 1 is common to all *B. pertussis* strains, with AGGs 2 through 6 being present in various combinations in different strains.

clinicians with cough for more than 14 days, 37% had serologic evidence of pertussis.[558]A study of 130 university students reporting to the student health service with a cough of 6 days' duration or greater found that 26% had serologic evidence of recent *B. pertussis* infection.[894] A subsequent study with this population found that 15% of 319 students with cough of ≥5 days' duration had serologic evidence of *B. pertussis* infection; 17 of these individuals also had serologic evidence of coexisting viral, *Chlamydophila pneumoniae*, or *Mycoplasma pneumoniae* infections.[633] A study of US Marine Corps trainees who reported 7 or more days of cough found that 17% had acute *B. pertussis* infection.[643] A study of 51 healthcare workers who were evaluated annually from 1984 to 1989 for rises in antibody titers to four pertussis-specific antigens (PT, filamentous hemagglutinin [FHA], pertactin, and fimbriae) found that 90% had a significant increases in antibody titer to one or more pertussis antigens between two consecutive years during the 5 years of the study.[351] Among 246 German adults with a coughing illness of greater than 14 days' duration, evidence of *B. pertussis* infection was found in 64 (26%), 5 had positive nasopharyngeal cultures and 59 were diagnosed on the basis of serology and/or PCR.[1150] A study in Denmark of 201 patients with coughs persisting for a mean of 6.5 weeks found that 4 patients (2%) were culture-positive for *B. pertussis* and 11 (5.5%) were positive by PCR (including the 4 culture-positive patients).[128] Serologic evidence of pertussis infection was also demonstrated in 100 adults aged ≥65 years who were studied over a 3-year period. Between 3.3% and 8% of this population had pertussis infections each year, and between 37.5% and 50% were symptomatic as a result of the infection.[591] A study of 178 adults in Denmark with a duration of coughing between 2 weeks and 3 months reported that 3.4% to 12.4% were seropositive for pertussis, indicating recent infection.[322]

The clinical spectrum of pertussis in adults has been described in several studies and case reports. In a UCLA study, the two principal clinical diagnoses entertained for those individuals found to have pertussis were nonspecific upper respiratory tract infection (39%) and bronchitis (48%).[894,1039] In a study conducted in Germany on 64 adults with documented *B. pertussis* infection, only 39% were thought to have pertussis and 14% were thought not to have pertussis clinically; other diagnoses included upper respiratory tract infections, nasopharyngitis, pharyngitis, adenoiditis, and sinusitis. However, of the 64 patients, 70% had a paroxysmal cough, 38% had an inspiratory whoop, and 17% experienced post-tussive vomiting.[1150] Another German study of 84 adults with pertussis found persistent (>21 days) cough in 81%, spasmodic coughing in 65%, and choking in 56%.[1042] Eighteen patients had complications, including otitis media, pneumonia, urinary incontinence, cervical lymphadenopathy, acute hearing loss, rib fractures, and inguinal hernia. Pertussis-associated

encephalopathy has also been documented in adult pertussis.[548] Although uncommon, acute *B. pertussis* infections in adults have been described; in one case, acute pertussis was diagnosed in a 53-year-old man who acquired the organism from his son, who had been immunized, and had a mild case of whooping cough.[1201] *B. pertussis* has also been isolated from adults with underlying disease such as HIV infection. Ng and coworkers isolated *B. pertussis* from BAL and transbronchial biopsy specimens of three patients with AIDS.[945] These organisms were recovered on media for isolation of *Legionella*. Doebbeling et al. reported the isolation of *B. pertussis* from the upper respiratory tract of a 25-year-old HIV-positive patient with a 4-month history of paroxysmal cough; a similar case was also reported in a 60-year-old patient with AIDS in Belgium.[292,360] Despite these case reports, pertussis is believed to be relatively rare in HIV-infected individuals, with an estimated prevalence of nasopharyngeal carriage of less than 6.5 cases per 100,000 patients.[289] *B. pertussis* has also been isolated from blood cultures of immunocompromised hosts on at least three occasions. All three patients had underlying diseases, including Wegener's granulomatosis and multiple myeloma; in two patients the infection was fatal.[217,642,1266]

Pertussis Vaccines

The original whole-cell pertussis vaccine was administered along with diphtheria and tetanus toxoids and aluminum-coated adjuvants (DTwP). After licensure, widespread use of whole-cell vaccine resulted in declines in pertussis in countries employing mandatory immunization, and additional evidence of efficacy accrued as a result of diminished immunization programs in some countries. Diminished public acceptance of immunization in Great Britain beginning in

the mid-1970s resulted in pertussis reaching epidemic proportions among children less than 5 years of age in the late 70s. While experience suggested clinical efficacies of greater than 80%, variations in the performance of whole-cell vaccine preparations became more apparent during prospective clinical efficacy studies conducted with potential acellular pertussis vaccines. Publicity surrounding reports of encephalopathy and permanent neurologic sequelae that were temporally related to receipt of DTwP vaccine accelerated efforts to develop acellular or "subunit" vaccines. Acellular pertussis vaccines contain PT and FHA, and some also contain pertactin (69-kDa protein) or both fimbriae and pertactin.[308,526] Pertussis vaccines are combined with diphtheria and tetanus toxoids in the DTaP vaccine for children, and the Tdap for adolescents and adults (see below). In the United States, three DTaP vaccines are licensed:

- Infanrix (GlaxoSmithKline Pharmaceuticals): contains PT, FHA, and pertactin
- TriPedia (Sanofi Pasteur, Inc.): contains PT and FHA
- DAPTACEL (Sanofi Pasteur, Inc.): contains PT, FHA, pertactin, and fimbriae types 2 and 3

The immunization schedule consists of three doses of DTaP by intramuscular injection at 4- to 8-week intervals, typically at 2 months, 4 months, and 6 months of age, with a fourth dose at 15 to 18 months of age. A fifth dose is recommended at 4 to 6 years of age. In general, studies have demonstrated that multicomponent vaccines containing pertactin and fimbrial antigens in addition to PT and FHA elicit a more robust immune response than vaccines containing only PT and FHA.[1040] These vaccines also appear to be highly immunogenic when administered along with other childhood immunizations. DTap is also available in combinations with inactivated polio vaccine (KINRIX, GlaxoSmithKline Pharmaceuticals), *H. influenzae* type b vaccine (TriHIBit, Sanofi Pasteur), inactivated polio and hepatitis B vaccines (Pediarix, GlaxoSmithKline Pharmaceuticals), and inactivated polio and *H. influenzae* type b vaccines (Pentacel, Sanofi Pasteur, Inc.). KINRIX is only used for dose 5 of DTaP, which occurs at age 4 to 6 years, when the child is due for the inactivated polio vaccine booster. TriHIBit is licensed for the 15- to 18-month dose, which can be given after the first three DTaP and hepatitis B vaccine series.

Acellular pertussis vaccines have been investigated for immunogenicity in previously vaccinated or unvaccinated adults. Studies in adult populations have shown that these vaccines, given alone or combined with diphtheria and tetanus toxoid, produce minimal adverse effects and engender brisk antibody responses to PT, FHA, pertactin, and fimbrial antigens present in the vaccine with no interference with the responses to either of the toxoids. The combination "Tdap" vaccines are combinations of tetanus toxoid, diphtheria toxoid, and acellular components of *B. pertussis*, including PT, FHA, pertactin, and fimbrial antigens. As indicated by the lowercase "d," the concentration of diphtheria toxoid has been reduced in these "adult" formulations to prevent adverse effects, while the "a" in "aP" indicates that acellular pertussis components are present. Acellular pertussis booster vaccines formulated specifically for adolescents and adults have now been licensed for use in the United States and include Boostrix (GlaxoSmithKline Biologicals) and ADACEL (Sanofi Pasteur). Boostrix contains detoxified PT, FHA, and pertactin, while ADACEL contains PT, FHA, pertactin, and fimbriae types 2 and 3 as the acellular pertussis components. Boostrix is licensed for use in adolescents aged 10 to 64 years of age, while ADACEL is licensed for adolescents and adults aged 11 to 64 years.[783] The Advisory Committee on Immunization Practices (ACIP) has recommended that all adolescents scheduled for a diphtheria/tetanus booster (recommended every 10 years) and any adult receiving a tetanus toxoid booster receive Tdap instead.[181,735] Tdap can also be

administered as prophylaxis for tetanus wound management. Five years between doses of tetanus toxoid or doses of tetanus toxoid and Tdap is the current standard since frequent exposure to tetanus toxoid can cause local reactions. People who will be in contact with young infants are encouraged to get Tdap even if it has been less than 5 years since the last tetanus booster to reduce the risk of infants being exposed to pertussis. ACIP has also recommended that all healthcare workers receive Tdap.[783]

Clinical Significance of Other Bordetella *Species*

Bordetella parapertussis. *B. parapertussis* is associated with a pertussis-like illness in humans as well, but it is generally less severe in clinical presentation. Children with *B. parapertussis* infection had shorter durations of coughing, bronchospasm, whooping, vomiting, apnea, and cyanosis.[114,850] However, outbreaks of *B. parapertussis* have been reported where the illness has been quite severe and resulted in death, particularly in very young children. Wirsing von Konig and Finger compared the severity of disease in 33 children with *B. parapertussis* infection with 331 patients with *B. pertussis* infection and found that the frequency of whooping paroxysmal coughing, whooping, and vomiting were almost identical in both groups.[1358] Heininger and colleagues also compared *B. pertussis* infection in 76 patients with *B. parapertussis* infection in 38 patients matched by age and sex. They also found that the illness caused by *B. parapertussis* was typical of pertussis but much less severe, and that, unlike pertussis, lymphocytosis was not a characteristic of the infection, presumably due to the absence of the lymphocytosis-promoting activity of PT.[571] In a study conducted in Germany after introduction of the acellular pertussis vaccine, Liese and colleagues documented 180 *Bordetella* infections in small children.[787] Of these 180 cases, 116 (64%) were caused by *B. pertussis* and 64 (36%) by *B. parapertussis*. Paroxysmal cough, inspiratory whooping, and vomiting for ≥21 days were found in 53%, 22%, and 8% of all *B. pertussis* cases and in 22%, 5%, and 0% of all *B. parapertussis* cases, respectively. Of the 116 *B. pertussis* cases, 81 (70%) of the children had received at least one dose of acellular pertussis vaccine, and of the 64 cases of *B. parapertussis* infection, 56 (87.5%) had received at least one vaccine dose. Typical pertussis was present in 29 (83%) of 35 unvaccinated *B. pertussis* cases, in contrast to 33 (41%) of 81 vaccinated *B. parapertussis* cases.

Bordetella bronchiseptica. *B. bronchiseptica* causes respiratory tract infections in various animal species (e.g., infectious tracheobronchitis or "kennel cough" in dogs,

pneumonia in cats, atrophic rhinitis and bronchopneumonia in pigs and piglets,

pneumonia and otitis media in rabbits and guinea pigs) and may be isolated as a commensal from the human upper respiratory tract. Humans may acquire the organism from domesticated animals, and nosocomial transmission from patient to patient may also occur.[617,1066] *B. bronchiseptica* is an unusual cause of infections primarily in immunocompromised hosts, including patients with hematologic malignancies, solid organ transplants, human stem cell transplant recipients, cystic fibrosis, and COPD.[489,495,498,617,866,943,1339] Since 1991, several cases of *B. bronchiseptica* pneumonia, sinusitis, and pleural infections have been reported in patients with AIDS.[382,468,866,1304] Bacteremia, meningitis following head trauma, peritonitis, bronchitis, and pneumonia caused by *B. bronchiseptica* have been reported in patients with underlying liver disease, alcoholism, asplenia, hematologic malignancy, chronic renal failure, asthma, systemic lupus erythematosis, and severe hypertension.[105,1028,1338,1361] *B. bronchiseptica* may occasionally cause severe infections, such as pneumonia with shock, in immunocompetent patients.[1241] Rare reports of endocarditis caused by *B. bronchiseptica* have also appeared in the literature. In one such instance, the patient presented with fever and a dermatitis around a recent surgical incision.[1189] The patient's dog frequently nipped and licked the area of dermatitis surrounding the incision, suggesting a canine origin for the organism.

Bordetella avium. *B. avium* causes rhinotracheitis in turkeys and other avian species.[696] *B. avium* infections in humans have been described in patients with cystic fibrosis.[1216] This organism was also isolated from BAL fluid from a 68-year-old man with pneumonia and hemoptysis following a 10-day history of increasing shortness of breath and cough, and from a 61-year-old homeless man with COPD.[561] The isolate from the latter patient showed 11 base pair differences and 15 base pair differences compared with the type strain of *B. avium* and the type strain of *B. trematum*, respectively; so the isolate was termed *B. avium* like.

Bordetella hinzii. *B. hinzii* was proposed as a new species in 1995, although the organism had been described informally when it was isolated from sputum and multiple blood cultures collected from a 42-year-old patient with AIDS who did not have any respiratory tract symptoms.[303,1288] This *B. avium*-like organism has been isolated from the respiratory tract of chickens and turkeys in addition to respiratory tract specimens from humans. *B. hinzii* strains were isolated on eight occasions over a 3-year period from an adult patient with cystic fibrosis in association with acute exacerbations of his chronic pulmonary disease; the organism could not be eradicated from the respiratory tract of the patient despite appropriate chemotherapy.[462] In 2000, fatal *B. hinzii* bacteremia was reported in a 69-year-old man with cholestasis, and *B.*

hinzii was isolated along with *Nocardia asteroides* complex organisms from BAL fluid of a patient with AIDS.[466,686] Although avian exposure of the respiratory tract could be ruled out in the patient with AIDS, possible acquisition from avian exposure or gastrointestinal colonization was suggested as possible modes of pathogenesis in the bacteremic patient. *B. hinzii* has been isolated from biliary tract specimens collected from a liver transplant recipient with chronic cholangitis and from blood cultures obtained from a patient with myelodysplastic syndrome.[69,457] In 2008, sepsis with *B. hinzii* was documented in a 36-year-old woman with EBV viremia, human herpes virus type 6 viremia, and diffuse large B-cell lymphoma. In this case, *B. hinzii* was also isolated from sputum cultures.[612] Another as yet unnamed "*B. avium*-like" isolate was recovered in mixed culture from a patient with chronic otitis media. This isolate was distinct in its antimicrobial resistance pattern, protein and fatty acid composition, and both DNA:DNA and DNA:rRNA hybridizations.

Bordetella holmesii. *B. holmesii*, described originally as CDC nonoxidizer group 2 (NO-2), has been isolated from blood cultures of patients with acute febrile illnesses, endocarditis, sickle cell anemia complicated by arthritis, diabetes, prior splenectomies, Hodgkin's disease, and respiratory insufficiency.[1343] Of the 15 patients described in this report, underlying disease was present in seven cases, three occurred in asplenic patients, and one patient had been bitten by a dog. The strains described in this 1995 report had been isolated in Switzerland, Saudi Arabia, and the United States. In 1996, Lindquist and colleagues isolated *B. holmesii* from multiple blood cultures of a 12-year-old boy who presented with fever, headache, and a history significant for splenectomy 4 years earlier.[792] Although the normal habitat of *B. holmesii* is unknown, the patient had frequent and prolonged contact with hunting dogs and had recently suffered a fishhook injury to his thumb. Tang and others at the Mayo Clinic reported the isolation of *B. holmesii*-like organisms from three patients with septicemia, endocarditis, and respiratory failure.[1245] Risk factors in these three patients included underlying asplenia, Hodgkin's disease, and severe cardiomyopathy, respectively. *B. holmesii* was also isolated from a 10-month-old infant who presented to the emergency room with fever and irritability.[910] Originally diagnosed as having a viral syndrome, *B. holmesii* was recovered from a single aerobic blood culture within 36 hours. Follow-up found the infant to be afebrile and asymptomatic. *B. holmesii* has also been isolated from multiple blood cultures of a 24-year-old man who presented with dry cough and fever, and a history remarkable for neonatal-onset sickle cell anemia, hepatitis C, tibial osteomyelitis at age 6 years, and frequent abdominal crises requiring multiple transfusions until the age of 8 years.[948] *B. holmesii* was isolated from blood, pleural fluid, and lung biopsy specimens from a previously healthy 14-year-old girl with a 6-month history of weight loss and dyspnea.[1122] *B. holmesii*

bacteremia has also been documented as a cause of fever and bacteremia in patients with asplenia, sickle cell disease, and nephrotic syndrome.[364,868,998,1183] *B. holmesii* bacteremia may also occur as a complication of hemodialysis.[516] *B. holmesii* has also been isolated from respiratory tract specimens of patients suspected of having pertussis. Of 10,996 specimens submitted from January 1995 through December 1998 to the Massachusetts State Laboratory Institute in Boston for culture of *Bordetella* species, 32 specimens grew *B. holmesii*, while 740 and 96 specimens were positive for *B. pertussis* and *B. parapertussis*, respectively.[865,1399] A study conducted on French patients with suspected pertussis found *B. holmesii*

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DNA by real-time PCR in 20.3% of patient specimens.[947] However, a 2006 survey of 2,804 nasopharyngeal swabs from Finnish patients and 8,515 swabs from Dutch patients with suspected pertussis using a specific, real-time PCR assay for *B. holmesii* failed to detect any specimens that were positive for this agent.[48]

Bordetella trematum. *B. trematum*, another recently described species, has been recovered from human wound specimens, diabetic leg ulcers, and patients with chronic otitis media.[332,1287] This species was found to be genetically and chemotaxonomically more closely related to type strain of genus *Bordetella* (*B. pertussis*) than to the type strain of the genus *Alcaligenes* (*A. faecalis*).[1287]

Bordetella petrii. *B. petrii* was first isolated in 2001 from an anaerobic dechlorinating bioreactor culture enriched with sediment from river water.[1307] Subsequently, two strains of *B. petrii* were isolated from a patient with mandibular osteomyelitis and from a patient with chronic suppurative mastoiditis.[458,1219] Similar strains have also been isolated from respiratory tract specimens from patients with COPD and bronchiectasis. In these patients, the organism may persist for years and be isolated from purulent sputum each time the patient had an exacerbation of symptoms.[770] The environmental origin of *B. petrii* is also supported by its recovery from marine sponges and grassroot microenvironments.

Bordetella ansorpii. *B. ansorpii* is a species that was first described in 2005. This novel species was isolated from the purulent exudate of an epidermal cyst on the neck of a 19-year-old female in Korea who was hospitalized for chemotherapy due to rhabdomyosarcoma.[711] This same organism was isolated in the United Kingdom in 2007 from two sets of blood cultures that had been collected through a Hickman catheter that had been placed 6 months prior.[459] The patient was an 88-year-old man

who was receiving chemotherapy for acute myeloid leukemia.

Culture and Isolation of Bordetella pertussis

Since *B. pertussis* preferentially attaches to the ciliated epithelium in the upper respiratory tract, the specimen of choice is a nasopharyngeal aspirate. Nasopharyngeal aspirates are optimally collected with an infant-size feeding tube connected to a mucus trap. The tip of the feeding tube is threaded into one nostril along the floor of the nasopharynx until it reaches the posterior pharynx. Once the tube is in place, gentle suction is applied. Following aspiration, saline (1 mL) is flushed through the tubing into the trap.[546] For swab specimens, a small-tipped, nasopharyngeal swab is passed posteriorly through each nostril until the swab tip reaches the posterior nasopharynx. Each swab is left in place for 30 seconds to one minute to allow organisms to adsorb onto the swab. Cotton-tipped swabs should not be used, because cotton material may be inhibitory to the organism. In addition, PCR-based assays for direct detection of *B. pertussis* may be inhibited by calcium alginate fibers and aluminum; specimens processed for detection of *B. pertussis* by PCR should be collected with Dacron swabs with plastic shafts.[1310] Nasopharyngeal swabs collected along with an aspirate may provide the highest yield of positive cultures. Regular throat cultures are not appropriate for isolation of *B. pertussis*. Specimens for bordetellae also require special transport media for optimal organism recovery. Regan–Lowe (RL) transport medium may be used for aspirates and swab specimens and has the advantage of serving as both a transport and an enrichment medium (see below).

Special medium is required for the isolation of *B. pertussis.* The classical medium used for this organism is Bordet–Gengou (BG) agar. This medium is prepared from potatoes to impart a high starch content to the medium. The starch neutralizes toxic materials that may be present in the agar or in the specimen itself. Peptones are omitted from the medium, since these proteins are also inhibitory. BG agar also contains glycerol as a stabilizing agent. Although "homemade" BG agar base is superior, dehydrated basal medium is commercially available (Remel Laboratories; BD Biosciences). The base is prepared ahead and stored under refrigeration. If a patient is suspected of having pertussis, the laboratory must be notified in advance of receiving the specimen, since the final medium must be freshly made. Although optimal, this is impractical for most laboratories. To prepare the medium for use, the potato/glycerol agar base is melted, and 30 mL of defibrinated sheep blood per 100 mL agar base medium (approximately 23% blood w/v) is added. Cephalexin (final concentration of 40 µg/mL) should be added to some of the medium to inhibit contaminating gram-positive organisms that may be present in the specimen; methicillin (2.5 µg/mL) or oxacillin (0.625 µg/mL) may be substituted for the cephalexin. Both nonselective and selective medium should be inoculated, since some *B. pertussis* strains may be slightly inhibited by cephalexin.

In 1977, Regan and Lowe described a medium containing charcoal and horse blood that has demonstrated superiority to BG agar in several studies (Image Plate 9-5G and 9-5H).[1070] Although originally described as a transport/enrichment medium, RL agar formulations are available both as a semisolid transport/enrichment medium and as a solid medium for organism isolation. The formula for RL medium is shown below (per liter distilled water):

Charcoal agar (Oxoid CM 119)	51 g
Horse blood, defibrinated	100 mL
Cephalexin	0.04 g
Amphotericin B (optional)	0.05 g
Final pH	7.4

The semisolid transport/enrichment medium is identical in formula to the isolation medium shown above, except that the charcoal agar is present in half strength (i.e., 25.5 g/L) and is dispensed into sterile screw-capped tubes rather than into 100 mm Petri dishes. It is also recommended that some of the medium be prepared without cephalexin so that both selective and nonselective media are available for recovery of the organisms. Optimally, media should be directly inoculated at the time of specimen collection onto both selective (methicillin- or cephalexin-containing) and nonselective media. If a transport system is used, the RL semisolid

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medium is optimal, since Stuart and Amies transport medium formulations are not suitable for maintaining the viability of *B. pertussis*. Specimens transported in semisolid RL medium can be subcultured on receipt to RL isolation medium or BG medium with and without cephalexin (or methicillin). The transport medium is then incubated along with the primary plate and subcultured to RL agar after 48 hours' enrichment at 35°C in ambient atmospheric air. Contrary to widely held beliefs and practices, ambient air is superior to a CO_2-enriched incubation environment. Plates should be incubated for at least 7 to 10 days. In a study conducted in Canada, however, 7 (16%) of 44 *B. pertussis* isolates and 2 (50%) of 4 *B. parapertussis* isolates were obtained only after 12 days of incubation.[689] *Bordetella* species will also grow on BCYE agar used for the isolation of *Legionella* species.[945]

Although culture is considered the "gold standard," the availability of sensitive molecular assays has demonstrated that the diagnostic performance of culture methods is highly dependent on the stage of the disease when specimens are collected, previous

antimicrobial administration, vaccination status, the techniques used to collect the specimens, transport conditions, and the experience of the laboratory in handling pertussis specimens and cultures.[248] The earlier in the clinical course that specimens are obtained, the greater the yield obtained with culture. Under optimal conditions, the sensitivity of pertussis culture can be 80% to 90%, but in practice, sensitivity usually ranges from 30% to 60%.[1406] The yield of *B. pertussis* in culture declines after 2 or more weeks of cough illness, after antimicrobial treatment, or after previous pertussis vaccination.[1291] By the time patients have been symptomatic for 2 to 3 weeks, the sensitivity of culture falls to less than 5%.[248]Although *B. pertussis* can be isolated in culture as early as 72 hours after plate inoculation, cultures may require 10 days' to 2 weeks' incubation before they can be definitively called negative. However, culture is required to obtain isolates for antimicrobial susceptibility testing or molecular typing.

Direct FA Test

Direct FA (DFA) tests have been used to detect *B. pertussis* and *B. parapertussis* directly on smears prepared from nasopharyngeal specimens (Image Plate 9-5E). While DFA tests do provide rapid results, culture on appropriate media is more sensitive than DFA and far less sensitive than molecular methods (i.e., PCR). Depending on when in the clinical course the specimens are collected, the DFA test may not detect small numbers of organisms that may be detected by culture or by PCR. Historically, the sensitivities reported for DFA have ranged from 11% to 68%, with corresponding specificities of 99.6% to 100%.[797,1336] Halperin and associates found that only 6 of 20 patients who were positive by culture were DFA-positive, and that only 4 of the 12 DFA-positive, culture-negative cases were confirmed by serologic testing of acute and convalescent serum speciemens.[547] Tilley and colleagues evaluated culture, two PCR assays, and DFA for diagnosis of pertussis with 637 nasopharyngeal swabs and aspirates submitted for pertussis evaluation.[1257] DFA reagents included polyclonal antipertussis and antiparapertussis reagents (BD Difco FA Pertussis and BD Difco FA Parapertussis, BD Biosciences), fluorescein isothiocyanate-labeled monoclonal anti-LPS antibody, and "Accu-MAb Plus" *B. pertussis/B. parapertussis* monoclonal antibody reagents (Delta Biotech, Inc., British Columbia, Canada). The sensitivities of the polyclonal DFA reagent, the anti-LPS antibody reagent, and the commercial monoclonal antibody reagent were 11.4%, 5.2%, and 8.3%, respectively, while the corresponding specificities were 94.6%, 98.1%, and 98.4%, respectively. In contrast, the two PCR assays used in this study had sensitivities of 95.0% and 89.2%. DFA smears were often difficult to interpret due to the small size of the bacteria and the presence of artifacts. Owing to poor performance and the availability of superior diagnostic approaches, the DFA test for detection of *B. pertussis* should no longer be performed in clinical microbiology

laboratories.[1336]

Cultural Characteristics and Identification of Bordetella *Species*

On culture, colonies of *B. pertussis* may be observed after 2 to 4 days. Growth is usually apparent sooner on antibiotic-free medium, but this is not always the case. Plates should be examined under a dissecting microscope (10×) with oblique incident light to determine colony characteristics. Fresh clinical isolates of *B. pertussis* on BG agar appear as smooth, shiny colonies with a high, domed profile. Classically, the colonies are described as resembling small droplets of mercury. Colonies on BG agar may be slightly β-hemolytic, particularly in the more confluent areas of growth or after prolonged incubation. On RL agar medium, colonies are small, domed, and shiny, with a white mother-of-pearl opalescence. *B. parapertussis* colonies grow more rapidly, are more β-hemolytic on BG agar, and are gray or slightly brown in color. *B. bronchiseptica* colonies are apparent within 24 hours, are large, are flatter, and have a dull rather than a shiny appearance. The latter species resembles a nonfermentative gram-negative rod (like an *Alcaligenes* species) in the appearance of its colonies and the production of a distinct "nonfermenter" odor. Gram's stain morphology of these organisms also differs. *B. pertussis* appears as small, pale-staining coccobacilli, while *B. parapertussis* and *B. bronchiseptica* are more definitely rod-shaped (Image Plate 9-5F). Because of the pale staining of the organisms, the safranin counterstain should be left on the slide for at least 2 minutes.

Once isolated, *B. pertussis* can be identified by the FA test or slide agglutination, since the organism is quite inert in biochemical identification tests (Table 9-16). For the FA test, organisms from plates should be suspended in phosphate-buffered saline (PBS), and known cultures of both *B. pertussis* and *B. parapertussis* should be used as controls. For slide agglutination, an organism suspension equivalent to a McFarland no. 3 turbidity standard should be prepared in PBS, and positive and negative controls should be included. Serologic methods may be used to identify *B. parapertussis* as well, but biochemical methods may provide a presumptive identification. *B. pertussis*, *B. bronchiseptica*, *B. hinzii*, and *B. avium* are oxidase-positive with Kovac's oxidase reagent, while *B. parapertussis*, *B. holmesii*, and *B. trematum* are oxidase-negative. *B. parapertussis*, *B. bronchiseptica*, *B. hinzii*, and *B. holmesii* are catalase-positive, while *B. pertussis* is variable for catalase production. Both *B. parapertussis* and *B. bronchiseptica* are urease-positive, with the latter species producing a positive test in less than

TABLE 9-16 Phenotypic Characteristics for Identification of *Bordetella* Species

Characteristic	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*	*B. hinzii*	*B. holmesii*	*B. trematum*	*B. petrii*	*B. ansorpii*
Oxidase	+	–	+	+	+	–	–	+	–
Catalase	+	+	+	+	+	+[a]	+	NA	NA
Motility	–	–	+	+	+	–	+	–	+
Nitrate reduction	–	–	+	–	–	–	V	–	–
Denitrification	–	–	–	–	–	–	V	+	
Urease	–	+ (24 hours)	+ (4 hours)	–	V	–	–	–	–
Citrate	–	V	+	V	+	–	–	NA	NA
Brown pigment in heart infusion agar with L-tyrosine (1 g/L)	–	+	–	–	–	+	–	–	NA
Growth on:									
BG agar	2–6 days	1–3 days	1–2 days	2 days	2 days	2 days	2 days	+	+
RL agar	3–6 days	2–3 days	1–2 days	1–2 days	2 days	2 days	2 days	+	+
Blood agar	No growth	1–3 days	1–2 days	2 days	2 days	2 days	2 days	+	+
Chocolate agar	No growth	1–3 days	+	2 days	2 days	2 days	2 days	+	+
MacConkey agar	No growth	+	+	+	+	No growth	+	+	+
Major cellular fatty acids	C16:1ω7c	C16:0, C17:0cyc	C16:0, C16:1ω7c, C17:0cyc	C16:0, C17:0cyc	C16:0, C17:0cyc	C16:0, C17:0cyc	C16:0, C17:0cyc	NA	NA

[a] Weak/delayed reaction.
+, positive reaction; –, negative reaction; V, variable reaction.

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4 hours. *B. bronchiseptica* can be misidentified as *Oligella ureolytica* or *Ralstonia paucula*, but *B. bronchiseptica* reduces nitrate to nitrite (*R. paucula* is negative), does not reduce nitrite to nitrogen gas, and is penicillin susceptible (*O. ureolytica* reduces nitrite to nitrogen gas and is penicillin-resistant). *B. hinzii* is variable for urease production, while *B. pertussis*, *B. holmesii*, *B. avium*, and *B. trematum* are urease-negative. Both *B. parapertussis* and *B. holmesii* produce a soluble brown pigment on agar media, while *B. pertussis*, *B. bronchiseptica*, *B. hinzii*, *B. trematum*, and *B. avium* do not. Other characteristics for the identification of *Bordetella* species are shown in Table 9-16.

Molecular Methods for Detection and Identification of Bordetella *Species*

With the genetic characterization of *B. pertussis* and other *Bordetella* species, molecular assays have been developed for direct detection of these organisms in nasopharyngeal specimens and for identification of organisms recovered in culture. PCR-based assays have, in fact, become the tests of choice for diagnosis of pertussis because of their exquisite sensitivity and specificity. Nucleic acid target sequences specific for *B. pertussis* and other species have been identified and sequenced, and primers and probes have been designed for detection of these targets by PCR. Targets identified for potential use in direct detection include the PT gene promoter region (*ptxA*), outer membrane porin (PO) genes, the adenylate cyclase gene, the *recA* gene, and sequences determined to be species-specific based on the entire *B pertussis* genome sequences.[1045,1305]

Most molecular techniques described for *Bordetella* species involve the detection of

insertion sequences such as IS*481*, IS*1001*, and IS*1002*. *B. pertussis* carries up to 80 to 100 copies of IS*481,* and this sequence is found in *B. pertussis, B. holmesii*, and occasionally *B. bronchiseptica.* IS*1001* is found in *B. parapertussis* and occasionally in *B. bronchiseptica* and *B. holmesii*. Reischl and colleagues designed a real-time PCR assay targeting IS*481* and found that specificity of the assay was compromised by the finding of similar IS*481* sequences in *B. holmesii*.[1077] Sloan and colleagues also designed a multiplex, real-time PCR assay for detecting IS*481* and IS*1001* of *B. pertussis* and *B. parapertussis*, respectively, and also noted that false-positive results may occur with *B. holmesii*.[1198] This approach was also used by other investigators, who also noted the presence of IS1001 in *B. holmesii*. Differentiation of *B. parapertussis* and *B. holmesii* was accomplished by sequencing of the amplification product. Poddar designed a LightCycler assay for *B. pertussis* and *B. holmsei* targeting IS*481*, and differentiation of the two species was accomplished by PCR probe-target melting profile analysis, which was able to detect the single nucleotide difference in the IS*481* sequences of the two organisms.[1037] Menard and colleague described a similar assay for *B. pertussis* and *B. parapertussis* based on amplification of the IS*481* and IS*1001* of *B. pertussis* and *B. parapertussis*, respectively, with species identification again confirmed by melting-curve analysis.[882] In this study, one specimen grew *B. bronchiseptica* while the PCR assay was positive for *B. pertussis*. The *B. bronchiseptica* strain harbored an IS*481* insertion sequence, which is found in less than 1% of *B. bronchiseptica* isolates. Rooda and colleagues designed a real-time PCR assay for detection and discrimination of all clinically relevant *Bordetella* species.[1107] This assay detected IS*481*, IS*1001*, and IS*1002*. Amplification of both IS*481* and IS*1002* confirmed detection of *B. pertussis*, amplification of both IS*1001* and IS*1002* confirmed detection of *B. parapertussis*, and amplification of IS*1001* only confirmed detection of *B. bronchiseptica*. Amplification of IS*481* only detected *B. holmesii* and the rare isolate of *B. bronchiseptica* harboring the IS*481* element. Knorr and colleagues devised two real-time LightCycle PCR assay for *B. pertussis* where specimens were tested for detection of IS*481* and, if positive, for the presence of IS*1001*.[710] Specimens positive for IS*481* and negative for IS*1001* were confirmed to be *B. pertussis*. No *B. holmesii* strains (where IS*481* and IS*1001* are both present) were detected in this study. Xu and coworkers designed a single-tube, "triplex" real-time PCR assay that used three targets for amplification: *ptxP* (PT promoter region) and IS*481* for *B. pertussis*, and the insertion sequence IS*1001* for *B. parapertussis*.[1377] The lower limits of detection in this assay were 1 to 5 cfu for *B. pertussis* and 1 cfu for *B. parapertussis*. Even though IS*1001* is also found in some *B. holmesii* strains, the primers and probes used in this triplex assay were specific for *B. parapertussis.* Specific detection of *B. holmseii* directly in clinical specimens has been accomplished by designing real-time PCR assays targeting a 50 base pair segment

of the housekeeping gene *recA* that is polymorphic among *B. holmesii* and all other *Bordetella* species.[538]

Serologic Tests for Diagnosis of Pertussis

Prior to the vaccine efficacy trials, serologic tests for pertussis were performed with inactivated whole cells, whole-cell sonicates, or crude acellular extracts of *B. pertussis* as antigens and interpretation of these tests was often difficult. For vaccine trials conducted with the WHO, pertussis serology was developed as a semistandardized EIA procedure using purified antigens and was included in the case definition of pertussis.[1359] These antibody assays used purified antigens (e.g., PT, FHA, pertactin, fimbrial antigens) in an EIA format. Antibody directed against PT is specific for *B. pertussis*, while anti-FHA antibodies are also produced following infection with *B. parapertussis*. Other organisms may also engender antibodies that cross-react with *B. pertussis* FHA (e.g., nontypeable *H. influenzae*). Following natural infection, antibodies usually appear 10 days to 2 weeks after the onset of symptoms, with anti-PT and anti-FHA IgG antibodies being detected in over 90% of patients and IgA antibodies being detected in 20% to 40% of infected individuals. Immune responses to other antigens (e.g., pertactin, fimbrial antigens) tend to be more variable and may be found in 20% to 60% of infected persons. Therefore, the most specific serologic assay is IgG to PT as it is unique to *B. pertussis* and the titer rapidly falls below cutoff values within 4 to 5 months' post infection and, in 82% of patients, with 1 year post infection.[85,316,1325]

The immune response to pertussis is also influenced by vaccination status and age of the host. In the case definition developed by WHO, serologic confirmation required paired sera (showing seroconversion from negative to positive, or a fourfold titer increase), along with clinical criteria

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(i.e., a spasmodic cough for more than 21 days).[1359] Trollfors et al. performed pertussis serology on a group of unvaccinated Swedish children with clinical (i.e., cough ≥21 days and cough <21 days) and nonserologic evidence of *B. pertussis* infection (i.e., positive culture, PCR).[1265] Among those with cough for ≥21 days, levels of anti-PT and anti-FHA increased 400-fold and 68-fold, respectively. More than 90% of the children had significant titer increases to both antigens if the acute specimen was obtained within 14 days of disease onset. Serology may not be useful with very young acutely ill patients, as some culture-positive individuals, particularly infants less than 3 months of age, do not develop measurable antibodies.[248] The highest sensitivity and specificity for the serologic diagnosis of pertussis are the

demonstration of at least a twofold rise in titer against PT between an acute and a convalescent specimen.[856] In vaccinated children, the secondary immune response results in rapid increases in antibody titer; so a significant titer changes between sequential specimens may not be seen. Serologic testing on older, vaccinated populations may be diagnostically useful since a high titer from the secondary immune response in a single serum specimen may indicate a current infection.[856] This approach may be useful for diagnosing pertussis in adolescents and adults, since pertussis often is not included in the initial differential diagnosis of nonpediatric respiratory infections; consequently, attempts at culture or PCR are made late in the clinical course when these two assays may be negative.[248]

Treatment of Pertussis

Pertussis in children is generally treated with erythromycin (estolate form, 40 to 50 mg/kg/day) in four divided doses for 14 days to prevent relapse. For adults and adolescents, 2 g/day is administered for 14 days. Shorter courses (e.g., 7 to 10 days) may not eliminate the organism from the upper respiratory tract. Administration of antimicrobial therapy even during the paroxysmal stage shortens the severity and the duration of illness. Strains of *B. pertussis* are susceptible to erythromycin in vitro, having MICs ranging from 0.02 µg/mL to 0.12 µg/mL. Erythromycin resistance in *B. pertussis* was not seen until 1995, when the first case of pertussis caused by an erythromycin-resistant *B. pertussis* strain was reported in a 2-month-old child in Yuma, Arizona.[785] After the child failed to improve clinically on a 12-day course of erythromycin, disk testing of the isolate showed no zones of inhibition around the erythromycin, clarithromycin, and clindamycin disks. Agar dilution testing revealed an erythromycin MIC of >64 µg/mL. Erythromycin-resistant strains of *B. pertussis* remain relatively uncommon, but their prevalence may be underestimated. In a survey of 47 isolates from children collected in the intermountain west region of the United States from January 1985 through June 1997, only one strain was erythromycin-resistant (MIC = 32 µg/mL) on agar dilution testing.[725] Screening of 1,030 *B. pertussis* isolates at the CDC using a disk diffusion method revealed 5 strains that displayed a heterogenous resistance phenotype in which isolated colonies grew within the zones of inhibition around an erythromycin disk after 5 to 7 days' incubation.[1352] When these colonies were subcultured and retested, only the resistant phenotype grew, and erythromycin susceptibilities of these isolates were shown to be >256 µg/mL by Etest. Retesting of isolates demonstrating heterogenous susceptibility to erythromycin consistently produced resistant colonies after 5 to 7 days that were not apparent after incubation for 3 to 5 days. Erythromycin resistance in these strains is apparently due to a mutation that alters the erythromycin binding site in the 23S rRNA gene.[92] Other drugs have been used in open noncomparative studies and successfully eliminate the

organism from the respiratory tract. Azithromycin for 3 to 5 days or clarithromycin for 7 days is able to eliminate *B. pertussis* from the nasopharynx and does not have the attendant gastrointestinal side effects of erythromycin.[28] TMP-SMX administered for 7 days is able to eradicate *B. pertussis* from the nasopharynx and has been used to successfully treat patients with erythromycin-resistant isolates. While TMP-SMX may be a useful alternative to erythromycin, the dosage and duration of therapy have not been ascertained. Fluoroquinolones also have generally good activity against *B. pertussis*, but clinical trials have not been performed since fluoroquinolone use is contraindicated in children. These agents may be useful for treatment of adults with pertussis infection.[504]

Antimicrobial Susceptibility Testing of Bordetella *Species*

Although CLSI-approved, standardized procedures are not available, several methods have been evaluated for in vitro antimicrobial susceptibility testing of *Bordetella* species. Agar dilution susceptibility testing has been performed with a number of media, including Müeller–Hinton agar with 5% horse blood, BG agar with 5% or 20% horse blood, and RL agar without cephalexin.[587,605] Hoppe and Paulus evaluated BG agar with 5% horse blood, Oxoid charcoal agar with 5% horse blood, and Müeller–Hinton agar with 5% horse blood for in vitro agar dilution testing. They found that Müeller–Hinton with horse blood provided the most consistent results.[605] MICs determined on charcoal-containing media were one- to fivefold higher for clinical isolates, control strains, and *S. aureus* ATCC 29213, due to inactivation of some antimicrobial agents by charcoal. Using the agar dilution method, *B. pertussis* was susceptible to β-lactams (i.e., ampicillin/amoxicillin), macrolides (i.e., azithromycin, clarithromycin, erythromycin, roxithromycin), sulfonamides (i.e., SXT), rifampin, and fluoroquinolones (i.e., ciprofloxacin, ofloxacin, temafloxacin, trovafloxacin).[604,606,725] Among the fluoroquinolones, levofloxacin was more active against *B. pertussis* (MIC= 1 μg/mL) than against *B. parapertussis* strains (MICs >2 μg/mL).[606] Most *B. pertussis* strains were resistant to tetracycline. Hill et al. tested a disk diffusion procedure using RL agar with a standardized inoculum (0.5 McFarland standard) and found that this method accurately detected erythromycin resistance. Resistant strains (MIC >256 μg/mL) produced no zones around the standard erythromycin disk, while susceptible strains (MIC ≤0.06 μg/mL) produced zones of at least 42 mm.[587]

Etest strips (bioMérieux, Inc.) and modified disk diffusion methods have also been evaluated for antimicrobial susceptibility testing of *B. pertussis*. Korgenski and Daly found essential agreement between agar dilution MICs and Etest MICs for 89.1% of 46 isolates (i.e., the Etest MIC was within

+/− 1 doubling dilution of the agar dilution MIC, while the remaining 10.9% had Etest MICs that were more than 1 doubling dilution different than the agar dilution MIC).[725] Hill and colleagues also evaluated the Etest using both BG agar with 20% horse blood and RL agar and found that the Etest method produced similar results to those of their "reference" agar dilution procedure (using BG agar with 20% horse blood) for erythromycin, rifampin, and chloramphenicol; SXT MICs using Etest were lower than those obtained with agar dilution.[587] Etest was employed in a erythromycin susceptibility survey of 99 *B. pertussis* isolates collected from 1971 through 2006 in Australia.[1190] All *B. pertussis* isolates were susceptible to erythromycin, with MICs of <0.12 μg/mL. Galanakis and Englund used Etest and Müeller–Hinton agar with 5% sheep blood to test 100 *B. pertussis* isolates from a regional laboratory in Washington state for susceptibility to macrolides (erythromycin, azithromycin), β-lactams (amoxicillin, meropenem), fluoroquinolones (ciprofloxacin, moxifloxacin), clindamycin, SXT, and tigecycline.[467] All isolates were susceptible to all agents. Elevated MICs were seen for clindamycin (3 strains) and amoxicillin (1 strain); MICs for azithromycin were slightly lower than those for erythromycin. All other agents, including tigecycline, were very active against all strains tested. A study of 27 *B. pertussis* isolates in Taiwan also found that all strains were susceptible to macrolides and SXT using Etest with Müeller–Hinton agar with 5% horse blood.[1397]Using Etest with Müeller–Hinton agar containing 5% sheep blood, Gordon and colleagues in Iowa tested 45 *B. pertussis* isolates and found that all isolates were susceptible to all agents tested, including macrolides (i.e., erythromycin, azithromycin, clarithromycin), clindamycin, fluoroquinolones (ciprofloxacin, gatifloxacin, and trovafloxacin), and SXT.[504] Similar results were reported by Fry and colleagues using Etest and Müeller–Hinton agar with 5% horse blood for testing of 583 *B. pertussis* isolates collected from 2001 through 2009.[460] Standardization of susceptibility testing for these organisms is needed for investigation of new antimicrobial agents against the bordetellae, and it may gain additional clinical relevance if erythromycin resistance becomes more widespread.

The other *Bordetella* species tend to be more resistant to antimicrobial agents than *B. pertussis*. As a part of the Multicenter Pertussis Surveillance Project, agar dilution antimicrobial susceptibility data were collected on 46 *B. parapertussis* and 11 *B. bronchiseptica* isolates.[741] Although the *B. parapertussis* strains were susceptible to erythromycin, SXT, and ciprofloxacin, most were resistant to amoxicillin and all were resistant to rifampin and tetracycline. Levofloxacin, ciprofloxacin, and cefpirome, a broad-spectrum cephalosporin, were more active against *B. pertussis* than against *B. parapertussis*.[606] Among the macrolides, both erythromycin and azithromycin are active against *B. parapertussis*, with 90% of strains having MICs for erythromycin and azithromycin of 0.5 μg/mL and ≤0.06 μg/mL, respectively.[604] In the multicenter surveillance project, 82% of *B. bronchiseptica* isolates were susceptible to SXT, 27%

were susceptible to ciprofloxacin, and all *B. bronchiseptica* strains were resistant to amoxicillin, erythromycin, rifampin, and tetracycline.[741] *B. hinzii* isolates are resistant to ampicillin, ampicillin sulbactam, cefazolin, ceftriaxone, cefotaxime, cefuroxime, cefotetan, aztreonam, ciprofloxacin, and tobramycin, and are susceptible to cephalothin, ceftazidime, cefepime, imipenem, tetracycline, SXT, levofloxacin, gentamicin, and amikacin.[462,686] Isolates of *B. holmesii* are susceptible to amoxicillin, amoxicillin clavulanate, cephalosporins (i.e., cefazolin, cefotaxime, ceftazidime), aminoglycosides (gentamicin, tobramycin, amikacin), imipenem, tetracycline, erythromycin, rifampin, ciprofloxacin, and SXT, but are resistant to penicillin.[792,948,1245]

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CHAPTER 10

Legionella

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Introduction

During the summer of 1976, an explosive outbreak of pneumonia of unknown etiology occurred among persons who attended an American Legion convention in Philadelphia.[66] Individuals in whom the multisystem illness (which included pneumonia) developed were said to have Legionnaires' disease.[24] A total of 182 infected patients were documented, of whom 29 died. By early January 1977, the etiologic agent had been isolated by Dr. Joseph McDade of the Centers for Disease Control (CDC).[110] Thus, within 6 months, a major medical mystery was solved and a new family of bacteria, the *Legionellaceae,* was discovered. The history of *Legionella* and Legionnaires' disease has been thoroughly reviewed.[61,113,149,171] Information is also available through the CDC (http://www.cdc.gov/legionella/index.html) and other web sites.[11]

Taxonomy and Characteristics of the Genus *Legionella*

In 1979, Brenner, Steigerwalt, and McDade classified the bacterium that caused the Philadelphia outbreak of Legionnaires' disease as *Legionella pneumophila,* in the family *Legionellaceae.*[21] At present, 50 validly published species and subspecies and a total of 71 serologic types of *Legionella* have been isolated from either human specimens, environmental sources, or both (Table 10-1).[81,173] Several recently described species are obligate intracellular parasites of free-living amoebae and can be isolated only by cocultivation with amoebae.[2,37] These amoeba pathogens were initially recognized by Rowbotham,[134] who called them *Legionella*-like amoeba pathogens (LLAPs). The most frequent of these species appears to be *L. lytica* (formerly called *Sarcobium lyticum*).

Legionella species are non–spore-forming, narrow, gram-negative bacilli, 0.3 to 0.9 µm in width. In length the bacteria vary from short forms, 1.5 to 2 µm in length, to longer filamentous forms. They are usually short and thin or coccobacillary when seen in direct smears of clinical specimens, but are more variable in length after growth in suboptimal culture media, when forms longer than 20 µm are not unusual. Legionellae stain much more readily with the Diff-Quik, Giemsa, or Gram–Weigert stains than they do with the traditional Gram's stain in touch preparations of fresh tissue imprints, smears of bronchial alveolar lavage (BAL) fluid, or sputum. The addition of 0.05% basic fuchsin to the safranin counterstain in the Gram's procedure, however, yields

TABLE 10-1 Species of the Genus *Legionella* (Number of Serogroups)

Species Isolated From Humans and From the Environment	Species Isolated From the Environment Only
L. anisa	*L. adelaidensis*
L. bozemanii (2)	*L. beliardensis*
L. birminghamensis	*L. brunensis*
L. cincinnatiensis	*L. busanensis*
L. dumoffii	*L. cherrii*
L. erythra (2)	*L. drancourtii*

L. feelei (2)	*L. drozanskii*
L. gormanii	*L. fairfieldensis*
L. hackeliae (2)	*L. fallonii*
L. jordanis	*L. geestiana*
L. lansingensis	*L. gratiana*
L. longbeachae (2)	*L. gresilensis*
L. londiniensis	*L. israelensis*
L. lytica	*L. jamestowniensis*
L. maceachernii	*L. moravica*
L. micdadei	*L. nautarum*
L. oakridgensis	*L. pittsburghensis*[a]
L. parisiensis	*L. quateirensis*
L. pneumophila—pneumophila (15)	*L. quinlivanii (2)*
L. pneumophila—fraseri	*L. rowbothamii*
L. pneumophila—pascullei	*L. rubrilucensa*
L. santicrucis	*L. shakespearei*
L. sainthelensi (2)	*L. spiritensis*
L. tucsonensis	*L. steigerwaltii*
L. wadsworthii	*L. taurinensis*
	L. waltersii[a]
	L. worsleiensis

[a] The pathogenicity of this organism for humans is unclear.

far better staining of *Legionella* spp., as well as many other poorly staining gram-negative bacilli.

Except for three species that are nonmotile, *L. oakridgensis*, *L. nautarum,* and *L. londinensis*, the remainder of *Legionella* species are motile by means of one or more polar or subpolar flagella.[173] The *Legionellaceae* are aerobic and nutritionally fastidious. They require L-cysteine and iron salts for growth. R.E. Weaver first cultivated *L. pneumophila* on Müeller–Hinton agar supplemented with 1% IsoVitalex and 1% hemoglobin. Isolates may grow very slowly on the chocolate agar used for gonococcus isolation as well.[38] The optimal growth medium is a variation on the charcoal yeast extract agar developed by the late James Feeley.[58] The yeast extract in the medium supplies the necessary nutrients. Activated charcoal removes toxic oxygen radicals produced by exposure of many media to light.[80] *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer has a pK of 6.9, the optimum for growth of *Legionella* spp. Addition of ACES buffer and α-ketoglutarate yields buffered charcoal yeast extract (BCYE) agar.[41]

Growth on BCYE agar with no growth on blood agar is one of the most useful presumptive clues that an isolate could be a species of *Legionella.* Another gram-negative organism that also grows on BCYE but not on ordinary blood agar is *Francisella tularensis.* In contrast to *Francisella* spp., which produce acid from carbohydrates, *Legionella* spp. neither ferment nor oxidize carbohydrates. Certain thermophilic spore-forming bacilli and *Bordetella pertussis* are also capable of growing on BCYE agar; differences in morphology, serologic features, and cellular fatty acids aid in differentiating these organisms. *Legionella* spp. produce characteristic branched-chain fatty acids in their cell walls.[173] Most species are weakly catalase positive and peroxidase positive. The sodium hippurate hydrolysis test, which is positive for *L. pneumophila* and negative for the majority of other *Legionella* species isolated from clinical materials, provides a useful presumptive procedure for differentiation between *L. pneumophila* and the other *Legionella* species. Phenotypic characterization of *Legionella* isolates using biochemical tests is of only limited value for presumptive identification of isolates to the species level. Serotyping of isolates using immunofluorescent antibody (IFA) testing, however, is a practical way to differentiate isolates presumptively as species of *Legionella.* Definitive identification of *Legionella* species may require nucleic acid studies and other chemotaxonomic reference procedures.[49,147]

Clinical and Pathologic Spectrum of Legionellosis

Legionnaires' disease occurs both sporadically, in the form of community-acquired pneumonia, and in epidemics.[23,57,56] In addition to Legionnaires' disease, a mild form of illness called Pontiac fever occurs.[72] Illness may also involve anatomic regions of

the body outside the chest cavity. Thus, the term *legionellosis,* including Legionnaires' disease, Pontiac fever, and extrapulmonary involvement with *Legionella* species, is used in this chapter to refer to any infection caused by bacteria of the family *Legionellaceae.* Approximately 85% of documented patients with legionellosis have *L. pneumophila* as the causative factor. Serogroups 1 and 6 of *L. pneumophila* account for up to 75% of the legionellae reported to cause human illness.[127] In addition to *L. pneumophila,* many other species have been isolated from clinical specimens collected from humans (Table 10-1). An international survey of patients with culture-confirmed infections disclosed that the most frequent non-*pneumophila* species were *L. longbeachae* and *L. bozemanii.*[174] *L. micdadei*[92,36] and *L. dumoffii*[156,85] have also been responsible for sporadic and epidemic disease in certain locales. Multiple species and/or serogroups may be present in water systems, either environmental or potable, and the same patient may be infected with more than one serogroup or species simultaneously.[101]

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TABLE 10-2 Clinical Manifestations in Two Types of Legionellosis

	Legionnaires' Disease	**Pontiac Fever**
Mortality	15%–30%	0%
Incubation period	2–10 days	1–2 days
Symptoms	Fever, chills, cough, myalgia, headache, chest pain, sputum, and diarrhea. Confusion or other mental status changes have occurred in some patients.	Similar to influenza: fever, chills, and myalgia. Cough, chest pain, and confusion have been reported in some patients.
Lung	Pneumonia and pleural effusion. Occasionally, pneumonic disease will progress to a lung abscess.	Pleuritic pain; no pneumonia, no lung abscess
Kidney	Renal failure (proteinuria, azotemia, and hematuria in some)	No renal manifestations
Liver	Modest liver-function abnormalities	No liver-function abnormalities
Gastrointestinal tract	Watery diarrhea, abdominal pain, and nausea and vomiting	No abnormalities

Central nervous system	Somnolence, delirium, disorientation, confusion, and obtundation. Rarely, seizure has been documented.	No central nervous system manifestations

Legionellosis has most commonly been recognized as a form of pneumonia. The earliest symptoms typically include fatigue, muscle aches, and a slight headache. During the first day, patients commonly experience a rapid onset of dry cough and elevated temperature (i.e., 102°F to 104°F or higher) with chills. Abdominal pain and gastrointestinal symptoms (i.e., nausea, vomiting, and diarrhea) occur in many patients. A summary of clinical manifestations is given in Table 10-2. Investigators have repeatedly demonstrated their inability to distinguish among the various etiologies of pneumonia based on clinical history, physical examination, or traditional laboratory tests.[131] However, the introduction of the *Legionella* urinary antigen test and nucleic acid amplification assays has improved our ability to definitively diagnose patients with legionellosis.

At onset, chest roentgenograms typically show patchy infiltrates, which may progress to five-lobe consolidation.[152,54,112] Figure 10-1 illustrates a chest roentgenogram of a patient with legionellosis. Infiltrates are bilateral in two-thirds of patients, and abscess cavities may be present, particularly in immunocompromised patients. Laboratory findings commonly include, in varying combinations, the following: a moderate leukocytosis with a left shift, proteinuria, hyponatremia, azotemia, elevated serum aspartate aminotransferase (AST), and a high erythrocyte sedimentation rate. As mentioned previously, legionellosis may also take the form of a mild, self-limited illness of short duration, known as Pontiac fever. This disease presents with elevated temperature, myalgia, malaise, and headache, but with few or no respiratory findings and no pneumonia.[59,63] Table 10-2 provides a comparison of the clinical aspects of Legionnaires' disease and Pontiac fever. Both pneumonic disease and nonpneumonic disease may result from exposure to the same environmental source.[71]

The clinical spectrum of legionellosis has expanded from that which was originally defined. The illness may involve essentially any organ system of the body, with or without pneumonia. Examples of selected manifestations of extrapulmonary involvement follow. Bacteremia has been reported, but data on its frequency are lacking.[43,98,130] Nearly half the patients with Legionnaires' disease show central nervous system manifestations such as headache, lethargy, confusion, stupor, and other less frequent manifestations, including ataxia, coma, and seizures.[84] A painful, nonpruritic, macular rash, limited to the pretibial surfaces of the legs, has been reported; however, dermal manifestations are uncommon.[78] *L. pneumophila*

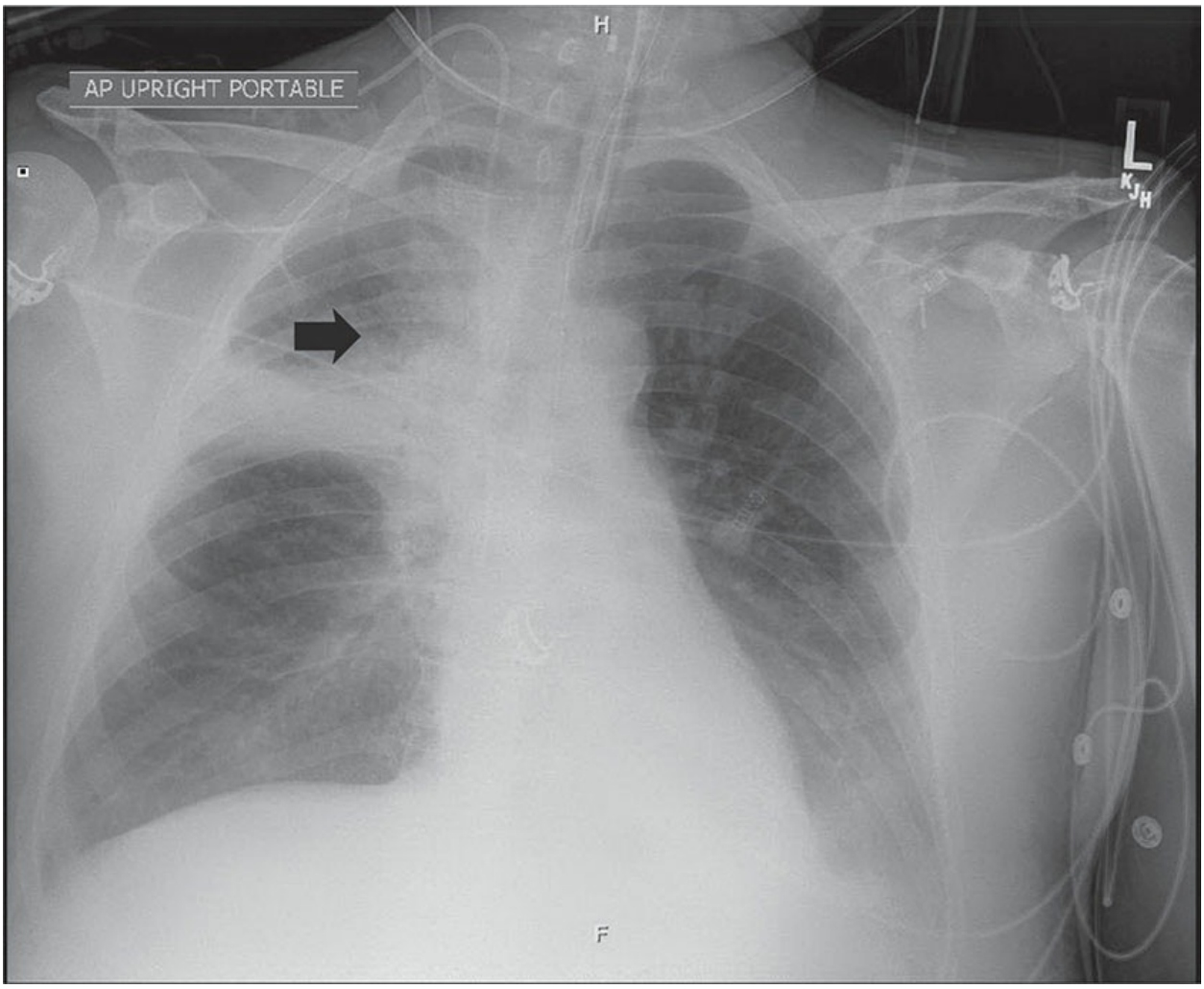
serogroup 1 has been demonstrated in lymph nodes, spleen, kidney, and bone marrow and has been documented in acute myocarditis,[166,172] prosthetic valve endocarditis,[109] pericarditis,[108] and hemodialysis fistula infections.[89] Arnow and associates[7] reported the isolation of *L. pneumophila* serogroup 3, mixed with multiple species of anaerobic bacteria, from a perirectal abscess. *L. pneumophila* serogroup 4 was shown by direct immunofluorescence in lesions of acute pyelonephritis in a patient who had both pneumonia and pyelonephritis associated with this organism.[35] Cutaneous legionellosis, caused by *L. pneumophila* serogroup 8, has been described in a 27-year-old woman who was immunosuppressed secondary to an allogeneic stem cell transplant.[122] In general, however, reports of extrapulmonary manifestations have been more commonly associated with *L. pneumophila* than with other species. For example, *L. micdadei* was the only organism isolated from a cutaneous leg abscess of a 62-year-old woman who had been receiving prednisone and cyclophosphamide for rapidly progressive glomerulonephritis.[4] This organism has also been described as causing a neck mass in a 9-year-old girl.[124] There are a number of excellent reviews of the manifestations of illness produced by *Legionella* species other than *L. pneumophila*, for those interested in further reading.[56,57,112–114]

The high seroprevalence of antibody to legionellae in some populations suggests that asymptomatic or subclinical infection may be common. This assumption is bolstered by the documentation of unrecognized infection (defined by seroconversion) that occurred in a group of patients who had undergone renal transplantation.

Predisposing Factors

Three factors are required or enhance the likelihood of symptomatic infection:

1. The presence of virulent legionellae in an environmental source.



■ **FIGURE 10-1** This chest roentgenogram was from a patient with legionellosis. It was interpreted as having a right upper lobe infiltrate *(arrow)*, as well as a small left-sided pleural effusion.

2. An efficient mechanism for dissemination of bacteria from the environment to human subjects.
3. Diminution of host defense mechanisms that are effective against this organism.

Persons who have Legionnaires' disease are usually middle-aged or older (mean age, about 55 years); however, the disease can occur in persons of any age, including children.

Legionellosis must be included in the differential diagnosis of immunosuppressed patients in whom fever and pulmonary infiltrates develop. It should be considered in patients in whom pneumonia is not responsive to penicillins, cephalosporins, or aminoglycosides; or in any patient with severe pneumonia, especially when there is no other readily apparent alternative diagnosis. In individuals receiving hemodialysis,

recipients of organ transplants, and other surgical patients, for example, Legionnaires' disease has been a major cause of morbidity and mortality.[123,96]

Other potential predisposing conditions include diabetes mellitus, alcoholism, chronic obstructive pulmonary disease, and cardiovascular disease. Cigarette smoking was suggested as a predisposing factor in the Philadelphia outbreak and in some subsequent outbreaks. A prerequisite is exposure to concentrated virulent *Legionella* organisms in the environment. Thus, legionellosis has occurred with higher frequencies among travelers to a site with an ongoing hyperendemic, which often is unrecognized.[12] Infections have also occurred on cruise ships, usually associated with the use of whirlpools or hot tubs.[83]

As a result of the prerequisites outlined above, sites where elderly or debilitated individuals congregate are most likely to be problem areas. Certainly healthcare facilities have been a major problem, partially thanks to the unwitting

collaboration of engineers who place cooling towers upwind from intake vents for fresh air.[34]

Pathology and Pathogenesis

Pathologic features of human infection with several species of *Legionella* other than *L. pneumophila* are similar to those found in *L. pneumophila* infections. A multifocal lobular pneumonia often becomes confluent and may assume a lobar appearance. Small abscesses are common; less frequently, large abscesses that can be visualized radiographically occur.

Histologically, neutrophils, macrophages, and large amounts of fibrin fill airspaces, and septic vasculitis of small blood vessels has been observed. Pulmonary fibrosis and decreased pulmonary function may occur as a long-term sequela.[27] Legionellae do not stain well with hematoxylin and eosin (H&E) in formalin-fixed paraffin sections but may be visualized if large numbers are present. A silver impregnation method, such as the Dieterle, Steiner, or Warthin–Starry method will stain the organisms reliably.[159] Silver impregnation techniques are nonspecific and stain virtually all microorganisms present in addition to *Legionella.* The Brown–Hopps tissue Gram's stain will also demonstrate the bacteria, but careful attention to detail and appropriate controls are essential.

Whenever possible, fresh imprints of lung biopsy material should be prepared, because visualization and analysis of all bacteria are easier in smears than in histologic sections. Legionellae can be demonstrated with Giemsa, Gram–Weigert, and Gram's procedures, especially if 0.05% basic fuchsin is added to the traditional safranin

counterstain in Gram's procedure (Image Plate 10-1) The organisms are facultative intracellular pathogens and may be found within macrophages and neutrophils or extracellularly.

A distinctive characteristic of *L. micdadei* is the presence of acid-fastness in clinical material, a property that is lost after cultivation on agar. Modified decolorization (i.e., a Fite stain), as used for *Nocardia* spp. may be necessary. Instances have been recorded in which *L. pneumophila* was isolated from a specimen in which acid-fast bacilli had been demonstrated.[13] It is difficult, however, to be certain that a co-infection with *L. micdadei* was not present, as this species is more difficult to isolate than is *L. pneumophila.*

Considerable progress has been made toward an understanding of the pathogenesis of legionellosis.[67,151,52,60,82] The bacteria are facultative intracellular pathogens and replicate predominantly within cells of the monocyte/macrophage system, including prominently pulmonary alveolar macrophages. In the natural environment, a variety of free-living amoebae support the growth of legionellae. These organisms are often phagocytized by polymorphonuclear neutrophils, but they do not appear to replicate in these cells. Within macrophages, legionellae inhibit phagolysosomal fusion and acidification of the phagosome; they continue multiplying until the host cell ruptures, releasing the organisms, which can then infect other phagocytic cells. The detailed pathogenesis is a subject of intensive ongoing investigation. Cell-mediated immunity rather than humoral immunity appears to play the central role in the host defense against legionellae.

Epidemiologic and Ecologic Aspects of Legionellosis

Legionella species are widespread in both natural and man-made environments. Legionnaires' disease and Pontiac fever may be contracted by exposure to a wide variety of environmental sources, but there is no convincing evidence of a carrier state in humans, or of person-to-person transmission. Thus, there is no evidence that patients who have or have had Legionnaires' disease are "contagious." Furthermore, *Legionella* spp., once isolated in the laboratory, have not proven to be any more hazardous to laboratory personnel than bacteria routinely isolated in the clinical microbiology laboratory. Inhalation of aerosolized organisms from environmental sources or possibly the aspiration of organisms present in water or in oropharyngeal contents are the most likely routes of spread. An early review by Broome remains relevant.[23]

Incidence

Legionellosis has been documented in countries around the world, but the morbidity and mortality associated with both the epidemic and sporadic cases of legionellosis are underreported in public health statistics. Most countries lack a disease-oriented surveillance system for tracking the disease. Additionally, clinicians and microbiology laboratories may overlook the infection because of lack of awareness on the part of clinicians and/or failure of laboratories to use proper diagnostic methods.[73]

In general, it has been estimated that less than 5% to 12% of cases of pneumonia are caused by *Legionella* species, a number which has increased with the introduction of improved diagnostic techniques.[39] In outbreaks of Legionnaires' disease (as opposed to Pontiac fever), attack rates for the exposed high-risk population are usually low, but have been reported to be as high as 30%.[6] In one review, *Legionella* species (6.7% of etiologic agents) ranked as the third most common etiologic agents (following *Streptococcus pneumoniae,* 15.3%, and *Haemophilus influenzae,* 10.9%) for community-acquired pneumonia among 359 patients admitted to university, community, and Veterans Affairs hospitals examined in a US multicenter study.[56] In other countries, the frequency of sporadic cases of community-acquired pneumonias caused by *Legionella* species varied from 2% in the United Kingdom to 3% to 4% in Germany and 10% in France.[135] The incidence of Pontiac fever in the general population is not known. Outside an outbreak setting, sporadic cases of Pontiac fever are probably almost always unrecognized. In any event, the definition of Pontiac fever is partially epidemiologic; differentiation of sporadic nonpneumonic cases of "Legionnaires' disease" and Pontiac fever is impossible clinically. Patients with pneumonic and nonpneumonic disease (i.e., Pontiac fever) have been reported from the same exposure to environmental bacteria, presumably reflecting differences in host defenses and/or number of bacteria inhaled.

When evaluating studies of incidence, it is important to recognize the inherent bias introduced by the methods chosen by the investigators, by the feasibility of diagnosing some causes of pneumonia, and probably also by the inherent interests of those collecting the data. In a comprehensive study of pneumonia acquired in the outpatient setting, *Legionella* spp.

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were far down the list of etiologic agents.[17] In all studies, the most common etiologic agent has been "unknown."

Legionellaceae *in the Environment*

As mentioned earlier, species of the genus *Legionella* are present in diverse natural

and man-made habitats. Numerous studies have been focused on the ecology of *Legionella* species in man-made habitats, such as cooling towers that use evaporation of water and potable (drinking) water systems within buildings. Man-made habitats probably serve as "amplifiers" or disseminators of legionellae that originated in natural environments.

Natural Habitats. *Legionella* spp. have been found in natural waters from around the world, including ground water.[129] They are widespread in lakes, ponds, streams, and both cold and warm springs. They have been isolated from aquatic habitats with temperatures ranging from 5.7°C to 63°C[64] and in hot spring water used for hydrotherapy.[18] There appear to be higher concentrations of *Legionella* species in warmer waters (30°C to 45°C) than in water at cooler temperatures.[162] In Puerto Rico, legionellae were recovered from marine waters and from epiphytes in trees.[121]

Although soil (e.g., wind-blown dust from an excavation site) was implicated epidemiologically (but not studied microbiologically) as a source of *Legionella* in one of the early outbreaks of Legionnaires' disease, there have been few reports of attempts to isolate *Legionella* spp. from soil. *L. pneumophila* and *L. bozemanii* were isolated from samples of wet soil soon after recognition of the organism.[111] Steele et al.[146] isolated *L. pneumophila, L. longbeachae* serogroup 1, and *L. micdadei* from potting soils in Australia. *L. longbeachae* serogroup 1 was also isolated from natural soil and from pine sawdust. Epidemiologic and microbiologic studies from South Australia in association with an outbreak of legionellosis due to *L. longbeachae* serogroup 1 suggested that soil, and not water, could be the natural habitat of *L. longbeachae* serogroup 1 and that soil could be the source of this organism in human disease. In this study, gardening in soil, rather than exposure to water contaminated with *L. longbeachae,* appeared to be the major environmental risk factor associated with legionellosis.[145] Much more work on the ecology of *Legionella* spp. in natural aquatic and terrestrial habitats is needed.

Aquatic Habitats Made by Humans (Artificial). There have been numerous reports that associated the presence of *L. pneumophila* in institutional potable hot water with the occurrence of legionellosis. Legionellae appear to survive the usual chlorination procedures of municipal water treatment facilities, and thus, not unexpectedly, may be present in potable water supplied to homes, apartment buildings, hotels, hospitals, and other buildings.[3,150] Potable hot water, especially if it does not exceed 55°C, has contained high concentrations of *Legionella* in some instances. In addition to water temperature, the construction of the plumbing system also seems to play an important role; for example, the presence of certain kinds of resins in gaskets; the presence of dead ends or cul-de-sacs, where there is stasis, obstruction, or stagnation in water

flow; and the presence of biofilms or slime layers on the surface of pipes containing other commensal bacteria, protozoa, and algae may favor the presence of legionellae.[99,144] Many of these studies were performed before the importance of environmental protozoa was realized, and it is not clear whether some of the associations may have been fortuitous rather than causal. Some of the factors associated with enhanced replication of the bacteria in homes have been defined,[30] but addressing the problem at the residential level will be all but impossible. It is worth noting that apparently nosocomially acquired infection may, in actuality, have been acquired in the patient's home.[138]

In modern society, the mechanisms by which bacteria in water may be aerosolized and transmitted to unwitting respiratory tracts are legion. In 1990, the CDC reported an outbreak of community-acquired Legionnaires' disease involving 33 persons that was associated with a grocery store mist machine.[103] The mist machine continuously generated a tap water aerosol (presumably containing *L. pneumophila*) in respirable (2 to 5 μm) droplets over the produce display. The system used ultrasonic transducers located in the humidifier's tap water reservoir to create the mist.

Other public sources of infection have included ornamental fountains.[79] Whirlpool spas have been implicated frequently as the source of the organism for patients with legionellosis.[55,74] Dramatically, a large outbreak at a flower show in the Netherlands was traced to whirlpool spas used in the exhibits.[32] An isolate of *L. pneumophila* was also recovered from a sprinkler used in the exhibits, but the genotype of this strain was different from that of the two whirlpool spas and the infected patients. Such experiences emphasize the importance of bacterial culture and molecular analysis of clinical and environmental strains during the investigation of an epidemic.

Although legionellae have been isolated from shower heads and shower water on repeated occasions, the epidemiologic evidence that bathing or showering in such water leads to legionellosis has been rather weak.[65,114] Supportive evidence reported by Breiman and associates from an investigation of an outbreak of nosocomial Legionnaires' disease at a hospital in South Dakota strengthened the hypothesis that aerosolized shower water can serve as the vehicle for spread of *L. pneumophila* to patients.[20]Perhaps the most bizarre instance of water-associated Legionnaires' disease was a neonatal infection that was acquired during a home birthing in a domestic spa bath.[118]

Factors that promote the growth of legionellae in plumbing systems are poorly known in spite of a large accumulation of literature. Legionellae are fastidious organisms that require enriched media for growth in the laboratory, and it is unlikely that drinking water supplies all their nutritional requirements for growth and energy. Rowbotham was the first to report that *Legionella* species multiply in close association with free-living aquatic and soil amoebae of the genera *Acanthamoeba* and *Naegleria*

(see Chapter 22).[133] Others have confirmed and extended these observations, not only with *Acanthamoeba* and *Naegleria,* but also with other amoebae, such as *Hartmannella* and the ciliate *Tetrahymena.*[62,119,161] Thus, amoebae or ciliates phagocytize legionellae, as they do other bacteria in nature, and the legionellae then survive and multiply within nutritionally deficient habitats by living

parasitically within the protozoa. In addition, it has been suggested that the amoebae, which form cysts, might offer further protection to legionellae within the cysts against the effects of chlorine.[90,143] Scientists have taken advantage of this association to enhance recovery of legionellae from the environment, and one species, *L. lytica,* consists of strains that grow only intracellularly in amoebae.

Legionellosis in Travelers

Since the 1976 outbreak of Legionnaires' disease, which occurred among travelers to Philadelphia, travel-associated epidemics and sporadic instances of Legionnaires' disease have been recognized in many countries on all continents.[88,132] The true incidence of travel-associated disease is not clear. Infections are likely to be underreported not only because of underdiagnosis and lack of awareness of the epidemiologic significance, but also conceivably because of concerns about the effect of adverse publicity on tourism. Clusters and sporadic infections occur each year, associated especially with hotels and with hotel water systems contaminated with legionellae. Not surprisingly, the outbreaks may be recurrent and persistent until the nature of the problem is recognized and corrected. Clusters of infections have also been observed among travelers who have been on board cruise ships.

Sporadic disease and clusters of travel-associated *Legionella* infection should be reported to state and federal public health agencies and should lead to investigations of the source and magnitude of the outbreak to prevent further spread among the traveling public. As demonstrated first in the 1976 Philadelphia epidemic and confirmed repeatedly, it may only be the collection of multiple individual reports of disease by a central repository, such as a public health facility, that triggers the recognition of the problem.

Apparently sporadic disease that is not linked to travel may also be, in actuality, related to an unrecognized epidemic focus.[15] Bhopal has suggested approaches that may be useful for public health authorities to detect problems in their catchment areas.[14]

A recent twist to the theme of travel-associated infection has been the recognition that participants in holiday cruises are at risk of contracting this as well as other

infections.[26] As has been true for resorts on dry land, these infections have been traced to contaminated water systems, such as whirlpool spas.

Nosocomial Outbreaks of Legionellosis

Within hospitalized populations are patients who may be compromised and are very susceptible to infections in general. These patients are at risk of acquiring legionellosis should they be exposed to virulent organisms.

The usual source of *Legionella* in hospitalized patients is water[140] (mainly the hot water system), especially from showers or baths.[53] In addition, patients may be infected from cooling towers that are part of or adjacent to the healthcare complex.[1,70] Other documented sources have included nasogastric tubes (with microaspiration of legionellae in contaminated water), humidifiers, respiratory therapy equipment (e.g., masks and hand-held nebulizers washed with contaminated tap water), whirlpools, and other less common sources. Early diagnosis of legionellosis and epidemiologic surveillance of infections within the hospital are needed not only for prompt and effective therapy but to aid in instituting control measures to prevent subsequent infections. With the high mortality rate for nosocomial Legionnaires' disease (range, 30% to 50%), it would seem prudent that efforts be taken to prevent the spread of *Legionella* species from the hospital environment to patients who are likely to be most susceptible to infection.

Nosocomial Legionnaires' disease has also been specifically linked to the use of tap water to clean nebulizers used for delivery of medication and to other respiratory therapy equipment. In a report by Mastro et al.,[107] the use of tap water contaminated with *L. pneumophila* to wash medication nebulizers was a major factor that led to an outbreak of nosocomial Legionnaires' disease in patients with chronic obstructive pulmonary disease. In one particularly instructive cluster of infections, the disease was more common in patients who were both receiving corticosteroids and exposed to nebulizers or room humidifiers filled with tap water.[8]

Some instances of nosocomial Legionnaires' disease have been linked to microaspiration of water contaminated with *Legionella* species in patients with nasogastric tubes.[16,44,106,105] A different twist was documented at Stanford University Medical Center when wound infections caused by legionellae developed in postsurgical patients who had been bathed with tap water. The bacteria were clearly introduced directly into the wounds,[100] rather than into the respiratory tract with subsequent dissemination, a sequence of events that has also been documented.

Given all of these occurrences and the relative ease with which sterile water may be used in individuals at high risk, the continued use of tap water is difficult to justify or defend.[5]

Laboratory Diagnosis

The mainstay of diagnosis has traditionally been culture, which is absolutely specific and provides isolates for epidemiologic study and molecular typing. Detection of urinary antigen is a useful adjunctive technique, particularly in patients who do not produce adequate sputum for culture. It is highly sensitive, but urine specimens remain positive for an extended time following the resolution of disease. Detection of nucleic acids has not yet reached the stage of widespread commercial availability and practicality, but will probably be a major diagnostic resource in the future.[116] Traditionally, serologic diagnosis required seroconversion, which provided only retrospective information; it was particularly useful for investigation of epidemics. Advances in serologic testing afford the differentiation of *Legionella*-specific immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies, which is helpful in confirming the diagnosis of legionellosis. Although this does represent an advance, other tests, such as the *Legionella* urinary antigen, polymerase chain reaction (PCR), and culture, would be positive in acute disease prior to seroconversion. The limitations of select diagnostic tests have been reviewed.[164] The use of more recently introduced diagnostic tools, such as PCR and urinary antigen testing, has vastly improved the ability of the laboratorian

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to meaningfully contribute to the diagnosis of legionellosis in particular and community-acquired pneumonia in general.[10,104]

Selection, Collection, and Transport of Clinical Specimens

The broad clinical spectrum and severe morbidity and mortality of Legionnaires' disease emphasize the need for rapid and accurate laboratory diagnosis. When legionellosis is suspected clinically, lower respiratory tract specimens should be collected for both culture and/or *Legionella* PCR. Although some still use direct fluorescent antibody (DFA) testing, this is inferior to PCR in both sensitivity and specificity. Appropriate specimens include expectorated sputum, materials collected using bronchoscopy (e.g., bronchial brush, biopsy, lavage, or washings), transtracheal aspirates, lung biopsies, fine-needle aspirates of lung, and pleural fluid. In most situations, it is appropriate to test specimens for *Legionella* spp. only upon specific request. It would be appropriate, however, to include legionellae in the spectrum of pathogens sought when culturing lung tissue, in those collected postmortem, if the cause of death remained undetermined. Such routine culture can serve as one part of the warning system that these water-associated pathogens are a problem in a particular

institution.

Primary isolation of *Legionella* species on solid media has been successful from closed- and open-lung biopsy material, pleural fluid, transtracheal aspirates, BAL samples, and sputum. A reasonably semi-effective selective medium is available for the primary isolation of *L. pneumophila* from sputum and contaminated bronchial materials.[40,42,167]

Specimens should be collected carefully to avoid aerosols and transported to the laboratory at ambient temperature in sterile, leak-proof containers, preferably within 2 hours after collection. Specimens to be sent to a reference laboratory should be refrigerated or packed on wet ice if a delay of less than 2 days is anticipated. Specimens that are to be stored for days or weeks should be maintained at 70°C or colder. These may be shipped on dry ice. Specimens that must be shipped to a reference laboratory should be packaged and mailed in accordance with federal regulations (see Chapter 1). If specimens are being submitted only for pathologic study and are to be fixed in buffered neutral formalin before shipment, they need not be refrigerated or frozen during transport. Specimens for PCR analysis should be handled in a manner similar to culture.

L. pneumophila has been isolated from blood cultures on a few occasions using conventional media supplemented with L-cysteine and ferric pyrophosphate, and in the distant past using BACTEC (Becton Dickinson Instrument Systems, Sparks, MD) radiometric aerobic and anaerobic media with no special supplements.[48] Blind subculture of the BACTEC aerobic and anaerobic bottles onto BCYE appears to be necessary for isolation of legionellae. As mentioned previously, legionellae may also be encountered, albeit rarely, in extrapulmonary sites. The practical value of seeking *Legionella* spp. in blood cultures or extrapulmonary sites on a routine basis is low. However, the ability to perform cultures from nonrespiratory sites should be retained in the event it is needed.

In addition to the above specimens, urine (preferably an early-morning sample) should be collected for detection of *Legionella* antigen and serum should be submitted for serologic studies. An acute-phase serum sample should be collected early as an adjunct to other tests that may enable a more rapid diagnosis. This should be tested for *Legionella*-specific IgM and IgG antibodies. The presence of *Legionella*-specific IgM with or without the presence of *Legionella*-specific IgG is supportive of acute disease. Antibodies may not be detectable very early in the course of disease, so a convalescent study should be performed if the cause of the infection was not determined. Serologic studies are particularly useful in patients who produce minimal sputum.[167] Unfortunately, the development of a diagnostic antibody titer can be slow and may occur in no more than 75% to 80% of patients who ultimately are shown to have Legionnaires' disease.[167] In addition, there are individuals who have seroconverted but have remained asymptomatic; these patients likely had a subclinical

infection.

Direct Examination of Clinical Specimens

Gross Examination. The gross examination of lung specimens may aid the pathologist in selecting the best areas for culture or histologic examination. Direct smears of exudates or touch preparations of fresh lung biopsy material should be prepared. Frozen sections of lung samples are also useful in diagnosis. Frozen sections and touch preparations should be placed in methanol for fixation if Gram's stain, H&E, Giemsa, Diff-Quik, or modified-Kinyoun acid-fast stains are desired. The Giemsa stain is useful for demonstrating legionellae and other bacteria in fresh touch preparations of lung or bronchoscopic materials. Gram's stain should also be performed, because of the differential information it provides.

Microscopic Examination. Various specimens (e.g., transtracheal aspirates, pleural fluids, aspirates from thoracic empyema, fine-needle aspirates of lung, and touch preparations of lungs) can be stained with Gram's stain. Methanol fixation is better than heat fixation, and the intensity of staining can be improved by increasing the time of safranin staining to 10 minutes or longer. Alternatively, 0.05% carbolfuchsin to the safranin improves the intensity of staining with the counterstain. The Gram–Weigert, Diff-Quik, or Giemsa stain may reveal more organisms than can be seen with routine Gram's staining. In formalin-fixed, paraffin-embedded histologic sections, *Legionella* organisms cannot be readily seen in the H&E, Gram, Brown–Brenn, Brown and Hopps, or MacCallum–Goodpasture stained preparations. The Gomori methenamine silver (GMS) stain and periodic acid–Schiff (PAS) stain are not useful for demonstrating legionellae. A modified acid-fast stain, as performed for *Nocardia* spp., is useful for demonstrating *L. micdadei*.[117] All of these stains may be done on either impression smears or tissue sections but are most satisfactory on thin smears. A very sensitive histochemical method for demonstrating bacteria of all types is one of the silver impregnation stains, developed originally for spirochetes. A modified Dieterle stain was originally used, wherein the organisms stain black to dark brown. Other silver impregnation techniques, such as

the Steiner and Warthin–Starry methods, will also work and stain the organisms similarly. These silver stains are usually performed on histologic sections. Although sensitive, the deposited silver grains in these stains obscure the bacterial morphology and, of course, the Gram reaction cannot be determined. The morphology of *L. pneumophila* is shown in Image Plate 10-1A, B, and D.

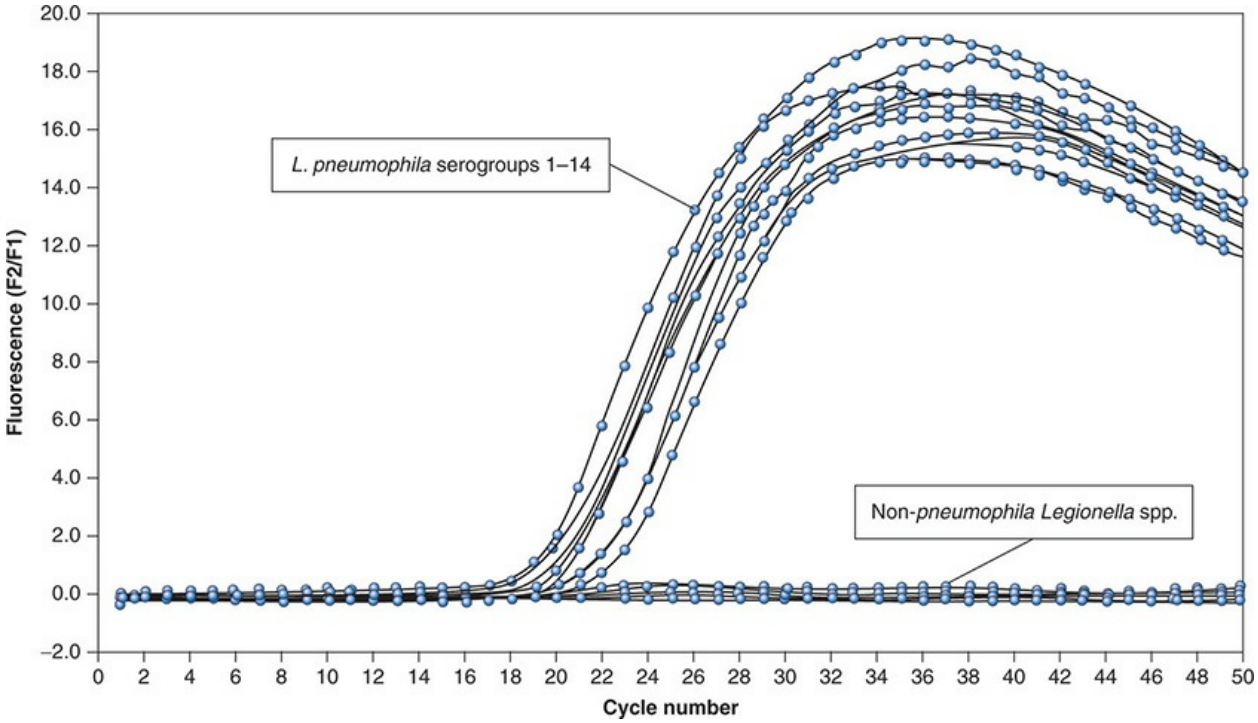
Legionella PCR and Other Molecular Methods

As with many other fastidious microorganisms, PCR or other nucleic acid amplification-based assays have proven more sensitive than culture for the detection of *Legionella.* Although there are some commercially available assays, many assays are laboratory developed tests.[76,125,128,158,165] Figure 10-2 demonstrates the amplification curves for an *L. pneumophila*-specific PCR. In addition, these assays are also available through high-quality reference laboratories.

Hayden et al.[76]studies archived BAL specimens from patients who were proven by culture to have legionellosis with both PCR and DFA. The sensitivity and specificity for PCR were each 100%, whereas for DFA these were 44% and 100%, respectively. We reviewed the 22,345 tests for *Legionella* performed at the Cleveland Clinic over a 4-year period. These included the results of an *L. pneumophila*-specific PCR, the *Legionella* urinary antigen test, and *Legionella* culture. There were 20 patients with laboratory-confirmed legionellosis during that time frame based on having at least two of these three tests positive. Of these, 50% (10/23) were positive by all tests, 45% were culture negative, but PCR and *Legionella* urinary antigen positive, and 5% (1/20) were positive only by PCR and culture.[28] In this setting, PCR was the most sensitive assay, followed closely by *Legionella* urinary antigen; culture disappointingly detected only 55% of the infected patients. There were no non-*pneumophila Legionella* species detected in this time frame.

Direct Fluorescent Antibody Testing. Direct immunofluorescence assays, originally developed by Cherry et al.[29] at the CDC, have been used for rapid detection of *Legionella* species in clinical specimens from the respiratory tract. Compared with culture and PCR, the DFA has a low sensitivity (25% to 70%).[173] The specificity has been high, but when there is a low prevalence of *Legionella* infection, false-positive results are unacceptably frequent. The DFA test for *L. pneumophila* has been positive in a few healthy individuals.[22] In addition to potential immunologic cross-reactions, contamination with environmental bacteria appears to have caused some false-positive results, even when scrupulous care is exercised when the test is performed. Use of immunofluorescence for diagnosis directly on patient specimens cannot, therefore, be recommended.

Immunofluorescence is a useful method, however, for identifying isolated bacteria. Several commercial suppliers market fluorescein isothiocyanate (FITC)-conjugated polyvalent antisera, as well as control sera and other reagents for *Legionella* DFA testing.[46] As a note of caution, some monoclonal DFA conjugates have failed to detect *L. pneumophila* in hot and cold storage tank water and in swab samples from showers and a water tap[160]; thus, these cannot be recommended for DFA studies of potable water or other samples



■ **FIGURE 10-2** This real-time PCR amplification curve for a *Legionella pneumophila*-specific PCR demonstrates that the 14 serotypes of *L. pneumophila* are detected, whereas non-*pneumophila Legionella* species are not detected in this assay.

from man-made water systems. PCR-based methods are superior for environmental testing.

In performing the DFA test, specific antibody in the form of FITC-labeled polyvalent antiserum (conjugate) directed against the antigen(s) to be detected is usually purchased commercially. The directions of the manufacturer of the kit should be followed. Antigen (present on or in *Legionella* organisms) is fixed on a slide, and then overlaid with FITC-labeled antibody. The antigen binds the FITC-labeled antibody, forming an antigen–antibody complex. This complex is not washed away when gently rinsed with buffer. When the antigen(s) of a *Legionella* species has reacted with FITC-conjugated antibody, exposure to ultraviolet light causes the FITC molecule to emit longer wavelengths of light in the yellow-green region of the color spectrum, and using a fluorescence microscope, the bacteria can be seen as brilliantly fluorescing yellow-green rods.

***Legionella* Antigen Detection.** Procedures for detecting *Legionella* antigens in urine, developed by Kohler and associates[95] at the Indiana University Medical Center, have included radioimmunoassay, enzyme-linked immunosorbent assay, and latex

agglutination. Commercially available immunoassays for detection of antigenuria caused by *L. pneumophila,* serogroup 1 have a very high specificity that approaches 100%. A number of high-quality assays are commercially available for the detection of antigen of *Legionella* in the urine. This test is very useful, especially for patients who do not produce adequate sputum for culture. It should be performed in conjunction with *Legionella* PCR and/or culture on all patients suspected to have legionellosis. The major limitations of the assay is that it is restricted to a single serogroup, albeit the most common human pathogen in the genus. Another potential limitation is the prolonged excretion of antigen in some patients, which may be up to 1 year. In particular, immunosuppressed patients with delayed resolution of fever are likely to excrete antigen for more than 60 days.[142] Detection of urinary antigen must, therefore, be carefully correlated with the clinical history and other laboratory and radiologic findings before ascribing the current infection to *Legionella.*[94] Occasional cross-reactions among serogroups of *L. pneumophila* have also been described.[93]

Detection of *Legionella* in Clinical Specimens

Isolation of Legionella *Species From Clinical Specimens*

Bacteriologic culture of *Legionella* is a method used in many laboratories to diagnose legionellosis.[176] Although culture is one and one-half to three times more sensitive than the DFA test, it is less sensitive than PCR-based methods and *Legionella* urinary antigen testing.

The cultivation of a *Legionella* species is usually of clinical significance, because these bacteria are not routinely isolated from the upper respiratory tract normal microbiota.

The recommended nonselective solid medium for isolation of legionellae is BCYE agar, which contains L-cysteine, ferric pyrophosphate, ACES buffer, α-ketoglutaric acid, and activated charcoal. The medium is available commercially from several manufacturers. In addition to BCYE, one should consider the use of one or more selective media to avoid overgrowth by normal microbiota. Antibiotics have been added to the BCYE base, resulting in reasonably effective selective media. One useful selective medium contains BCYE base supplemented with cefamandole, polymyxin B, and anisomycin; this is known as BMPA. Another option contains glycine, vancomycin, polymyxin B, and anisomycin; this medium is referred to as "modified Wadowsky–Yee" or MWY medium. The cefamandole in the former modification will inhibit *Legionella* species that do not produce β-lactamase, whereas the MWY medium is not as selective as BMPA. BCYE-based selective media are widely available commercially. Because the selective media may inhibit some legionellae, such

media should be used in conjunction with nonselective BCYE.

Tissue Specimens. To inoculate BCYE or BMPA media, the fresh lung tissue should be gently applied (i.e., dabbed) in the first quadrant and a sterile inoculating loop used to transfer this inoculum to the other quadrants for primary isolation. Alternatively, a sterile tissue grinder can be used to homogenize 1- to 2-mm pieces of minced tissue in 0.5 to 1 mL of a sterile broth, such as trypticase soy broth or enriched thioglycolate medium. After homogenization, the plating medium is inoculated with approximately 0.1 mL of the homogenate and streaked for isolation. In addition, slides for Gram's stain should be prepared from the same specimen.

Pleural Fluid and Transtracheal Aspirates. Pleural fluid and transtracheal aspirates are inoculated directly onto the selective and nonselective media as for tissue homogenates. The BCYE and BMPA plates are incubated in a 5% to 10% CO_2 incubator at 35°C and examined daily for 5 days; they may be examined further every 2 to 3 days for up to 2 weeks. Other media for lower respiratory tract specimens (e.g., sheep blood agar, chocolate agar, and MacConkey agar) are inoculated and incubated in the usual way. The same specimen used for culture is further processed by making smears for Gram's stain.

Acid-Wash Decontamination Procedure. The selective media currently available for primary isolation of legionellae are not completely selective, thus permitting breakthrough growth of other bacteria (e.g., *Bacillus* spp. or *Pseudomonas* spp.). In addition, growth of legionellae on BCYE may be inhibited by other bacteria in clinical specimens, even when antibiotics are incorporated into the selective media. The treatment of contaminated respiratory specimens such as sputum, bronchial washings, bronchial lavages, and tracheal aspirates with an acid-wash solution before inoculation of BCYE and BMPA plates has been reported to improve isolation of *Legionella.*[25] The protocol outlined in Chart 10-1may be used for processing specimens that contain normal microbiota. The use of acid-wash decontamination has not become commonplace in the clinical laboratory. It has, however, been shown to increase the yield of legionellae from environmental specimens when compared with direct

inoculation of selective or nonselective media with and without the inclusion of concentration methods.[97] Heat treatment of specimens produced only a marginal increase in the yield of *Legionella* isolates and was not recommended for routine use.[51]

Blood Cultures. Collection of blood for *Legionella* culture may occasionally be useful in certain clinical settings. Broth culture methods and lysis-centrifugation have both been used. Blind subculture or staining with a sensitive stain, such as acridine orange, of broth cultures must be performed, because legionellae do not regularly activate the indicators of growth in commercial systems. Patients with positive blood cultures had significantly higher concentrations of legionellae in respiratory specimens than those with negative blood cultures. The usefulness of routine blood cultures in less severely ill patients is not clear.

Identification of Legionella *Species*

Colonies of *Legionella* typically appear on BCYE after 2 to 3 days of incubation in areas that have been heavily inoculated. If only a few organisms are present and if the plates have been lightly inoculated, however, isolated colonies may take several more days to develop. The colonies are variable in size, ranging from punctate or up to 3 to 4 mm. They are glistening, convex, circular, slightly irregular, and have an entire margin (Image Plate 10-1C). When examined through a dissecting microscope (7× to 15×), *Legionella* colonies appear to have crystalline internal structures within the colonies or a speckled, opalescent appearance similar to that of *Fusobacterium nucleatum* (see Chapter 16, and Image Plates 10-1C and D). Some species show a blue-white fluorescence under long-wave (366 nm) ultraviolet light (Image Plate 10-1E); others exhibit a red fluorescence (Table 10-3). Colonies suspected of being *Legionella* should be subcultured onto an ordinary unsupplemented 5% sheep blood agar plate or BCYE lacking L-cysteine and onto BCYE medium that contains L-cysteine. Organisms that grow on 5% sheep blood, on BCYE that lacks L-cysteine, or on other routine media (such as MacConkey agar) are probably not *Legionella.* Occasional isolates of *Legionella* spp. may be recovered on enriched chocolate agar. Isolates of gram-negative bacilli with typical colonial characteristics of *Legionella* that grow on BCYE or BMPA medium after 48 hours or longer of incubation, but do not grow on BCYE that lacks L-cysteine or on sheep blood agar should be characterized. Most such isolates belong to *L. pneumophila* serogroup 1, but there is geographic variation in the species and serogroups recovered. The most convenient laboratory test for confirming a suspected *Legionella* isolate is the DFA test. Colonies can be tested with the fluorescent antibody conjugates mentioned previously to serogroup the isolate. Occasionally, organisms will be found that resemble *Legionella* but do not react with the serologic reagents used in the DFA test. Biochemical tests are unfortunately of limited use (Table 10-3); characterization of cellular fatty acids is useful if the laboratory has the capability of performing this analysis (Table 10-4). For most laboratories, however, the appropriate action will be submission of the isolate to a reference laboratory to determine if it represents a serogroup for which reagents were not available or even a previously unrecognized species or serogroup. It should

be noted that there are non-*Legionella* species that require L-cysteine, fail to grow on blood agar, and may resemble *Legionella* in colony and microscopic morphology.[173] Tests used in definitive characterization of *Legionella* species in some reference or research laboratories include serologic tests, gas-liquid chromatography of cellular fatty acids, and nucleic acid genetic studies. DNA sequencing of the *mip* gene may also be used to identify the *Legionella* to the species level.[47,147] Selected characteristics for laboratory recognition of *Legionella* species are shown in Image Plate 10-1.

Antimicrobial Susceptibility and Treatment

The mortality rate among patients with legionellosis caused by *L. pneumophila* has varied from 0% to 30%, depending on the clinical setting and patient population. Erythromycin has been effective in reducing the case-mortality rate and historically has been the drug of choice for legionellosis. Mortality from *Legionella* infection correlated both with delay in initiation of erythromycin therapy after admission to the hospital and with total delay in starting the antibiotic.[77] Other agents that achieve high intracellular concentrations, such as other macrolides, quinolones, tetracyclines, ketolides, and tetracyclines, are the other options. Although β-lactams and aminoglycosides demonstrate in vitro efficacy, these are not useful clinically. Although no randomized trials have been performed for the treatment of patients with legionellosis, the newer macrolides and quinolones are often selected for first-line therapy.

Because *Legionella* spp. are facultative intracellular pathogens, which replicate inside monocytes and macrophages, antimicrobial agents that are concentrated within these cells are most likely to be successful in treatment. The newer macrolide antimicrobial agents (e.g., azithromycin and clarithromycin), and the fluoroquinolones (e.g., ciprofloxacin and perfloxacin) are more active in vitro and in experimental models than erythromycin, although clinical experience with these agents is still limited. Rifampin is known to be very active in vitro[155] and could be given in addition to erythromycin or another macrolide to some patients who are seriously ill or fail to respond to macrolide therapy alone; rifampin, however, should not be given alone. Alternatively, doxycycline in combination with rifampin has been recommended for moderately or severely ill patients. Trimethoprim-sulfamethoxazole with or without rifampin is another potential option. The penicillins (e.g., penicillin, carbenicillin, oxacillin); first- (e.g., cephalothin, cefazolin), second- (e.g., cefamandole, cefoxitin), and the third-generation cephalosporins; aminoglycosides (e.g., gentamicin, tobramycin, amikacin); and vancomycin are not effective in treatment. In vitro antimicrobial susceptibility testing of *Legionella* isolates has not been standardized and does not correlate with the clinical response to antibiotic therapy in patients. Therefore, performance of in vitro antimicrobial susceptibility testing on *Legionella*

isolates in the hospital diagnostic laboratory is not recommended because the results are not readily interpretable by either the microbiologist or the clinician.

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TABLE 10-3 Characteristics of Selected Pathogenic *Legionella* Species[a]

Legionella Species	Modified Acid-Fastness in Tissue	Browning of Tyrosine-Containing Media	Hippurate Hydrolysis	Gelatin Liquefaction	β-Lactamase Production	Oxidase	Motility	Autofluorescence
L. anisa	–	+	–	+	+	+	+	BW
L. birminghamensis	–	–	–	+	+	±	+	–
L. bozemanii	–	+	–	+	±	±	+	BW
L. cincinnatiensis	–	+	–	+	+	–	+	–
L. dumoffii	–	+[b]	–	+	+	–	+	BW
L. feelei	–	+(w)	±	±	–	–	+	–
L. gormanii	–	+	–	+	+	–	+	BW
L. hackelii	–	+	–	+	+	+	+	–
L. jordanis	–	+	–	+	+	+	+	–
L. lansingensis	–	–	–	–	–	+	+	–
L. longbeachae	–	+	–	+	±	+	+	–
L. maceachernii	–	+	–	+	–	+	+	–
L. micdadei	+	–[c]	–	–	–	+	+	–
L. oakridgensis	–	+	–	+	+(w)	±	–	–
L. parisiensis	–	+	–	+	+	+	+	BW
L. pneumophila subsp. *pneumophila*	–[d]	+	+[e]	+	+	+/±	+	–
L. pneumophila subsp. *fraseri*	–	+	±	+	+	+/±	+	–
L. pneumophila subsp. *pascullei*	–	+	+	+	+	+/±	+	–
L. sainthelensi	–	+	–	+	+	+	+	–
L. tuconsensis	–	–	–	+	+	–	+	BW
L. wadsworthii	–	–	–	+	+	–	–	–

[a]All species are gram negative, show no growth on unsupplemented blood agar, and require L-cysteine (present in BCYE agar) for primary isolation; an exception to this exists for laboratory-adapted strains of *L. oakridgensis*, which lose the requirement for L-cysteine. These organisms are catalase positive or peroxidase positive. None reduce nitrate to nitrite, produce acid from D-glucose, or produce urease.
[b]One strain negative.
[c]Negative on initial isolation; may be positive after transfer on BCYE agar.
[d]Rare strains have been isolated from patients with modified acid-fast bacilli in clinical specimens.
[e]A few strains are negative.
+, positive; –, negative; +(w), weak reaction; w, weak; BW, bluish white autofluorescence.
Adapted from reference 173.

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TABLE 10-4 Major Cellular Fatty Acids of Select Pathogenic *Legionella* Speciesa[a]

Species	Cellular Fatty Acids[b]
L. anisa	a-$C_1$5:0
L. birminghamensis	a-$C_1$5:0, i-C_{14h}

L. bozemanii	a-$C_1$5:0
L. cincinnatiensis	i-$C_1$6:0, i-C_{16h}
L. dumoffii	a-$C_1$5:0
L. feeleii	a-$C_1$5:0, n-$C_1$6:1
L. gormanii	a-$C_1$5:0
L. hackeliae	a-$C_1$5:0
L. jordanis	a-$C_1$5:0
L. lansingensis	a-$C_1$7:0, a-C_{15h}
L. longbeachae	i-$C_1$6:0
L. maceachernii	a-$C_1$5:0
L. micdadei	a-$C_1$5:0
L. oakridgensis	i-$C_1$6:0
L. pneumophila	i-$C_1$6:0
L. sainthelensi	i-$C_1$6:0
L. tucsonensis	a-$C_1$5:0, n-C_{14h}
L. wadsworthii	a-$C_1$5:0

[a] The major cellular fatty acids, as determined by gas-liquid chromatography, of select, pathogenic *Legionella* species are listed. These bacteria are unusual compared with the other gram-negative bacteria because of their relatively large amounts of cellular branched-chain acids. The most abundant acid of *L. pneumophila* and some strains of *L. longbeachae* is i-$C_1$6:0. However, strains of *L. longbeachae* may have either i-$C_1$6:0 or $C_1$6:1 as the major acid, or the two acids may be present in roughly equal amounts as the two most abundant acids. Conversely, the major fatty acid of *L. bozemanii, L. micdadei, L. dumoffii, L. gormanii, L. jordanis*, and certain other species is a saturated, branched-chain, 15-carbon acid (a-$C_1$5:0).

[b] The numbers before the colon represent the number of carbon atoms contained in each of the different fatty acids; the numbers after the colon are the number of double bonds; i indicates a methyl (-CH_3) branch at the iso carbon atom (i.e., the next to last carbon atom); a indicates a methyl branch at the anteiso carbon atom (i.e., the second from last carbon atom).

Adapted from reference 173.

Serologic Studies for Legionella

The serum indirect IFA test is recommended as an adjunct for diagnosis of legionellosis, particularly for patients who do not or cannot provide adequate respiratory specimens (such as sputum) for culture. The sensitivity of the IFA test is 75% to 80%, and the specificity is 106%.[154,168,170] Clinicians must wait, however, 2 to 6 weeks for patients to develop a fourfold rise in antibody titer. Reagents for *Legionella* serodiagnostic tests should detect antibodies of IgG, IgM, and IgA classes. In some patients, a rise in IgM titer may occur without a detectable rise in IgG or IgA titers or may occur earlier in the course of illness than a rise in IgG titer.[175] IgM antibody may persist for as long as 1 year, so its presence cannot be used to diagnose a recent infection.

As documented by Wilkinson et al.,[168,170] the serum IFA test using *Legionella* antigens may cross-react with the serum of patients who have certain other infections. Sera of patients who had outbreak-associated *Mycoplasma pneumoniae* infections and patients with Q fever cross-reacted with *L. longbeachae* serogroup 2 and *L. jordanis.* Also, cross-reactions were noted between sera of patients who had outbreak-associated tularemia (*Francisella tularensis*) and *L. jordanis* antigen. In addition, false-positive cross-reactions have been observed in patients with *Bacteroides fragilis, Proteus vulgaris, Rickettsia* spp., and *Citrobacter* spp.[75] Some cross-reactions can be eliminated by absorption of antisera with *E. coli,* albeit with some diminution in the anti-*Legionella*titer.[169] Cross-reactions with *Campylobacter* spp. can be removed by absorption of sera with that bacterium.[19]

Seroconversions (fourfold increase in antibody titer) to reciprocal titers of 1:128 or greater are required for serodiagnosis of recent infection with *Legionella.* Single elevated reciprocal IFA titers of 256 or greater may be suggestive of *Legionella* infection during an outbreak. In sporadic cases of pneumonia, however, a 1:256 or higher titer does not necessarily indicate recent infection because such high titers can persist in healthy persons with no current clinical evidence of legionellosis. As a practical matter, it is unlikely that the follow-up required to demonstrate seroconversion will happen in most sporadic cases.

Environmental Microbiology Studies

Molecular Detection of Legionella *in Environmental Samples*

Given the inadequacy of traditional methods for the recovery of legionellae, it is not surprising that molecular methods have been widely employed. Amplification methods have been successfully used to detect legionellae in a variety of environmental samples. Lund et al.[102] have used both culture and quantitative PCR for the detection of *Legionella* in biologic waste water. Culture disclosed a 16% (21/130 cultures) positivity rate legionellae; overall 9% (12/130 culture) were *L. pneumophila.* Conversely, 99% (433/437 analyses) were positive for some type of legionellae; overall, 46% (218/470 analyses) disclosed *L. pneumophila.* Others have questioned the utility of molecular amplification methods, because conceivably the detection of nucleic acids from dead organisms is possible.[33]

Isolation and/or Detection of Legionella *in Environmental Samples*

The widespread presence of *Legionella* spp. in both natural and man-made waters, in the absence of patients with clinical illness, has been observed repeatedly and argues against routine microbiologic culture of water. Conversely, considerable evidence has accumulated that cites potable water as the source of the organism in many patients who have had infection with *L. pneumophila.* Institutional hot water has been implicated frequently in legionellosis; however, more studies

are needed on the magnitude of *Legionella* contamination in home potable water and hot water systems and the frequency of association with legionellosis.[45] For instance, factors that increase the likelihood that *Legionella* spp. will colonize home water systems were defined, but these factors did not appear to increase the incidence of legionellosis acquired in the community. Given the high probability that large numbers of healthy persons are frequently exposed to these organisms in nature, in homes, the workplace, and in private and public buildings of all sorts, it appears that many legionellae in natural and municipal waters are not highly virulent, that exposure to the organism is minimal in extent, or that most persons are not susceptible hosts, or perhaps all are true. Thus, microbiologic enumeration of *Legionella* species in potable or other waters (such as cooling towers) has no clinical or epidemiologic relevance to disease in humans, unless associated infections are documented clinically. Unfortunately, there are not sufficiently adequate markers of virulence to distinguish environmental isolates that are "pathogenic" from those that are unlikely to be pathogens, nor are there appropriate tests that predict in which human hosts legionellosis will develop versus who will be resistant when exposed to *Legionella* in the environment. Furthermore, the degree and extent of exposure to the organism cannot be measured accurately. Therefore, microbiologic surveillance of environmental waters is especially difficult to interpret in the absence of associated

infections, and is even difficult to interpret in the presence of documented infections. Conversely, the clinical and epidemiologic surveillance of patients (e.g., by hospital infection control nurses) is recommended as part of a program to prevent nosocomial legionellosis.

In outbreaks of nosocomial legionellosis, hyperchlorination of the hospital water to a level between 2 and 6 mg/L of free residual chlorine and raising the temperature of the hot water to more than 70°C or a combination of hyperchlorination and heat flushing are methods that have proven effective for suppressing the growth of *Legionella.*[115,141,143] To accomplish heat flushing or thermal eradication, the hot water is circulated throughout the building water system at a temperature of 70°C to 75°C. All shower heads and faucets are flushed with hot water with the objective to kill the *Legionella* in these sites. Protocols for hyperchlorination and thermal eradication have been published elsewhere.[115,141,143] Unfortunately, there is a risk of scalding from the hot water, and precautions should be taken to minimize this possibility (e.g., warning signs or heating the water for relatively short periods of time when most patients are asleep). This is complicated by regulations in some states that require the water temperature in hospitals to be less than 120°F (48.9°C) to prevent scalding of patients. Continuous chlorination (i.e., to achieve 1.5 parts per million free residual chlorine levels) may be unduly corrosive and destructive for some plumbing systems and equipment; there is also a potential risk of toxic exposure to trihalomethane. If the heat and/or chlorination treatments are not done continuously, treatment should be recurrent because recolonization of the water is highly predictable and tends to recur following cessation of either heat shock or chlorination. Other methods, including ultraviolet light sterilization, ozonation, addition of amebicidal agents, and addition of trace metal ions (e.g., silver, copper), are being investigated as alternative water system treatments.

If nosocomial infections with *Legionella* are documented, a highly focused microbiologic investigation of environmental samples may be undertaken to aid in determining the most likely source of the *Legionella*. Control measures such as heat and/or chlorination may be designed in an effort to eliminate or suppress the organism, and follow-up environmental cultures may help determine the effectiveness of these measures. For hospitals with an outbreak or a hyperendemic *Legionella* problem, periodic microbiologic surveillance of the environment, combined with ongoing or repetitive control measures, should be considered unless it can be shown that no new cases of legionellosis have occurred.

The methods outlined in the protocol of Barbaree and coworkers[9] are recommended for persons who wish to isolate and identify *Legionella* species from environmental water. In this protocol, water samples (1 to 2 L) are collected and concentrated using 0.2-mm (pore size) polycarbonate filters. Viable counts are performed with and without prior acid treatment on BCYE and medium that contains

BCYE base supplemented with glycine, polymyxin B, vancomycin, and anisomycin.

Typing of Legionella *Isolates*

Because of the widespread distribution of *L. pneumophila* serogroup 1 in the environment, serogrouping with the DFA reagents described previously has had only limited epidemiologic usefulness in investigating the source of different strains. A number of investigators have developed methods to type or subgroup strains of *L. pneumophila* serogroup 1. Analyses of subtypes within *L. pneumophila* serogroup 1 using panels of monoclonal antibodies,[87,86] genetic structural analysis of *L. pneumophila* using a multilocus enzyme electrophoresis technique,[50,139] and determination of plasmid contents[120] of different isolates from different sources have all been investigated. Other techniques have included electrophoresis of *L. pneumophila* outer membrane proteins[148] and a typing method based on the use of cloned biotinylated DNA probes for analysis of restriction-fragment-length polymorphisms (RFLPs).[137] One of the more promising typing methods is random amplified polymorphic DNA (RAPD) profiling of *L. pneumophila* by PCR; this method appears to be faster and less costly than RFLP typing. RAPD profiling has been reported to be more discriminatory than RFLP typing of some *Legionella* isolates, and is capable of detecting differences among isolates with identical RFLP types.[136] An investigation of an epidemic or analysis of apparently unrelated strains can be strengthened by comparison of the molecular pattern of isolates from patients and from the environment. Although sequencing of nucleic acid directly from specimens may prove feasible for epidemiologic studies eventually, at present isolated strains are required, emphasizing once again the importance of culture in the diagnosis of these infections. It is important to remember that demonstration of "indistinguishable" strains from patients and from an environmental site means that the environmental site is "in contention" as the source of the infections, but does not prove the association. Thorough epidemiologic analysis and consideration of all possibilities are essential. Unfortunately, some of those possible sources may not yet be apparent to anyone. The classic example of this phenomenon was

TABLE 10-5 Correlation of Monoclonal Antibody Patterns With Epidemiologic Analysis

Monoclonal Antibody Pattern	Clinical Strains	Environmental Strains

A	Epidemic-associated infections	Cooling tower
B	Sporadic nosocomial infections	Hospital potable water
C	Sporadic community	—

Adapted from reference 85.

the recognition that potable water was the source of infections at a Veterans Affairs hospital after initial assumptions that cooling towers were the source proved to be unfounded.

In general, characterization of nucleic acids is more discriminatory than analysis of antigenic structure using monoclonal antibodies. Interestingly, antigenic types and genomic variants are not always concordant.[31] The ultimate test for any typing technique is discriminating strains into groups that make sense epidemiologically. The patterns must show enough differences to separate strains into related groups, but not too many that the excessive discrimination (i.e., the demonstration of meaningless variation) occurs. On the one hand, apparently similar strains might appear different if another typing tool was used. On the other hand, consensus agreement may be necessary to decide how many of what kind of differences actually define real-world differences with a particular technique. Whenever possible, it is optimal to analyze strains with more than one technique.

At its best, molecular or antigenic typing can rationalize an epidemic or endemic problem. Analysis of a group of clinical and environmental strains from one location over a period of years produced three clusters of strains, as summarized in Table 10-5. These strains were characterized with a panel of monoclonal antibodies, relatively nondiscriminatory reagents. Amplified-fragment-length polymorphism analysis has been used successfully by a group of European investigators.[69] The polymorphism types of *L. pneumophila* serogroup 1 have been defined and validated by proficiency testing.[68] Another group used automated ribotyping, using reference strains, and concluded that the procedure was useful for genomic analysis, but not sufficiently discriminatory to be used for epidemic analysis. The brightest promise involves the so-called next-generation sequencing. This technology, which can be used to rapidly and efficiently either sequence the entire bacterial genome or simultaneously determine the sequence of multiple taxonomically important genes, has been used in a variety of outbreak investigations. The precise role this will play in the future remains to be determined, but it is fairly certain that this technology will play a role in this area.[153]

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CHAPTER 11

Neisseria Species and *Moraxella catarrhalis*

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Introduction

Gonorrhea was recognized at least as early as the time of Galen (2nd century AD), who named the disease after the Greek words *gonor* ("seed") and *rhoia* ("flow"), suggesting that the disease was related to the flow of semen. References to this infection are also found in the Old Testament and in written histories of several cultures. Although well recognized as a sexually transmitted infection by the 13th century, gonorrhea was not distinguished from syphilis until the mid-19th century. In 1897, *Neisseria gonorrhoeae*, the etiologic agent of gonorrhea, was first observed in purulent exudates from the genital tract and conjunctiva by Albert Neisser at the University of Breslau in Germany. Subsequent isolation of the organism, inoculation

into human volunteers, and reisolation of the organism by Bumm in 1885 proved the causal relationship between the organism and the disease (see Koch's postulates, Chapter 5).

Epidemic cerebrospinal meningitis was known early in the 19th century, but the etiologic agent was not described until 1884, when Marchiofava and Celli observed the organism in meningeal exudates. In 1887, Weichselbaum isolated the organism, now called *Neisseria meningitidis*, in pure culture and first described its characteristics and etiologic role in six patients with acute meningitis. Additional work by Kiefer in 1896 and Albrecht in 1901 established the existence of the meningococcal carrier state in healthy individuals. Over the period from 1929 through 1943, large outbreaks of meningococcal disease in Chile and US cities focused scientific attention on this organism. With subsequent outbreaks among military recruits in the United States and abroad, a better understanding of the epidemiology, pathogenesis, chemoprophylaxis, and prospects for vaccine development began to emerge. During the late 1930s, sulfonamide agents were found to eradicate the meningococcal carrier state, thereby providing a means for prevention of disease spread. During the early 1960s, resistance to sulfonamides began to emerge among meningococci, along with the appearance of epidemic disease at two military installations in California. These outbreaks stimulated the development of effective polysaccharide vaccines against both serogroups A and C meningococci. At present, epidemic serogroup A *N. meningitidis* meningitis remains a serious problem in regions of Africa, and meningococcal disease is still seen among semi-closed military populations.[482] In both the United States and Europe, serogroup C meningococcal disease has increased in the community and on college and university campuses, resulting in marked increases in disease incidence among adolescents and young adults. Molecular techniques have enabled researchers to examine possible meningococcal virulence factors and to understand the "molecular epidemiology" of meningococcal serogroups, serotypes, subserotypes, and clonal virulence.

N. gonorrhoeae and *N. meningitidis* continue to be formidable and versatile pathogens that provide a challenge for both clinicians and clinical laboratorians. Although methods for isolation and identification of *Neisseria* species from clinical specimens have not changed significantly, new nucleic acid probe- and nucleic acid amplification-based techniques for direct detection of *N. gonorrhoeae* have greatly modified laboratory approaches to the diagnosis of gonococcal disease. The development of resistance to antimicrobial agents continues to be a major concern in the new millennium, particularly with the appearance of fluoroquinolone resistance among gonococci and meningococci and the emergence of gonococcal strains with diminished susceptibility to ceftriaxone. Recognition, treatment, and management of meningococcal disease in a community require the cooperation of clinicians, clinical laboratories, and epidemiologists in order to formulate the most appropriate

interventions. On occasion, the recovery of "nonpathogenic" and newly recognized *Neisseria* species from immunocompromised patients must also be considered by the microbiologist in formulating an approach to the detection and identification of *Neisseria* species and "*Neisseria*-like" bacteria in clinical specimens.

Taxonomy of the Family *Neisseriaceae* and the Family *Moraxellaceae*

Members of the genus *Neisseria* are classified in the family *Neisseriaceae*, order *Neisseriales* in the β-subgroup of the phylum *Proteobacteria*. The β-*Proteobacteria* also includes several families of nonfermentative, gram-negative bacteria (i.e., families *Alcaligenaceae*,*Burkholderiaceae*, *Comamonadaceae*, and *Spirillaceae*). The family *Neisseriaceae* also includes the genera *Allysiella*, *Aquaspirillum*, *Chromobacterium*, *Eikenella*, *Kingella*, *Simonsiella*, and *Vitreoscilla*. *Moraxella catarrhalis* is a member of the genus *Moraxella*, which is classified in the family *Moraxellaceae*, order *Pseudomonadales* in the γ-subgroup of the phylum *Proteobacteria.* The genus *Moraxella* comprises more than 20 species, with *M. catarrhalis* being the most clinically significant species recovered from human specimens. *M. catarrhalis* has a gram-negative diplococcal morphology similar to most of the *Neisseria* species, but the other members of the genus *Moraxella* are coccobacillary gram-negative organisms. The other *Moraxella* species are discussed in Chapter 7.

General Characteristics of the Genus *Neisseria*

Members of the genus *Neisseria* are coccal or rod-shaped gram-negative organisms that frequently occur in pairs or short chains. Diplococcal species have adjacent sides that are flattened, giving them a "coffee bean" appearance. All species in the genus *Neisseria* inhabit mucous membrane surfaces of warm-blooded hosts. These organisms are nonmotile and do not form spores, and most species grow optimally at 35°C to 37°C. The organisms are capnophilic and grow best in a moist environment. *Neisseria* species produce acid from carbohydrates oxidatively, and acid production from various carbohydrates constitutes part of the reference identification of these species. Most *Neisseria* species grow as gram-negative diplococci, except for the three *N. elongata* subspecies and *N. weaveri*, which are medium-to-large, plump rods that sometimes occur in pairs or short chains, and

N. bacilliformis, *N. animaloris*, and *N. zoodegmatis*, which are short bacilli or coccobacilli.[26,293,309] All species in the genus are oxidase-positive and (except for *N. elongata* subspecies *elongata, N. elongata* subspecies *nitroreducens,* and some isolates of *N. bacilliformis*) are catalase-positive.[89,281] Members of the genus that are found in humans include *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. sicca*, *N. subflava* (including biovars *subflava*, *flava*, and *perflava*), *N. mucosa*, *N. flavescens*, *N. cinerea*, *N. polysaccharea*, *N. bacilliformis*, and *N. elongata* subspecies. Among animal species, *N. canis* and *N. weaveri* are found as part of the normal respiratory tract flora of dogs. *N. macacae*, *N. dentiae*, and *N. iguanae* are found in the mouths of rhesus monkeys, cows, and iguanid lizards, respectively.[26,56,309] *N. denitrificans* is present in the upper respiratory tract of guinea pigs and was reclassified in the genus *Bergeriella* as *Bergeriella denitrificans* in 2005.[710] *N. animaloris* and *N. zoodegmatis* were formerly called the Centers for Disease Control and Prevention (CDC) eugonic fermenter (EF)-4a and EF-4b, respectively.[660] These species are found in the mouths of dogs and have been isolated from dog bite wounds. *N. wadsworthii* and *N. shayeganii* are new species that were isolated from archived clinical reference specimens.[702]The clinical significance, cultural characteristics, and procedures for differentiating these organisms are discussed later in this chapter.

Most *Neisseria* species found in humans are normal inhabitants of the upper respiratory tract and are not considered as pathogens, although on occasion these organisms may be isolated from infectious processes, particularly in the settings of underlying disease and immunosuppression. *N. gonorrhoeae* is always considered a pathogen, regardless of the site of isolation. *N. meningitidis* also causes significant and oftentimes severe disease but may also colonize the human nasopharnyx without causing disease. Although most *Neisseria* species are not exacting in their nutritional requirements for growth, the pathogenic species, *N. gonorrhoeae* in particular, are more nutritionally demanding. *N. gonorrhoeae* will not grow in the absence of the amino acid cysteine and a usable energy source (i.e., glucose, pyruvate, or lactate). Some strains display requirements for amino acids, pyrimidines, and purines as a result of defective or altered biosynthetic pathways. The *Neisseriae* are aerobic, but will grow under anaerobic conditions if low concentrations of an alternative electron acceptor (e.g., nitrites) are present.[368,369] Although the saprophytic *Neisseria* species can utilize amino acids for growth, glucose or another energy source is required by the pathogenic *Neisseria* for metabolism via the Krebs cycle (see Chapter 5). The growth of *Neisseria* species is also stimulated by CO_2 and humidity. Atmospheric CO_2 shortens the lag phase by being rapidly assimilated for initial nucleic acid and protein biosynthesis. In the clinical laboratory, use of enriched media and incubation of cultures in 5% to 7% CO_2 fulfills the requirements for the recovery of these organisms from clinical specimens.

The pathogenic *Neisseria* species possess several factors and characteristics that contribute to virulence. *N. meningitidis* strains isolated from serious infections usually possess polysaccharide capsules external to the outer membrane on the surface of the bacterial cells; 13 different capsular serogroups have been described (see later discussion). The capsules render the organisms resistant to phagocytosis, particularly in the absence of opsonizing antibodies.[35] Various outer membrane protein (OMP) antigens found in both *N. gonorrhoeae* and *N. meningitidis* also have functions associated with virulence. Both gonococci and meningococci also produce an immunoglobulin A (IgA_1) protease capable of splitting humoral and secretory IgA_1 into Fab and Fc fragments, potentially neutralizing the effects of secretory IgA and abrogating mucosal resistance to infection.[468] Nutritional and atmospheric conditions also contribute to the virulence of both gonococci and meningococci. Colonization and subsequent infection of mucosal surfaces by these organisms require iron, and both of these organisms have genetically regulated enzymatic methods for releasing iron from transferrin and lactoferrin, making free iron available for bacterial metabolism. Requirements for individual amino acids or other trace nutrients may also restrict the abilities of different gonococcal and meningococcal strains to cause certain clinical syndromes. Since *N. gonorrhoeae* strains are able to grow anaerobically in the presence of nitrite as an electron acceptor, this property may contribute to gonococcal virulence by allowing the organism to proliferate in anaerobic milieus, such as the endocervix, rectum, genital tract, and the pharynx, and would explain the pivotal role of this organism in pelvic inflammatory disease, in which the organism may be recovered in culture along with obligately anaerobic bacteria.[369]

Clinical Significance of *Neisseria* Species

Neisseria gonorrhoeae

Epidemiology. *N. gonorrhoeae* is the causative agent of gonorrhea, a bacterial infection of great public health significance. In the United States, the incidence of gonorrhea steadily increased during the 1960s and early 1970s, with the highest incidence—over 460 cases per 100,000 population—occurring in 1975.[132] The "gonorrhea epidemic" that occurred during that time was attributable to several factors, including an increasing at-risk population of young adults, importation of less susceptible gonococcal strains along with the return of servicemen from the Vietnam conflict, increasing use of nonbarrier contraceptive methods (i.e., the birth control pill and intrauterine devices), and improved screening, outreach, and contact tracing. Since the early 1980s and into the 1990s, the incidence of gonorrhea has steadily declined. This decline was largely due to changes in sexual behaviors, particularly among men

who have sex with men (MSM), in response to AIDS, and to more effective case-finding and contact tracing among women. Incidence rates during the mid-1990s and into the new millennium continued to decline, with 128 cases per 100,000 reported from 1998 through 2006, and 119 cases per 100,000 reported in 2007.[135] However, gonorrhea remains the second most common reportable disease in the United States.[138] The incidence of gonorrhea is still high among sexually active teenagers (ages 10 through 19 years) and young adults (ages 20 through 24 years) of all races, with the highest attack rates being among 15- to 19-year-old women.[132] Disproportionately high rates of gonococcal and chlamydial

infections are found among urban-dwelling African Americans, particularly women in this 15- to 19-year age group.[485] Over the past several years, the incidence of gonorrhea has dramatically increased among MSM in the United States and abroad. The reasons for this include behavioral responses related to the availability of highly active antiretroviral therapy (HAART) and the management of HIV infection as a chronic illness.[606] A study in Seattle demonstrated that the incidence of gonococcal infection in MSM more than tripled from 1995 to 2003, while the incidence in the Seattle population at large changed minimally.[355]Among heterosexuals, gonococcal infection is disproportionately seen among African Americans and reflects other social dynamics beyond socioeconomic status, including stability of partner relationships, participation in high-risk sexual behaviors, drug and alcohol use, social disenfranchisement, and incarceration.[235] Both social (i.e., urban residence, lack of education, access to health care, unmarried status, race/ethnicity, male homosexuality, prostitution, histories of other sexually transmitted diseases) and behavioral risk factors (i.e., unprotected intercourse, multiple partners, other high-risk partners, drug use) have been identified for targeting by outreach/intervention and STD control programs. Transmission of gonorrhea and maintenance of the infection in a population are related to a social subset of “core” or “high-frequency transmitters” who serve as reservoirs of infection. These individuals have frequent, unprotected sexual contacts with multiple partners during incubation periods subsequent to organism acquisition and prior to recognition of infection and treatment, thereby maintaining a pool of infection and transmission within a population.[100,593] On a worldwide basis, the highest incidence of gonorrhea and its complications is found in resource-limited countries, such as those in Africa and Latin America.

The risk of acquiring gonorrhea is multifactorial and is related to the frequency and sites of exposure. For heterosexual males, the risk of acquiring urethral infection from an infected female is about 20% for a single exposure and up to 80% for four

exposures.[313] Owing to anatomical considerations, the risk of infection of the female genital tract from a single exposure to an infected male is much higher, being on the order of 50% to 70% per exposure.[408] Transmission of rectal infection is also quite efficient, and studies among MSM have demonstrated that urethral infection following fellatio from an infected partner may account for as much as 26% of urethral infections diagnosed in this population.[385] Among women, use of hormonal contraceptive methods is associated with an increased risk of gonococcal infection, while barrier methods such as condoms and diaphragms used with spermicidal foams and gels (i.e., non–oxynol-9) exert a protective effect against infection.[418,419]

Infections Caused by *N. gonorrhoeae*. In males, *N. gonorrhoeae* causes an acute urethritis with dysuria and urethral discharge (Fig. 11-1).[435,454] The incubation period between acquisition of the organism and onset of symptoms ranges from 1 to 14 days or longer, with an average of 2 to 7 days. Infections in men are asymptomatic during the prodromal stages of infection, and conversely, 95% to 99% of men with urethral gonococcal infection will experience a discharge at some time. The discharge is purulent in 75% of cases, cloudy in 20%, and mucoid in about 5%; the consistency of the discharge at presentation is affected by the length of time that the infection has been incubating and whether the patient has recently urinated. About 2.5% of men presenting to sexually transmitted disease clinics are truly asymptomatic, and it is estimated that the prevalence of asymptomatic urogenital gonorrhea in men in the general population may be as high as 5%. This pattern of disease is often associated with infection by certain Por IA gonococcal serovars and with the arginine–hypoxanthine–uracil (AHU) strains and certain other auxotypes of *N. gonorrhoeae* (see below).[102,370,373,464] If left untreated, most cases of gonorrhea in men resolve spontaneously, but in less than 10% of cases, ascending infection may result in gonococcal epididymitis, epididymo-orchitis, prostatitis, periurethral abscess, and urethral stricture.[641] These complications are rarely seen in clinical practice in the United States.

■ **FIGURE 11-1** Male with purulent urethral discharge characteristic of *Neisseria gonorrhoeae* infection.

In females, the primary gonococcal infection is present in the endocervix, with concomitant urethral infection occurring in 70% to 90% of cases. After an incubation period of 8 to 10 days, patients may present with cervicovaginal discharge, abnormal or intermenstrual bleeding, and abdominal or pelvic pain; the latter suggests the presence of upper genital tract disease. The presence of dysuria indicates significant urethral involvement.[101] Gonococcal infection of the vaginal squamous epithelium of postpubertal women is uncommon, and in women with hysterectomies, the urethra is the most common primary site of infection. Although it has often been stated that most women with genital gonococcal infection are asymptomatic, this is probably not

true. This assertion was based on the detection of infected women during widespread screening in locales such as family planning clinics, and did not account for those women who presented to physicians or emergency rooms with a spectrum of symptoms referable to the genital tract (e.g., vaginal discharge, dyspareunia, menorrhagia) or the lower abdomen.[435]

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Symptoms of uncomplicated endocervical infection often resemble those of other conditions, such as cystitis or vaginal infections, and the symptomatology of gonococcal endocervicitis is often clouded by co-infection with *Chlamydia trachomatis*, *Trichomonas vaginalis*, and/or *Candida albicans*. While the genital tract may appear normal, careful endocervical examination often reveals areas of friable cervical mucosa that bleed on swabbing.[101] Only 10% to 20% of infected women, however, will present with an obvious mucopurulent endocervical discharge. Infection of the Bartholin's and Skene's glands may be seen in about one-third of women with genital tract infection. Careful manipulation of these areas can sometimes provide purulent material for direct examination and culture. Endocervical gonorrhea may also complicate pregnancy and is a recognized cofactor for spontaneous abortion, chorioamnionitis, premature rupture of membranes, and premature delivery.[341,435] Infants born to women with genital tract infection are at risk for developing gonococcal conjunctivitis ("ophthalmia neonatorum") or pharyngeal gonococcal infection.

Ascending gonococcal infection may occur in 10% to 20% of infected women and can result in acute pelvic inflammatory disease (PID) that is manifested as salpingitis (infection of the Fallopian tubes), endometritis, and tubo-ovarian abscess, all of which can lead to scarring of the Fallopian tubes, ectopic pregnancies, sterility, and chronic pelvic pain.[341,389,435]Symptoms of gonococcal PID include bilateral lower abdominal pain, abnormal cervical discharge and bleeding, pain on motion, fever, and peripheral leukocytosis. PID caused by *N. gonorrhoeae* generally occurs early, rather than late, in infection and often during or shortly after the onset of menstruation. Fever, leukocytosis, elevated erythrocyte sedimentation rates, and C-reactive protein (CRP) are seen in about two-thirds of patients, while chills, nausea, and vomiting are variable features of PID. Women with salpingitis may also develop a perihepatitis called the Fitz–Hugh–Curtis syndrome, which is characterized by direct extension of the organisms from the Fallopian tube to the liver and peritoneum, resulting in right upper quadrant pain and the finding of adhesions between the liver and the anterior abdominal wall on laparoscopy. The development of PID is influenced by many factors, including diagnosis and treatment for other genital tract infections (e.g., *C. trachomatis* infection, bacterial vaginosis), prior PID, the use of oral contraceptives,

intrauterine devices, vaginal douching, characteristics of infecting strains, and the immune competence of the host.[477,478] Obstruction of the Fallopian tubes, leading to infertility, occurs in 10% to 20% of women following a single episode of acute gonococcal PID and in 50% to 80% after three or more episodes.[693] Among women with ectopic pregnancies, up to 80% have a history of prior PID.[211] In pregnant women, gonococcal infection is associated with an increased risk of complications, including premature labor, premature rupture of the fetal membranes, spontaneous abortion, and infant mortality. Complications due to gonococcal infections are uncommon after the first trimester.

N. gonorrhoeae may also cause pharyngeal and anorectal infections.[596] Oropharyngeal gonococcal infection is seen in MSM and women who acquire the infection by engaging in orogenital sexual contact with an infected partner. Pharyngeal gonorrhea may also be seen occasionally in heterosexual men resulting from performing cunnilingus with an infected partner. The prevalence of oropharyngeal gonococcal infection is particularly high among MSM, patients seen at STD clinics, and HIV-positive individuals.[335,385,513] Patients with oropharyngeal gonococcal infection often have gonococcal infection at genital and/or rectal sites as well.[265,431] Among MSM, in particular, the oropharynx may be the source of transmission to sexual contacts.[385] Over 90% of oropharyngeal gonococcal infections are asymptomatic and are diagnosed by culture of the organism from the throat.[54,501] In many cases, pharyngeal gonococcal infections resolve without antimicrobial therapy. Anorectal infections are seen primarily in MSM who practice unprotected receptive anal intercourse. Kent and colleagues examined 174 rectal specimens from MSM seeking HIV testing and found a prevalence of rectal gonorrhea of 2.9%.[354] A second study conducted in Seattle found a prevalence of rectal gonococcal infection of 2.2% among 500 MSM.[412] Rectal gonococcal infection is frequently associated with HIV infection. In a study of 564 MSM, the prevalence of rectal gonorrhea was 7.1%. In this study, the prevalence of rectal gonorrhea among HIV-positive MSM was 15.2%, 3.5 times the prevalence observed in HIV-negative MSM.[361]Among MSM, gonococcal infection of the rectum may be the only infected anatomic site.[288]Women may also acquire rectal infections by receptive anal intercourse, but most rectal infections in women are due to perianal contamination with infected cervicovaginal secretions. Rectal gonococcal infections are often asymptomatic, but some individuals may experience symptoms ranging from mild pruritis to acute proctitis with anorectal pain and itching, a mucopurulent discharge, bleeding, tenesmus, and constipation 5 to 7 days following infection.[365,541] Anoscopic examination of the anal canal usually reveals an edematous and erythematous rectal mucosa and a purulent discharge associated with the anal crypts.[700]*Chlamydia* infection, herpes simplex virus infection,

and other sexually transmitted infections are included in the differential diagnosis of anorectal gonococcal infection.

In a small percentage (approximately 0.5% to 3%) of infected individuals, gonococci may invade the bloodstream, resulting in disseminated gonococcal infection (DGI).[167,450,596] This infection is characterized by low-grade fever (rarely above 39°C), hemorrhagic skin lesions, tenosynovitis, migratory polyarthralgias, and septic arthritis. Blood cultures are intermittently positive, so at least three blood culture sets should be obtained when a diagnosis of DGI is suspected. Women appear to be at a greater risk for developing DGI, particularly during menstruation and during the second and third trimesters of pregnancy. The skin lesions are exquisitely painful and initially appear as a papule that evolves into a necrotic pustule on an erythematous base (Fig. 11-2, Image Plate 11-1B). There may be as few as 5 or up to 30 lesions present, and the majority of them are on the extremities (i.e., toes, fingers).[168,450] Cultures of skin lesions and synovial fluids from patients with DGI are often negative, suggesting that immune processes (e.g., antigen/antibody complex deposition) contribute to the signs, symptoms, and pathogenesis of DGI. On occasion, patients may develop extensive macular, papular, or petechial rashes and vasculitis.[334,427] In 30% to 40% of cases, organisms localize in one or more joints to cause a purulent and destructive

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■ **FIGURE 11-2** Skin lesion of DGI on the index finger of the left hand. Skin lesions associated with DGI are usually located on the extremities.

gonococcal arthritis.[530] Joint involvement is usually asymmetric and most commonly involves the knee, elbow, wrist, fingers, or ankle joints.[530] Infected synovial fluid frequently contains 40,000 to 60,000 cells/mm^3, with greater than 80% being polymorphonuclear neutrophils.[258,530] Gram's stain and culture of joint aspirates are positive in only 10% to 30% of cases. Molecular methods (i.e., polymerase chain reaction [PCR], strand displacement amplification) have also been successfully applied to the detection of gonococci in synovial fluid and swab specimens obtained from skin

lesions.[362,525]

Complications of DGI include permanent joint damage, endocarditis, and rarely, meningitis. Gonococcal endocarditis develops in 1% to 2% of patients with disseminated infection and follows a rapid and destructive course.[577] The aortic valve is involved in 50% of cases and mitral valve involvement is present in 30% of cases; four cases of gonococcal tricuspid valve infection have also been reported.[11] Thompson and Brantley reported on gonococcal endocarditis in a 23-year-old man that required prosthetic valve placement due to severe aortic insufficiency and a prolapsed aortic cusp.[633] In 1997, the first case of gonococcal endocarditis associated with systemic lupus erythematosus (SLE) was diagnosed in a 24-year-old woman who presented with nonspecific symptoms of cough, chest pain, fever, and malaise without signs or symptoms of DGI.[637] Transthoracic and transesophageal echocardiograms showed a large vegetation on the pulmonary valve, and blood cultures were positive for *N. gonorrhoeae*. Nielsen and colleagues used the Gen-Probe APTIMA transcription-mediated amplification (TMA) test on surgically excised valvular tissue to successfully diagnose gonococcal endocarditis in a 45-year-old male.[484] Pericarditis and pericardial effusions are also complications of DGI, and adult respiratory distress syndrome, septic shock, and multisystem organ failure have been reported in association with gonococcal bacteremia.[386] Gonococcal meningitis is a rare complication of DGI that has features typical of meningitides caused by other organisms.[442] Other features associated with DGI (e.g., arthritis, tenosynovitis, and skin lesions) are also seen in these patients. Molecular approaches have been helpful in diagnosing gonococcal meningitis, as cerebrospinal fluid (CSF) cultures may be negative due to the fastidious nature of the organism.[104,106,442] Clinical entities that resemble DGI include Reiter's syndrome, pyogenic and crystal-induced arthritis, syphilitic and tuberculous arthritis, rheumatoid arthritis, Lyme disease, and rheumatic fever. Repeated bouts of DGI have been observed in individuals with certain complement deficiencies.[222] Disseminated disease may develop following infection at genital or extragenital sites. Studies of isolates recovered from cases of DGI have shown that these more invasive strains have unusual characteristics, including unique nutritional requirements (e.g., requirements for arginine, hypoxanthine, and uracil for growth [AHU strains]), defined PorIA serovar classification, and resistance to bactericidal activity of normal human serum.[218,464] With the decline in the prevalence of AHU/Por IA serovars over many years, increasing numbers of DGI infections have been associated with other auxotype/serovar classes.[628]

Ocular gonococcal infections, once seen primarily among neonates who acquired the organism during passage through an infected birth canal ("ophthalmia neonatorum"), occur in adults who become infected by autoinoculation from genital

secretions.[22,242] Laboratory personnel working with cultures may also become accidently infected if care is not taken to protect the eyes.[429] Conjunctival infection with *N. gonorrhoeae* is usually painful, with the presence of photophobia, markedly decreased visual acuity, eyelid edema, periorbital cellulitis, and a profuse purulent conjunctival discharge.[523] This infection may be uni- or bilateral and may be complicated by epithelial or stromal keratitis, ulcerative keratitis, corneal perforation, prolapse of the iris, and endophthalmitis.[173] Prompt antimicrobial therapy is required in these cases. The presence of underlying disease (e.g., HIV infection, SLE, hepatitis B infection with cirrhosis) can be associated with atypical presentations of gonococcal infection (e.g., endophthalmitis, bacteremia).[233,524,703] Gonococcal scalp abscesses have been reported in neonates as a complication of intrauterine fetal monitoring.[674] Periurethral and cutaneous gonococcal abscesses have been reported in both HIV-positive and HIV-negative individuals.[352,595]

Historically, gonococcal infections in children included only ophthalmia neonatorum, which is described above. Almost all cases of gonorrhea in children during the newborn period are the result of ocular contamination, although more serious infections with gonococci in newborns have been reported.[226] Transmission of gonorrhea from adults to children by fomites (e.g., shared towels) was proposed as a mode of transmission in older children. However, it is now recognized that gonococcal infections, including conjunctivitis, and other sexually transmitted diseases in children beyond the immediate neonatal period (defined as beginning at birth up through the first month of life) are indicators of sexual abuse.[58,292,423] With a careful, multidisciplinary approach, histories of sexual contacts can usually be obtained from older children. When a child with gonorrhea is

identified, investigation of both adult caretakers and other siblings frequently reveals infected adults and other infected children. Gonococcal infections in children resemble those in adults, with some notable differences. *N. gonorrhoeae* causes a vaginitis with a vaginal discharge, rather than a cervicitis, in prepubertal girls. The epithelium of the prepubertal vagina is composed of columnar epithelial cells, which are the cell types that *N. gonorrhoeae* preferentially infects. With the onset of puberty, these cells are replaced by a stratified squamous epithelium that is not susceptible to gonococcal infection. Urethral infection in male children, if present, resembles that seen in adults. Pharyngeal and rectal gonococcal infections, as in adults, are usually asymptomatic in children.

Neisseria meningitidis

Meningococcal Epidemiology: Serogroups, Serotypes, and Serosubtypes. *N. meningitidis* is a primary pathogen that causes an array of infectious processes, ranging from occult sepsis with rapid recovery to fulminant overwhelming fatal disease.[35,707] Some patients may manifest limited aspects of this clinical spectrum, while others may progress across this spectrum with alarming rapidity. A major virulence factor of all disease-associated isolates of *N. meningitidis* is the polysaccharide capsule. Thirteen meningococcal capsular polysaccharide serogroups (A, B, C, D, H, I, K, L, X, Y, Z, W135, and 29E) have been described, but most infections worldwide are caused by organisms belonging to serogroups A, B, C, X, Y, and W135. Serogroup B *N. meningitidis* strains cause a substantial proportion of endemic disease in many parts of the world, including the United States. Serogroup C and serogroup Y strains have been associated with outbreaks of disease in both the United States and other countries. Serogroup A strains occur primarily in the "meningitis belt" of sub-Saharan Africa and have been responsible for the largest meningococcal epidemics, with serogroups C, X, and W-135 also responsible for some disease in regional areas of Africa (e.g., Kenya, Togo, Niger), China, Argentina, and in Hajj pilgrims.[217,536,544,615,616,632,698,699,722] Because of the availability and widespread use of polysaccharide and conjugate vaccines against *N. meningitidis* serogroups A, C, Y, and W-135, serogroup B is now responsible for up to 90% of disease in some countries, particularly in Europe (Box 11-1).[643] Some data suggests that serogroup C may be associated with more hypervirulent clones and tends

BOX 11-1

Global Epidemiology of *N. meningitidis* and Meningococcal Disease: An Overview

Among the countries of Latin American, little data is available due to recognized underreporting, but a 2008 survey of almost 7,000 isolates collected from 2000 through 2005 from 19 Latin American countries (especially Brazil and Chile) reported that 69% of meningococcal isolated belonged to serogroup B, 26% were serogroup C, and serogroups Y and W-135 each comprised about 2% of isolates.[255]Brazil experienced epidemics of serogroup A disease in the 1920s through the 1940s, with subsequent overlapping serogroup A and serogroup C meningococcal epidemics occurring during the early 1970s.[185]During the late 1990s and into 2002, the annual incidence of meningococcal disease in Sao Paolo was 6 cases per 100,000 and the involved serogroups were serogroup B (59%) and serogroup C (36%).[183]During 2003 to 2005, annual disease incidence in Sao Paolo was 3.9 cases per 100,000, with almost two-thirds of isolates belonging to serogroup C. In southern

Brazil, similar incidence rates have been observed throughout the late 1990s and into the mid-2000s; however, the serogroup distribution in the southern regions of Brazil included a preponderance of serogroup B isolates (78.9%), along with serogroup C (14.1%) and serogroup W-135 (6.2%).[48]The prevalence of isolates belonging to serogroup W-135 in southern Brazil now exceeds 17%, with all isolates belonging to the ST-11 complex.[691]In Chile, incidence rates of meningococcal disease have remained stable for many years at 0.6 per 100,000, with isolates belonging to serogroups B (71%), C (12%), and Y (10%). Argentina reported decreases in the rates of meningococcal disease during the period from 1993 through 2005, with serogroup B and C isolates predominating.[152]During 2008, disease due to serogroup W-135 began to emerge, with this serogroup being identified in 28% of disease cases.[217]Cuba experienced a national outbreak of serogroup B meningococcal disease during the early 1980s, but disease incidence has continued to decline since that time to 0.6–0.7 cases per 100,000 from 1998 through 2000, with a further decline to 0.3 cases per 100,000 in 2003.[193] In Columbia, disease incidence data are not available, but a study of 434 isolates collected in Columbia from 1994 through 2006 showed that serogroup B isolates predominated (78%), followed by serogroup C (10%), serogroup Y (9%), and serogroup W-135 (2%); serogroup Y isolates comprised none of the isolates from 1994 and 50% of isolates collected in 2006.[324]

In certain regions of sub-Saharan Africa that are referred to as the "meningitis belt," meningococcal disease caused primarily by serogroup A strains occurs annually, with incidences as high as 1,000 cases/100,000 population.[536,632]This region extends from Senegal in the west to Ethiopia in the east. Epidemics of meningococcal disease occur in cycles of 5–12 years, beginning during the dry seasons in the region and ending with the onset of the rainy season. During the dry season, temperatures fall below 10°C at night, causing populations to congregate in enclosed areas and leading to efficient person-to-person

transmission of the organisms. In addition, the drying effects of strong winds from the Sahara Desert on the oropharyngeal mucosa further increase the risk of invasive meningococcal disease.[457]The last major epidemic caused by serogroup A *N. meningitidis* occurred in 1996 through 1997, causing over 250,000 clinical cases and over 25,000 deaths. This serogroup A strain belonged to the ST-5 complex and caused epidemics of disease in Ethiopia, the Sudan, Kenya, Tanzania, and Chad from 1988 until 1998. In 1995, a new serogroup A clone (ST-7) appeared in Algeria and subsequently spread throughout the meningitis belt, causing an outbreak of meningococcal disease in the Sudan in 1999.[483]Extension of the meningitis belt into the southern regions of Africa was heralded by outbreaks of ST-7 disease in refugee camps in Rwanda, Burundi, and the Republic of Tanzania. Until about 2000, other

meningococcal serogroups were uncommon agents of disease in Africa, with rare outbreaks due to serogroup C strains and small epidemics due to serogroup X isolates in Chad, Niger, Ghana, and Kenya.[120,202]Since 2002, serogroup W-135 strains have emerged in the region that were originally linked to strains infecting individuals participating in the 2000 Hajj pilgrimage to Mecca, Saudi Arabia.[517,615,616]These individuals had been vaccinated with serogroup A and C polysaccharide vaccines, setting the stage for the emergence of the serogroup W-135 strain belonging to the ST-11 complex. This strain apparently arose due to a capsular switch from serogroup C to serogroup W-135. In 2002, over 200 cases of disease due to this strain occurred in Burkina Faso, and isolates belonging to this clone were isolated from individuals within and outside of the meningitis belt.[32]Administration of a trivalent serogroup A/C/W-135 vaccine caused an abrupt decline in disease due to this strain and the emergence of a new serogroup A strain belonging to the ST-2859 complex in 2003.

In the Middle East, most meningococcal disease involvement has been associated with the Hajj pilgrimage to Mecca, Saudi Arabia. During the 1987 pilgrimage, an outbreak of serogroup A meningococcal disease occurred, and travelers returning to the United States from Saudi Arabia demonstrated high rates of group A meningococcal nasopharyngeal carriage.[462]After the 1987 pilgrimage, meningococcal A and C polysaccharide vaccination was made mandatory in order for pilgrimage participants to enter Saudi Arabia.[120]This resulted in virtual disappearance of this serogroup A strain. Beginning in 2000, serogroup W-135 strains emerged during the Hajj pilgrimage, causing over 400 cases of meningococcal disease.[615]When Hajj participants returned to their countries of origin, cases caused by the same serogroup W-135 clone were reported in England, France, Israel, the United States, and other countries.[697,698]The disease strain belonged to ST-11 and had a unique serotype/serosubtype that was similar to strains seen in the England in previous years, only the UK strains belonged to serogroup C and not W-135. It was postulated that mandatory immunization of Hajj participants with serogroup C vaccine had exerted selective pressure for the same ST-11 clone to undergo a capsular switch to serogroup W-135, with serotype and serosubtype markers remaining unchanged.[8,445]Subsequent introduction of the quadrivalent meningococcal vaccine into Saudi Arabia as a visa requirement for Hajj participants has eliminated outbreaks of meningococcal disease in the region.[697]

In Europe, meningococcal disease is associated with serogroup B and C isolates, and incidence rates have declined from 1.67 cases per 100,000 in 1999 to 1.01 per 100,000 in 2006.[229] As in the United States, the highest incidence of disease has been among children less than 1 year of age and teenagers 15 to 19 years of age. During the late 1990s and early 2000s, several countries, including the United Kingdom, Ireland, Spain, Belgium, Portugal, and the Netherlands, experienced increases in disease due to a hypervirulent serogroup C strain belonging to the ST-11 clonal complex. Intensive meningococcal serogroup C vaccination programs in these countries were

implemented from 1999 to 2006 and were highly successful in controlling the spread of this strain, which had been responsible for up to 40% of cases in the United Kingdom alone.[426,643]At the same time as the reduction in serogroup C infections, the proportion of laboratory-confirmed disease due to serogroup B strains increased to 87% in 2007 and 2008 from between 46% and 69% in previous years. These serogroup B strains belong to ST-40 and ST-44 clonal complexes and these strains are now responsible for most meningococcal disease in Europe and the United Kingdom.[93]A second serogroup B clone (ST-269) that was first described in the Netherlands during the 1970s emerged as a cause of invasive disease in the United Kingdom and Scotland in the late 1990s; this same serogroup B clone has also been identified in Quebec, Canada.[196,393]Isolates belonging to serogroup W-135 emerged in France in 2000 and these strains are now the third most common meningococcal serogroup in both France and the United Kingdom.[698]The influx of W-135 strains into these regions at that time reflected the return of pilgrims from the Hajj to their home countries subsequent to the outbreak of W-135 disease in Saudi Arabia.[283]Because of the contribution of W-135 strains to the emergence of disease in areas to which Hajj pilgrims were returning, the United Kingdom made an official recommendation that pilgrims attending Hajj receive the tetravalent meningococcal polysaccharide vaccine for serogroups A, C, Y, and W-135. Visa requirements established in 2002 made receipt of the quadrivalent vaccine compulsory.

During the 20th century, most meningococcal disease in China was due to serogroup A strains, with four epidemics occurring in 1959, 1967, 1977, and 1984. During these epidemics, disease incidence was as

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high as 500 cases per 100,000.[722]All of these epidemics were due to the ST-5 clone, which has existed in the region for over 60 years, although the serotypes and serosubtypes of these serogroup A ST-5 clonal isolates varied. During the epidemic periods, this clonal group spread from China to Russia, Scandinavia, Nepal, and Saudi Arabia. Institution of a national immunization effort with monovalent serogroup A or bivalent serogroup A and C polysaccharide vaccines in the early 1980s reduced the disease incidence in the region to 0.2–1.0 cases per 100,000 population.[722]The last epidemic in China associated with ST-5 began in 1993, with subsequent spread to Moscow and Mongolia by 1996.[632]Beginning in 2000, *N. meningitidis* strains belonging to serogroups B and C caused sporadic outbreaks of disease China, and 2003 saw the emergence of another hyperinvasive serogroup C strain belonging to the ST-4821 complex.[574]Serogroup A strains remain important causes of outbreaks in India and the Philippines.[471,707]Meningococcal disease is fairly rare in Japan, with

fewer than 30 cases being reported each year, with most disease being due to the ST-32 clone of serogroup B *N. meningitidis*.[314]Severe meningococcal disease appears to be rare in the Far East (i.e., Taiwan, Malaysia, Thailand), with a disease incidence of less than 0.2 per 100,000 population.[153,508,522]

to cause more severe disease than other serogroups.[196,691] This increased virulence manifests as more severe residual effects (e.g., cutaneous lesions with scarring, limb loss, renal failure) and a higher case-fatality ratio than with other serogroups.[227,543,587] In France, a serogroup C clone (ST-11) had a case-fatality ratio of 22% compared with 15% for other French serogroup C isolates. In 2007, this clone caused 5 of 9 disease clusters, with increased virulence as evidenced by extensive hemorrhagic rashes in infected patients.[177]

Meningococcal isolates can be further characterized by OMPs that are expressed on the cell surface termed PorA and PorB. PorB (formerly class 2 and class 3 OMPs) are immunogenic proteins that determine the meningococcal serotype, while PorA proteins (formerly class 1 OMPs) determine the serosubtype.[253,448,657] PorB genotyping can be performed by sequencing of the *porB* gene, while PorA genotyping is performed by sequencing of two variable regions (VR1 and VR2) of the *porA* gene. *porA* genotyping has largely replaced serologic methods for PorA subserotyping because the gene may be variably expressed and single point mutations in *porA* may have a significant impact on virulence. *N. meningitidis* isolates can be further divided into 1 of 13 immunotypes based on lipo-oligosaccharide (LOS) antigenic determinants.[644]Although these methods are helpful in determining the relatedness of strains locally and in defined clinical settings, none of them are able to discriminate and define genetic relatedness of meningococcal strains for global epidemiologic purposes. *N. meningitidis* is able to undergo antigenic variation via horizontal gene transfer with other meningococci or saprophytic neisseriae, by autologous recombination within its own genome, and by variable gene expression due to the presence/absence of insertion sequences, tandem nucleotide repeats, and frame-shifts in nucleic acid transcription.[31,411,562] Horizontal gene transfers may result in **capsular switching**, where a strain changes from one capsular serogroup to another. Isolates that have undergone this process will have the same genetic serosubtype and immunotype, but will express different capsular serogroups. Meningococcal serogroup antigen gene sequencing by sialytransferase gene PCR can be used to confirm serologic grouping methods and to determine the genetic grouping of serologically "nongroupable" strains containing capsular genes that are "switched off."[83,239,305] Capsular switching permits "immunologic escape" from immunity to the original serogroup expressed by a given strain, thereby enabling

outbreaks of meningococcal disease to be initiated and sustained over considerable periods of time.[610] Capsular switching often results in strains that express the serogroup C capsular antigen. In a study of 1,160 invasive isolates collected from 2000 to 2005 in the United States, serogroup C isolates were the results of 12.9% of the switching events, compared with 1.5% resulting in serogroup B and 0.9% resulting in serogroup Y isolates. When sequence types (STs, see below) of these serogroup C strains were determined, 97.2% were STs usually associated with serogroup B, suggesting that these strains had undergone a "B" to "C" capsular switch.[183,299] Serogroup C-to-B and capsular C-to-W-135 capsular switches have been documented in isolates from Portugal and New Zealand, respectively.[59,581] Due to the high frequency of antigenic variation in OMPs and the phenomenon of capsular switching, other methods have been developed to genetically define, identify, and track pathogenic *N. meningitidis* strains globally.[238,610] Techniques such as multilocus enzyme electrophoresis (MLEE) have been used for strain characterization, but this method has been largely replaced by multilocus sequence typing (MLST). MLST is performed by sequencing of portions of seven housekeeping genes and determines the genetic lineage of meningococcal isolates in the community.[239,428] A clonal complex is comprised of a group of STs that share at least 4 of the 7 loci in common with an "ancestral genotype."[657] MLST, when used in conjunction with *porA* and *porB* genotyping and LOS immunotyping, has helped scientists and epidemiologists identify a number of genetically-defined clonal groups that are responsible for both epidemic and sporadic, endemic meningococcal disease.[120] Although MLST has replaced MLEE as a principal method for strain characterization, meningococcal isolates in the epidemiology literature are often referred to by both their enzyme type (ET designation) and their sequence type (ST designation). Other molecular genetic methods (e.g., DNA fingerprinting, restriction fragment length polymorphisms, pulsed-field gel electrophoresis, ribotyping, repetitive element-based PCR analysis, random-amplified PCR,*porA* gene sequencing, and PCR-amplicon endonuclease analysis) have also been developed for monitoring the epidemiology of pathogenic

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N. meningitidis strains on a worldwide basis, but are not as widely used as MLST.

Clonal analysis of *N. meningitidis* has revealed many interesting characteristics related to virulence and disease. Virulent clones are distinguished by having high transmission rates and causing high rates of disease upon introduction into a susceptible population. Avirulent clones are found in asymptomatic carriers and rarely cause disease, even when transmitted at rates similar to those of virulent clones.[300] The presence of virulent clones coupled with high transmission rates, a susceptible

population, certain host factors (e.g., smoking, mucosal immunity, viral co-infections, congenital/acquired complement deficiencies), and environmental/behavioral factors (crowding, close contact, smoke exposure) leads to high carriage rates and the often explosive appearance of disease.[120,243,716] Eventually, circulating virulent clonal groups undergo genetic diversification via transformation and various selective environmental pressures; these clones may then disappear and/or reemerge at a later time.[120] Invasive clones within an ST complex may express different serogroups. For example, the ST-11 sequence type that expresses serogroup C is responsible for outbreaks of meningococcal disease in the United States, Europe, and Canada, while the same ST-11 clonal complex expressing the serogroup W-135 capsular antigen has caused disease in Africa and Saudi Arabia.[8,93,483,457] Mayer et al.[445] showed that an outbreak of W-135 was due to the clonal expansion of a strain, rather than the emergence of a new strain. Conversely, Shao et al.[574]reported a new serogroup C clone in the Anhui province in China.

Worldwide, various pathogenic clones of *N. meningitidis* composed of different serogroups, serotypes, serosubtypes, and immunotypes are responsible for both endemic and epidemic disease.[185,193,299] Endemic meningococcal disease occurs at rates of 1 to 3 cases per 100,000 persons in the United States, and in 10 to 25 cases per 100,000 persons in developing countries. In the United States, the annual incidence of meningococcal disease peaked in the 1990s with an incidence of 1.7 per100,000 population and, subsequently declined to 0.35 per 100,000 in 2007, due to decreases in all three of the most common serogroups in the United States (serogroups B, C, and Y).[299] Attack rates are highest among children aged 3 months to 1 year and among older teenagers and young adults. Major changes in meningococcal epidemiology during the 1990s included an increase in incidence of community-acquired serogroup C infections, and an increase in disease among teenagers and young adults of both high school and college age.[124,300,426,543] During the 1990s, outbreaks of invasive meningococcal disease occurred on college campuses and were associated with patronage of bars, drinking, cigarette smoking, and dormitory residence.[164,322,323] Substantial amounts of disease have also been seen among non–first-year college students and among students living in off-campus housing.[296,298] During the mid-1990s through 2002, 76 outbreaks of meningococcal disease were identified in the United States; these occurred in colleges, primary and secondary schools, nursing homes, and the community.[246,327] As mentioned above, most of these outbreaks were caused by serogroup C strains, but serogroup Y *N. meningitidis* belonging to the ST-23 complex emerged during the early 1990s, and by the mid-1990s accounted for more than 25% of meningococcal disease, with infants, the elderly, and the immunocompromised being at greatest risk. In Oregon, outbreaks of meningococcal disease occurred in the mid-1990s due to serogroup B strains belonging

to the ST-32 clonal complex.[195] At the present time, serogroup C and serogroup Y strains account for about 31% and 35% of isolates, respectively, followed by serogroup B (23%), and serogroup W-135 (11%).[301] In Canada, the current incidence of meningococcal disease is 0.56 to 1.2 per 100,000, with serogroups C and Y accounting for 25.1% and 21.9% of isolates, respectively.[300]Recently, serogroup B strains belonging to the ST-269 complex emerged in Canada, with serogroup B strains now accounting for over 43% of isolates.[393] The epidemiology of the outbreak of meningococcal disease in and around New Dehli, India, has been reviewed.[471] Box 11-1 contains a brief discussion of the global epidemiology of *N. meningitidis* and meningococcal disease.

Carriage of *N. meningitidis*. *N. meningitidis* may be asymptomatically carried in the oropharynx and nasopharynx of a variable percentage of individuals, and the rate of carriage is related to several factors, such as age, socioeconomic class, and the presence of actual disease in a community.[35,711] In general, carriage rates tend to be less than 3% in children less than 4 years and increase to 24% to 38% in the 15-year- to 24-year-old age group.[121,155] The duration of carriage may also vary with the individual and the serogroup of the colonizing strain, and carriage of meningococci in the nasopharnyx may be transient, intermittent, or persistent.[12] During periods when disease is present in a community, the carriage rates may not be very different from observed rates when no clinical disease is being reported, but the proportion of individuals carrying the more virulent strain has been noted to increase.[35] The carriage prevalence among household contacts of patients with meningococcal disease is usually higher than in the population at large and at least half of the household carriers will be colonized with the same strain as the household's patient.[109,579] Contrary to what has been promulgated for years, the rate of meningococcal carriage does not appear to be seasonal, although most meningococcal disease in developed countries tends to occur during late winter and early spring. Crowded living conditions facilitate respiratory spread of meningococci, and this crowding affects both the transmission of the organism and the occurrence of overt disease. This has been amply demonstrated by the large outbreaks that have occurred on military bases over the years, where large numbers of young, susceptible adults live together in close quarters for prolonged periods. In addition, among university students, meningococcal carriage rises rapidly during the first few weeks of the academic year, largely due to residence in close quarters and socialization in classrooms and gathering places.[474] In addition, persons with bacterial or viral respiratory tract infections also tend to have higher meningococcal carriage rates.[604]Other factors that are associated with increased meningococcal carriage include male gender, cigarette smoking or exposure to

secondary smoke, and low socioeconomic status.[122] Carriage strains may be encapsulated (groupable) or nonencapsulated (nongroupable). Meningococcal colonization of the upper respiratory tract results in a humoral immune response, with formation of serogroup-specific and broadly cross-reactive

bactericidal antibodies against several other outer membrane antigens within 7 to 10 days.[35,711] Even individuals who are colonized with nongroupable strains develop high titers of antibodies against groupable strains, probably owing to the presence of shared antigenic determinants. In one study of 38 individuals who carried nongroupable strains in the nasopharynx, 2% to 52% of the men developed group-specific antibodies, depending on the serogroup examined.[527] This response does not eliminate the carriage state, but it may protect the host from overt disease. The best culture-based method of detection of meningococcal carriage is accomplished by swabbing the posterior wall of the oropharynx, followed by immediate inoculation and incubation of selective medium (e.g., modified Thayer–Martin [MTM] medium).[535]

Infections Caused by *N. meningitidis*. In some individuals, the meningococcal strain that becomes established in the upper respiratory tract goes on to enter the bloodstream and initiate systemic disease. A low level of serum bactericidal antibodies is a principal host factor associated with increased risk of infection, and it appears that invasive *N. meningitidis* disease occurs in those persons who are newly infected with a strain against which the individual lacks bactericidal meningococcal serogroup-specific antibodies.[229] In a study among military personnel, Edwards and coworkers[216] found that 86% of 31 patients had negative nasopharyngeal cultures during the 2 weeks before becoming ill, and that four patients were culture-negative the day before disease onset.[216] However, another case report documented a 7-week interval between nasopharyngeal acquisition of the organism and onset of meningeal disease.[475] Concurrent viral or mycoplasmal infection of the upper respiratory tract may also facilitate systemic invasion by the organism because both sporadic and epidemic outbreaks of meningococcal disease have been associated with outbreaks of viral and mycoplasmal respiratory tract infections.[463] Low socioeconomic status and minority status are associated with an increased risk of meningococcal disease, and the risk of meningococcal disease is also higher among those carriers with deficiencies in the terminal complement components (e.g., C5, C6, C7, C8, and C9) or the properdin system.[244,538] Other underlying conditions, such as hepatic failure, SLE, multiple myeloma, HIV infection, and asplenia may also predispose to serious meningococcal disease.[35,223] Newer behavioral risk factors associated with organism acquisition and

emergent meningococcal disease were brought into focus with college-based disease outbreaks include smoking (both active and passive), kissing, dormitory residence in colleges/universities, patronage of drinking establishments (bars, nightclubs), and sharing of drinking glasses/bottles.[164,249,322,323,424]

The pathogenesis of meningococcal disease is poorly understood and, although much has been reported on the descriptive epidemiology of the organism and its disease states, very little is known about the dynamics of disease production.[508,605,648] Humans are the only natural host for *N. meningitidis* and the organism is spread by respiratory droplets. Meningococci colonize the upper respiratory mucosa and may subsequently invade the bloodstream. Initial attachment to the nasopharyngeal mucosa is apparently mediated via interaction of cell surface adhesins with the I-domain of integrin-α chains or membrane cofactor protein (CD46).[344] A variety of cell surface adhesins are found in meningococci and include pilus proteins (PilC, PilQ), OMPs (Opa, Opc, PorA, PorB), and LOS.[451] Antigenic variation at the adhesin and pilus levels regulates the avoidance of nonspecific host factors during initial adherence to the epithelial mucosa. Environmental factors such as smoking and concomitant viral upper respiratory tract infections increase the risk of colonization due to alterations in the mucosa or abrogation of local immunity.[600] Initially, the polysaccharide capsule blocks the adhesins other than pili, but, subsequently, OMPs bind to the C46 receptor and Opa proteins bind the heparin sulfate proteoglycan receptors, respectively, on endothelial and phagocytic cells. This stimulates the release of cytokines by these cells, with subsequent endocytosis of the organisms by the epithelial cells.[210,605,648] PorB proteins subsequently become inserted into the membrane of the phagosome, preventing fusion with lysosomes.[453] In this way, meningococci can avoid humoral immune mechanisms and survive in mononuclear leukocytes. Meningococcal intracellular survival is determined by many factors, including IgA_1 protease, which degrades lysosome-associated membrane proteins, preventing phagosomal maturation and stimulating upregulation of capsule expression. In addition, intracellular replication is augmented by the capacity of meningococci to acquire iron through specialized iron transport systems (e.g., hemoglobin-binding receptor [HmbR], transferring-binding protein [TbpAB], lactoferrin-binding protein [LbpAB]).[511] The principal meningococcal virulence factor associated with bloodstream invasion is the polysaccharide capsule. Resistance to complement-mediated bacteriolysis and phagocytosis is determined by the expression of the capsule and the LOS present in the outer membrane.[343] In addition, certain class 1 OMPs of *N. meningitidis* are able to downregulate the expression of various complement receptors on neutrophils, thereby inhibiting ingestion of the organisms.[73] Bloodstream invasion by meningococci also correlates with the systemic release of a variety of inflammatory cytokines (e.g., interleukin-1, interleukin-6, tumor necrosis factor-α) that contribute to the

pathogenesis of meningococcemia and meningococcal septic shock.[665] Some of these cytokines may increase the permeability of the blood–brain barrier and allow entry of meningococci into the CSF. In addition, meningococcal invasion of endothelial cells with resulting edema and induction of apoptosis may also release organisms into the CSF and other closed spaces.

At the present time, *N. meningitidis* is the second leading cause of community-acquired meningitis in the United States, largely due to the availability and widespread use of highly efficacious *H. influenzae* and *S. pneumoniae* conjugate vaccines.[115,215] *N. meningitidis* is the most frequent cause of meningitis and sepsis in young adults the world over.[283,605] Clinical presentations of disease may be quite varied and include meningoencephalitis, meningitis with or without meningococcemia, meningococcemia without meningitis, and bacteremia without septic complications.[35,115] Initial symptoms are usually nonspecific and severe disease may supervene within 6 to 8 hours, so recognition of meningococcal syndromes by the clinician is key to successful patient management. The onset of acute meningococcal meningitis

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is sudden, with fever and chills, myalgias, and arthralgias.[115] The classical signs of meningitis, such as confusion, headache, fever, and nuchal rigidity, may be seen in only about 27% to 50% of patients ultimately diagnosed with meningococcal meningitis.[35,301] Nausea and vomiting may also be a part of the clinical presentation, particularly in children. Meningococcemia and widespread dissemination of the organism are heralded by the rapid development of a rash, which is seen in about 50% to 60% of patients. This rash starts as a pink, maculopapular eruption and then becomes petechial. Initially, these petechiae appear on the mucous membranes (e.g., conjunctivae, hard palate), and then spread to the trunk and the lower extremities. Lesions on the truck may be clustered near the beltline where pressure is applied to the body. Skin lesions are indicators of bleeding complications and coagulopathies that are caused by the organism. Fulminant, rapidly progressive disease may result in formation of additional macules and papules that progress to purpuric or ecchymotic areas of cutaneous hemorrhage and necrosis. About 10% of patients with meningococcemia develop purpura fulminans, resulting in extensive areas of tissue destruction secondary to coagulopathy; aggressive monitoring of coagulation parameters and replacement of coagulation factors (e.g., fresh frozen plasma, platelet transfusions) may benefit some of these patients.[4] Progression of these lesions may lead to peripheral gangrene of the digits that sometimes require amputation. Diffuse neurologic involvement, rather than focal signs and symptoms, and cardiac involvement occur more frequently with meningococcal meningitis than with other

types of bacterial meningitides. Cardiac abnormalities, particularly myocarditis and myocardial dysfunction, are seen in more than half of patients who succumb to meningococcal infection. Clinically, these abnormalities are manifested by congestive heart failure, pulmonary edema, and high central venous pressure.[35]Purulent pericardial effusion with and without cardiac tamponade are complications of meningococcal disease that are seen in both adults and children and usually develop during convalescence.[57,190,290,295,318,720] In one case report, sepsis with a group B meningococcus triggered an acute myocardial infarction in a 42-year-old man with normal coronary arteries.[245]

Fulminant meningococcal shock often dominates the clinical picture of meningococcal meningitis and acute meningococcal sepsis.[35] The patient becomes unresponsive, with absence of superficial and deep tendon reflexes and a depressed sensorium. The presence of shock, a low white blood cell count, a rash, and altered mental status on presentation are associated with a poor clinical outcome in these patients. Mortality from meningococcal septic shock is also associated with low serum potassium and a negative base excess, reflecting generalized metabolic abnormalities. Low platelet and serum CRP levels, which are associated with durations of petechiae and purpura, are predictive of impending disseminated intravascular coagulation (DIC).[380] In fatal cases, autopsies reveal terminal myocarditis and/or lesions of DIC, with microthrombi and thromboses observed in many organs. Avascular necrosis of bone due to thrombosis in intraosseus blood vessels secondary to DIC has also been documented in severe meningococcal disease in both children and adults.[107,571]The classic finding of acute hemorrhagic necrosis of the adrenal glands represents the anatomic hallmark of the Waterhouse–Friderichsen syndrome.[6,585] Despite the availability of excellent therapeutic agents, meningitis with sepsis caused by *N. meningitidis* still may have a mortality rate of up to 30%. Among survivors of infection, up to about 23.9% may develop some degree of sensorineural deafness.[383]

N. meningitidis may also cause bloodstream infection (meningococcemia) without meningitis.[35] This presentation occurs in 5% to 20% of patients with meningococcal disease. Patients with meningococcal bacteremia usually present with a fever, headache, malaise, hypotension, and peripheral leukocytosis. Symptoms of a respiratory tract infection may also be present, but meningeal signs and symptoms are not. Some patients may present with cutaneous lesions, including purpura.[314,358] Meningococci are recovered from blood cultures, but the patient may be clinically well by this time, and no therapy or a short course of therapy is administered. With chronic meningococcemia, the patient is generally symptomatic, with a low-grade fever, rash or purpura, and occasionally arthritis. This presentation is clinically very similar to the gonococcal arthritis–dermatitis syndrome.[542] This form of meningococcal disease, with or without meningitis, has been noted to recur in some patients who have

inherited underlying deficiencies in C3, properdin, C5, C8, and other late complement components.[244] Individuals with underlying conditions that are associated with hypocomplementeric states, such as SLE, are at risk for serious meningococcal disease, and chronic meningococcemia has also been documented in HIV-infected patients.[7,41,240]

N. meningitidis may also cause other infections, some of which result from hematogenous dissemination. Blood-borne spread of the organism may seed other internal organs, leading to complications such as septic arthritis, osteomyelitis, cellulitis, endophthalmitis, and spontaneous bacterial peritonitis.[264,396,430] Septic arthritis may occur along with meningitis or meningococcemia, but primary septic arthritis, with isolation of the organism from joint fluid, may occur without evidence of meningitis or meningococcemia.[34,79,446,449,680] Arthritis usually affects the knees and ankles, but any joint may be affected. Some articular symptoms may also be immune-mediated and both septic and immune-mediated arthritis may occur in the same patient.[70] Osteomyelitis is a rare manifestation of meningococcal infection, with only eight cases reported in the medical literature.[103] In these cases, bone invasion was first detected by radiographic studies and most often involved the long bones (e.g., femur, humerus, tibia). In cases of meningococcal osteomyelitis, blood cultures are usually positive. Soft tissue cellulitis is also a rare clinical presentation of hematogenous meningococcal dissemination and may be observed in otherwise normal hosts or patients with underlying diseases, including diabetes, obesity, pulmonary hypertension, congestive heart failure, and poor lower-extremity circulation.[289,353,518] This complication often involves the periorbital region, but may also involve the neck and the extremities. Involved areas are erythematous, swollen, warm, and tender, and blood cultures are usually positive. Patients with this clinical manifestation have ranged in age from 4 months to 83 years.[145,518] Endogenous endophalmitis is also a rare complication of meningococcal meningitis and meningococcemia.[51,143,713]Patients initially present with blurred vision or loss of visual

acuity and may or may not have systemic signs of sepsis when they present with vision problems. Vitreous specimens are often culture-positive, as are blood and/or CSF cultures, and intravitreal and systemic therapy with third-generation cephalosporins are indicated. Complications may include anterior uveitis and retinal detachment.[5,151] Occasionally, *N. meningitidis* may be isolated from distant body sites without other clinical evidence of meningitis, meningococcemia, or other foci of meningococcal infection. Meningococcal endocarditis is rare, with only 12 cases reported since 1960. Patients usually are elderly and have underlying disease (e.g.,

diabetes, complement deficiencies), and have preexisting valvular disease or prostheses, although endocarditis has been described in a healthy 13-year-old male with no cardiac abnormalities.[19,38,62] Lastly, *N. meningitidis* is one of the long list of organisms that have been recovered from patients with peritonitis and bacteremia complicating continuous ambulatory peritoneal dialysis.[162]

Acute meningococcal conjunctivitis has also been recognized as a distinct clinical entity. This infection may arise as a primary or exogenous infection, or as a secondary endogenous infection.[30, 225,297] Primary infection may be invasive or noninvasive. In invasive primary infection, the conjunctival infection occurs first, followed by the development of systemic disease. In primary, noninvasive disease, only conjunctival infection develops. Secondary endogenous infection occurs as a subsequent complication of systemic meningococcal disease.[28,515] Most cases of meningococcal conjunctivitis occur as a part of systemic infection with *N. meningitidis*, and systemic meningococcal disease associated with conjunctival infection develops in 10% to 18% of patients.[55,221] Primary conjunctivitis caused by *N. meningitidis* has been reported in neonates, older children, and adults.[248,494] In two-thirds of cases, the infection is limited to only one eye. In a review of 84 cases of primary meningococcal conjunctivitis, systemic meningococcal disease (i.e., meningitis or meningococcemia) developed in 17.8%; among these patients the mortality was 13.3%.[55] Systemic infection occurred more frequently among patients who received topical, rather than systemic, antimicrobial therapy; in fact, the risk of developing systemic disease was almost 20-fold greater for patients receiving topical therapy versus systemic therapy. Complications of the infection that were limited to the eye included corneal ulcers, hypopyon, keratitis, subconjunctival hemorrhage, and iritis.[713] In a report of three cases of primary meningococcal conjunctivitis in the United Kingdom, a younger sibling of the index case developed meningococcal meningitis, prompting the authors of this report to suggest that prophylaxis for close contacts of cases of primary meningococcal conjunctivitis is warranted.[599] Saperstein and colleagues also reported a case of exogenous meningococcal endophthalmitis, in which the organism entered the eye via a leaky filtering bleb following cataract surgery instead of via bloodstream dissemination.[558] In the reported cases of meningococcal endophthalmitis, this localized infection often presents without signs or symptoms of meningitis.[356,717]

Meningococcal pneumonia is infrequently seen, but presents primarily as a community-acquired pneumonia that is indistinguishable clinically from other acute bacterial pneumonias.[35,270,701] The pathogenesis of pneumonia may involve hematogenous dissemination or aspiration followed by direct invasion of the lung parenchyma. This infection occurs primarily in middle-aged to older persons with preexisting illnesses, including viral respiratory tract infections, chronic obstructive

pulmonary disease, coronary artery disease, diabetes, HIV infection, and SLE.[526,680,701] Patients usually present with classical signs and symptoms, including fever, dyspnea, cough, and consistent radiographic findings.[701] In a study of 68 military recruits with meningococcal pneumonia proven by culture of transtracheal aspirates, fever, rales, and lobar infiltrates were common findings.[379]The patients were moderately ill, but there were no fatalities. In a review of 58 cases of community-acquired meningococcal pneumonia over the last 25 years, blood cultures were found to be positive in 79.3%, although symptoms and sequelae associated with meningococcemia failed to develop in these patients.[701] Sputum cultures were positive in 15 (83.3%) of 18 cases in which sputum specimens were collected, and 5 of the 58 patients died from their infection. The diagnosis of meningococcal pneumonia is complicated by the presence of the organism in the nasopharynx, resulting in oropharyngeal contamination of expectorated sputum specimens. In addition to pneumonia, fulminant bacteremic supraglottitis has been reported in association with serogroup B, C, and Y meningococcal infections.[382,476,532,568,586] These infections were characterized by sore throat, dysphagia, swollen suproglottic tissues, and cervical cellulitis. Five of the six patients described with this condition required intubation or emergency tracheostomy, indicating that meningococcal supraglottitis is a fulminant, potentially life-threatening infection.

N. meningitidis may occasionally be isolated from the male urethra, the female genital tract, and the anal canal. In these sites, it may cause infections that are clinically indistinguishable from gonococcal infections, such as acute purulent urethritis, cervicitis, salpingitis, and proctitis.[147,348,417,495,540] Orogenital, anogenital, and oroanal sexual practices are believed to be responsible for the presence of meningococci in these anogenital sites.[655] Primary neonatal conjunctivitis due to *N. meningitidis* was described in a newborn delivered to a mother with endocervical meningococcal infection.[248] The meningococcal isolates obtained from the conjunctival culture of the baby, an endocervical culture from the mother, and an oropharyngeal isolate from the father of the baby were identical by pulsed-field electrophoretic analysis.

Meningococcal Prophylaxis and Meningococcal Vaccines. The risk of secondary meningococcal disease in close household contacts of a primary case of meningitis or meningococcemia is 500- to 800-fold greater than that of the general population. Close contacts include members of the same household, child care center contacts, and any person directly exposed to the patient's oropharyngeal secretions through kissing, mouth-to-mouth resuscitation, or endotracheal tube placement and management. Close contacts may also include individuals who frequently sleep and/or eat in the same dwelling with an index case. Individuals with intimate contacts within closed

populations, such as college dormitories, military barracks, and long-term-care facilities, are also candidates for meningococcal chemoprophylaxis. It is the standard of practice to provide

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chemoprophylactic antimicrobial therapy to these contacts as soon after exposure as possible. Ideally, antimicrobial prophylaxis should be administered within less than 24 hours after identification of the index case. Secondary cases of meningococcal disease among close contacts to an index case usually occur within 10 days of exposure. Prophylaxis administered 2 weeks or more after exposure is not effective in preventing secondary cases. During the 1940s and 1950s, sulfonamides were effective in eradicating the meningococcal carrier state and in preventing disease. However, during the 1960s, resistance to the sulfonamides developed in *N. meningitidis* strains. High doses of penicillin transiently eliminate meningococci from the nasopharynx, but the organisms rapidly reestablish themselves after treatment is discontinued. Currently, oral rifampin (adults, 600 mg q12h for 2 days; children less than 1 month of age, 5 mg/kg for 2 days; children older than 1 month of age, 10 mg/kg for 2 days) is administered to eradicate carriage, although this drug may fail to eradicate the organisms in 10% to 20% of carriers. Rapid emergence of rifampin resistance among meningococci has also been noted, even during the time of drug administration. Ceftriaxone, administered as a single intramuscular dose (children, 125 mg; adults, 250 mg) will also eradicate meningococcal carriage for about 2 weeks.[578]Azithromycin (500 mg single dose) has also been shown to eradicate meningococcal carriage in adults, but is not one of the agents recommended for chemoprophylaxis at this time.[268] Ciprofloxacin (adults, 500 mg single dose) and ofloxacin (400 mg single dose) have also been shown to be 95% effective in eliminating nasopharyngeal meningococcal carriage for anywhere from 2 to 5 weeks.[260,266,521] In 2008, three cases of meningococcal disease due to ciprofloxacin-resistant serogroup B strains were reported among residents of North Dakota and Minnesota during 2007 and 2008.[136,708] The first case was epidemiologically linked to a case that occurred in the same region in 2006, except that the particular strain lacked the *gyrA* mutation that confers resistance to fluoroquinolones. Consequently, ciprofloxacin should not be used for meningococcal chemoprophylaxis in regions of the United States where resistant isolates have been isolated and identified, and alternative agents should be used for chemoprophylaxis. A case of meningitis due to a ciprofloxacin-resistant serogroup A strain was also documented in a traveler from Italy who acquired the infection in New Delhi, India.[388]

Because the virulence of *N. meningitidis* is closely associated with the group-specific capsular polysaccharides of the organism, it has been possible to develop

vaccines to protect against meningococcal disease. Univalent group A and group C polysaccharide vaccines and a tetravalent vaccine incorporating group A, C, Y, and W135 capsular polysaccharide material are available. The latter tetravalent polysaccharide vaccine is called MPSV4 (Menomune-A, C, Y, W-135, meningococcal polysaccharide vaccine, 4 serogroups, Sanofi Pasteur, Inc., Swiftwater, PA).[273,398] The polysaccharides in MPSV4 are T-cell-independent antigens, meaning that they stimulate mature B-lymphocytes and not T-lymphocytes. Because of this, serogroup A and C polysaccharides and MPSV4 do not elicit an immunologic memory, so the immune response is not long lasting and does not engender an anamnestic response on subsequent challenge with the same polysaccharide antigen. The monovalent polysaccharide vaccines and MPSV4 elicit immune responses in older children and adults, but are poorly immunogenic in children less than 2 to 3 years of age. While the serogroup A polysaccharide induces a modest immune response in children as young as 3 years, the serogroup C polysaccharide is poorly immunogenic, particularly in children less than 2 years of age.[274,279]Polysaccharides of serogroups Y and W-135 are immunogenic in children over 2 years old and in adults.[284] In addition to the variability of the immune response, the duration of the response to these native polysaccharide vaccines is short-lived and antibody titers fall rapidly over time following immunization.[398] Among infants less than 12 months of age vaccinated with the group A polysaccharide, a detectable antibody response was maintained for only 12 months and, among children immunized at ages 12 to 17 months, titers were detectable for 2 years.[349] In a 1998 study from Montana, only 18% of 1-year-olds, 32% of 2-year-olds, and 50% to 60% of children aged 4 and 5 years produced bactericidal antibody titers in response to the serogroup C polysaccharide vaccine.[443] In addition, native serogroup A and C polysaccharide vaccines may induce immunologic hyporesponsiveness (tolerance) in both children and adults when two doses are given during the first 6 months of life.[86,278,425] MPSV4, in particular, has been widely used in vaccination programs, is safe and well-tolerated, and adverse reactions are relatively uncommon.

In January of 2005, MCV4, a tetravalent meningococcal polysaccharide conjugate vaccine, was licensed for use in the United States (Menactra, Sanofi Pasteur, Inc.), and, in January of 2010, a second MCV4 vaccine was licensed (Menveo, Novartis).[176,514] Both vaccines consist of serogroup A, C, Y, and W135 meningococcal capsular polysaccharides conjugated to diphtheria toxoid. Conjugation to this protein carrier enables the antigen to engender a T-cell-dependent instead of a T-cell-independent immune response. Consequently, the initial immune response to the antigen is more substantial and a strong anamnestic "immunologic memory" response occurs on reexposure to the antigen. In January 2005, the Advisory Committee on Immunization Practices (ACIP) licensed MCV4 for use in persons 11 to 55 years of

age, and, in May of 2005 recommended routine immunization with one dose of MCV4 for persons 11 to 12 years old and those entering high school (i.e., 15-year-olds) if not previously vaccinated.[131] The ACIP recommendations were revised again in June of 2007 to include routine vaccination of all individuals aged 11 to 18 years with a single dose of MCV4, with persons aged 11 to 12 years being vaccinated as recommended previously.[134] ACIP, along with the American Medical Association (AMA), the American Academy of Family Physicians, and the Society for Adolescent Medicine stressed that the ideal time for vaccine receipt for adolescents would be during the healthcare visit recommended for children aged 11 to 12 years. Vaccination was also recommended for persons aged 19 to 55 years who are at increased risk for meningococcal disease, including college freshmen living in dormitories, microbiologists working in clinical laboratories, military recruits, travelers to or residents of countries with endemic meningococcal disease, and those individuals with anatomic/functional asplenia or terminal complement component deficiencies. College

freshmen should be vaccinated with MCV4 before college entry if they have not been vaccinated previously.[131] The feasibility of targeting vaccinations on college campuses has led some schools to recommend that all incoming freshmen be vaccinated. CDC published another update in September of 2009, recommending that persons previously vaccinated with either MSPV4 or MCV4 who are at risk for disease should be re-vaccinated with MCV4.[137] If vaccinated at or after the age of 7 years, revaccination should occur 5 years later, and, if vaccinated at ages 2 to 6 years, revaccination should occur after 3 years. After review of data on vaccine immunogenicity in high-risk groups, persistence of bactericidal antibody following immunization, vaccine efficacy, and cost-effectiveness of various vaccination strategies, the ACIP published another update in 2010 recommending routine vaccination of adolescents at age 11 or 12 years, plus a booster dose at age 16 years.[141] This update also recommended a two-dose primary series of MCV4 administered 2 months apart for those persons aged 2 years through 55 years who have complement component deficiencies, functional/anatomic asplenia, and adolescents with HIV infection.

With the availability of effective conjugate vaccines for meningococcal serogroups A, C, Y, and W135, and the conjugate vaccine against *H. influenzae* type b, serogroup B *N. meningitidis* has become the major cause of bacterial meningitis worldwide. The meningococcal serogroup B polysaccharide capsule is comprised of a linear polymer of α2-8 *N*-acetylneuraminic acid (sialic acid).[622] This unique chemical structure and antigenic properties render it poorly immunogenic in humans. This lack of antibody

response has been attributed to the homologies of the group B polysaccharide to the polysialated form of the neural cell adhesion molecules found in fetal brain tissue.[247] Polysaccharide vaccines prepared from group B strains are poorly immunogenic in both children and adults. Efforts to develop group B vaccines have focused on the organism's OMP antigens. Immunity to group B OMPs is type-specific rather than group-specific, so any potential vaccine would have to include the OMPs of several group B serotypes and serosubtypes involved in disease production. Another approach that has been investigated is modification of the polysaccharide structure by replacing the *N*-acetyl groups of the sialic acid residues with *N*-propionyl groups and then conjugating this molecule to tetanus toxoid.[336] A short clinical trial evaluating the safety and immunogenicity of this candidate vaccine found that both immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies specific for the *N*-propionylated capsular B polysaccharide were produced and no antibodies that were reactive with sialic acid were detected.[97] However, the antibodies appeared to lack functionality (e.g., opsonic activity) in bactericidal assays. A similar approach that has been used in other studies is the synthesis of peptides that mimic hyper-immunogenic epitopes that are unique to the meningococcal serogroup B polysaccharide. Removal of *N*-acetyl groups from the sialic acid residues of the capsular polysaccharides resulted in molecules that still contained immunodominant epitopes of the serogroup B polysaccharide, and antibodies against this material did not cross-react with glycoproteins in cultured cell lines.[278,456] With all of these approaches, the concern remains that the similarities between the native serogroup B molecule and neuraminic acid residues in the central nervous system (CNS) may engender autoimmunity or interfere with fetal neural development.[247]

Much of the serogroup B vaccine work has concentrated on the use of outer membrane vesicle (OMV) preparations. OMVs are prepared from blebs of meningococcal outer membrane that are released during growth. The OMV are extracted with detergents to release LOS and endotoxicity. OMV preparations prepared from wild type, circulating meningococcal strains have been used for the control of epidemics in localized outbreaks involving specific serogroup B meningococcal clones.[257,310,311,351] These OMVs generally produced brisk antibody responses, particularly if the vaccine and disease strains were similar antigenically. The immune response to meningococcal OMV preparations is predominantly against the PorA cell wall outer membrane antigen that are known to be highly variable. In order to broaden the protection afforded by OMV vaccines, more than one serogroup B strain may be included or OMPs prepared from mutant strains that express more than one PorA antigen may be used for OMV preparation.[114,156,555] Characterization of these antigens by the Netherlands Vaccine Institute resulted in recombinant OMV formulations of various PorA preparations and the production of 8-valent ("Hexa-

Men") and 9-valent ("Nona-Men") group B meningococcal vaccines.[155,310,661] These vaccines were estimated to engender the production of anti-PorA antibodies for more than 70% of circulating serogroup B meningococcal strains. However, the principal drawback with the use of the PorA antigens from *N. meningitidis* is the hypervariability of this antigen over time. OMVs have also been prepared from of *N. lactamica* for possible use in meningococcal serogroup B vaccines.[276,277]*N. lactamica* strains lack the PorA protein but have a number of antigens in common with *N. meningitidis.* The safety and immunogenicity of an *N. lactamica* OMV vaccine was evaluated in a phase 1 placebo-controlled double-blind clinical trial.[278] This OMV vaccine was immunogenic, and produced rises in titers of IgG against the vaccine *N. lactamica* strains. A modest cross-reactive response to the vaccine was also elicited against six diverse serogroup B *N. meningitidis* strains. This IgG reacted with meningococcal OMVs, produced serum bactericidal antibodies, and elicited opsonophagocytic activity. Genetic manipulation of *N. meningitidis* by insertion of genes for candidate vaccine molecules into the organism has also been investigated. Inserted genes are hyper-expressed in the meningococcal strain, and OMV vaccines are prepared from the modified meningococcal strain expressing the selected antigen(s). Koeberling and colleague inserted the gene for the lipoprotein, cofactor H binding protein, into a meningococcal strain, and OVS preparations containing the expressed protein produced a functional immune response in animals, whereas the purified, recombinant antigen did not.[377] Several serogroup B meningococcal antigens have been investigated as vaccine candidates and, generally, most of them have shown disappointing results. In most cases, the antigens are not highly immunogenic and the antibodies that are produced do not function well in bactericidal assays. Assessment of vaccine efficacy is hampered by the lack of an animal model for human meningococcal disease. While measurement of in vitro correlates of antibody protection (e.g., the serum bactericidal activity assay) are used as surrogates for protective immunity, a number of

individual protective mechanisms are involved in the immune response to meningococcal infection and the development of overt meningococcal disease.

Other Neisseria *Species*

Neisseria lactamica is an organism that is of special concern because it is able to grow on selective media for gonococci and meningococci and must be differentiated from them. *N. lactamica* is found more frequently in the oropharynges of children than in adults.[49,116]This organism may be isolated from throat cultures of children in the

course of investigations of alleged sexual abuse, and *N. lactamica* must be differentiated from *N. gonorrhoeae* by the clinical laboratory. *N. lactamica* has been isolated as a rare cause of meningitis and sepsis in both adults and children; the adult case was associated with a fracture of the cribriform plate.[186,390] This organism has been associated with recurrent otitis media and with septicemia in a 7-year-old child during immunosuppressive therapy for acute lymphocytic leukemia.[493,565] *N. lactamica* also has been recovered from the female genital tract in association with a persistent vaginal discharge.[631] Recent case reports have described *N. lactamica* as a cause of cavitary lung disease in an organ transplant recipient, septicemia and arthritis in a patient with myeloma on immunosuppressive therapy, and bacteremic pneumonia in a patient with cirrhosis.[231,688,719] In the latter case, the isolate had reduced susceptibilities to penicillin (minimal inhibitory concentration [MIC] 0.75 μg/mL) and ciprofloxacin (MIC ≥ 0.5 μg/mL).*N. lactamica* may act as a natural immunogen against *N. meningitidis* due to the presence of antigens that cross-react with meningococcal strains. Anti-meningococcal bactericidal antibody titers increase steadily in infants despite low rates of meningococcal carriage in the same population.[116] This has provided indirect support for the idea that childhood carriage of *N. lactamica* may subsequently confer some protection against meningococcal disease. *N. lactamica* may be useful for inducing protective antibodies against meningococcal infection by whole-cell kill vaccines, OMV vaccines, or purified OMP vaccines.[64]

Neisseria subflava (biovars *flava*, *subflava*, and *perflava*), *N. mucosa*, *N. sicca*, *N. polysaccharea*, and *N. flavescens* have been reported most frequently as uncommon causes of native and prosthetic valve endocarditis.[21,29,302,407,415,582] With native valve endocarditis, cardiac structural abnormalities caused by past infections (e.g., rheumatic fever) or prior surgery are frequently present. In some reports, the use of intravenous (IV) drugs was a significant risk factor for endocarditis, because oral secretions may be used to dissolve drugs or to clean the skin before injection. Native valve endocarditis due to *N. mucosa* was described in a healthy 20-year-old woman after having her tongue pierced 1 month prior to the onset of symptoms.[642] In addition to endocarditis, these normally saprophytic organisms have been isolated from other significant infections. *N. subflava* has been isolated from joint fluid of a child with septic arthritis, and as a cause of bacteremia, meningitis, discitis, and vertebral osteomyelitis.[14,42,52,342,486,685] At the University of Illinois, a *N. subflava* bv. *perflava* strain was repeatedly recovered from urine cultures of a 10-year-old boy with congenital structural abnormalities of the urinary bladder.[331] *N. subflava* bv. *perflava* peritonitis has also been reported as a complication of chronic ambulatory peritoneal dialysis (CAPD).[678] *N. mucosa* has been isolated as an unusual cause of meningitis, pulmonary abscesses, ocular infections, septic arthritis/bursitis, crepitant cellulitis, dialysis-related bacteremia, and continuous ambulatory peritoneal dialysis-associated

peritonitis.[113,267,320,410,575,576,607] *N. sicca* has been isolated as a rare cause of pneumonia, bronchiectasis, meningitis in compromised hosts, CSF shunt infection, sinusitis, osteomyelitis, septic arthritis, Bartholin's gland abscess, endocarditis, and peritonitis in patients maintained on long-term peritoneal dialysis.[66,220,263,285,315,461,479,559,659] *N. subflava* bv. *perflava* and *N. sicca* have also been isolated from blood cultures of patients with end-stage HIV disease.[465] *Neisseria polysaccharea* is found in the upper respiratory tract of about 0.5% of individuals and has not been described as part of a pathologic process.[88,533] These saprophytic *Neisseria* species may present therapeutic dilemmas because some isolates may be penicillin resistant due to the presence of altered penicillin-binding proteins.[254] In these cases, the isolates must be identified to species, and antimicrobial susceptibility tests should be performed. Furthermore, intergenic recombination between the *penA* genes of these commensal *Neisseria* species and *N. gonorrhoeae* may be responsible for the emergence of mosaic *penA* genes in *N. gonorrhoeae* that encode altered penicillin-binding protein 2 (PBP 2). Gonococcal strains with altered PBP 2 have decreased susceptibility to intramuscular (IM) and orally administered (PO) cephalosporins used for treatment (see below).

Neisseria cinerea is a saprophytic species of the upper respiratory tract that is of particular interest because of its cultural resemblance to *N. gonorrhoeae*, its occasional recovery from genital sites, and its association with syndromes similar to those caused by gonococci, such as purulent conjunctivitis and proctitis.[91,207,209,371,374] *N cinerea* has also been recovered as the cause of nosocomial pneumonia in a patient with AIDS and cavitary lung disease in a renal transplant patient.[92,346] *N. cinerea* bacteremia has been documented in a child with pneumonia and otitis media, in an alcoholic male with intra-abdominal disease, in a patient receiving hemodialysis, and in a 17-year-old male following a fist fight in which he suffered significant facial trauma.[339,363,591] In the latter case, the organism was also recovered from the CSF. *N. cinerea* was described as the cause of tricuspid valve endocarditis in an IV-drug user and peritonitis associated with CAPD.[63,613]

The rod-shaped members of the genus *Neisseria* include the three subspecies of *N. elongata* (subsp. *elongata*, subsp. *glycolytica*, and subsp. *nitroreducens*), *N. weaveri*, and *N. bacilliformis*.[26,27,89,281,293,309] Up until 1990, *N. elongata* included only two subspecies, *N. elongata* subsp. *elongata* and *N. elongata* subsp. *glycolytica*. These organisms are normally found in the human upper respiratory tract. Neither of these subspecies had been implicated in human infections until 1995, when *N. elongata* subsp. *glycolytica* was isolated from wound specimens of three patients and from blood cultures of a 57-year-old man with subacute bacterial endocarditis and aortic insufficiency.[27] Hombrouck-Alet and colleagues used 16S rDNA sequencing to identify *N. elongata* subsp. *glycolytica* bacteremia in a neutropenic patient with

squamous cell carcinoma.[312] In 1996, Nawaz and colleagues reported endocarditis caused by *N. elongata* subsp. *elongata* that was

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complicated by a ruptured mycotic aneurysm of the right brachial artery, and this organism was subsequently documented as a cause of endocarditis in a 65-year-old female with type II diabetes.[36,473] *N. elongata* subsp. *nitroreducens* was formerly called CDC group M-6.[281] This "*Moraxella*-like" bacterium was shown to be related to the *Neisseria* species, in general, and to *N. elongata* subspecies, in particular, on the basis of genetic studies, cellular fatty acid composition, and phenotypic properties. *N. elongata* subsp. *nitroreducens* is found normally in the oropharynx of humans and has been reported as an opportunistic agent of bacteremia, native and prosthetic valve endocarditis, and as a cause of osteomyelitis following oral surgery.[208,230,307,316,317,452,704] This organism has also been isolated from urine and appendiceal tissue.[281] *Neisseria weaveri*, a rod-shaped organism that was formerly called CDC group M-5, is part of the upper respiratory tract flora of dogs and cats and may be isolated from human wounds and blood cultures associated with animal bites.[26,111,309] In 2002, *N. weaveri* was isolated from bronchial washings and sputum of a 60-year-old male with bronchiectasis and from a wound infection in a 7-year-old girl following a tiger bite.[108,507] In 2010, *N. weaveri* was identified as a cause of peritoneal dialysis-associated peritonitis.[376] *N. bacilliformis* was first described in 2006.[293] This species has been isolated from a submandibular wound in a patient undergoing a mandibulectomy, sputum specimens from two patients with brain lymphoma and lung cancer, respectively, and a lung abscess in a patient with lymphoma. *N. bacilliformis* was subsequently isolated from blood cultures of a 47-year-old male with bicuspid aortic valve endocarditis.[444]

N. animaloris and *N. zoodegmatis* are the new species designations for the former CDC group EF-4a and EF-4b, respectively.[660] These organisms are found in the oral cavities of dogs and cats and are rare isolates from bite wounds associated with these animals. *N. canis*, *N iguanae*, and *N. dentiae* are animal strains isolated from the oral cavity and upper respiratory tracts of cats, guinea pigs, iguanae, and cows, respectively.[56,589] *N. canis* has been recovered from human wounds resulting from cat bites.[287,552] *N. wadsworthii* and *N. shayeganii* are new species that were isolated from archived human clinical specimens.[702]These organisms are most closely related to *N. bacilliformis*, *N. canis*, and *N. dentiae*.

Clinical Significance of *Moraxella catarrhalis*

M. catarrhalis has received much attention as an emerging human pathogen over the last two decades.[347] For many years, *M. catarrhalis* was believed to be part of the normal human upper respiratory tract flora, but studies by Vaneechouette and colleagues and Knapp and Hook showed that this organism is found in the upper respiratory tracts of only 1.5% to 5.4% of healthy adults and is more common in the respiratory tracts of healthy children (50.8%) and elderly adults (26.5%).[370,670] A Danish study also found that *M. catarrhalis* was not a significant member of the nasopharyngeal flora of adults and was rarely present in children less than 1 month of age.[219] However, 36% of children aged 1 to 48 months had *M. catarrhalis* as a part of their respiratory tract flora, and, in this same age group, the prevalence of *M. catarrhalis* in children with respiratory tract infections was 68%. A study of nasopharyngeal colonization with *M. catarrhalis* during the first 2 years of life showed that 66% of 120 serially cultured children became colonized during the first year and that 77.5% were colonized by the end of the second year.[232] A study conducted among newborns and infants in Gambia, West Africa, found a 70% prevalence of *M. catarrhalis* carriage; carriage was about 57% during the first week of life and gradually increased to above 80% by 21 weeks of age.[384]Among healthy adults, the colonization rate of *M. catarrhalis* is about 4%, which correlates with the lower infection rates seen in adults compared with children. When isolated from adults with respiratory tract disease, the organism is more frequently found in specimens that represent lower respiratory tract secretions than in specimens with oropharyngeal contamination. Increased rates of respiratory tract colonization in adults are associated with chronic bronchitis, bronchiectasis, and chronic obstructive pulmonary disease.[469] Among adults with chronic airway disease who were followed over a 27-month period, Klingman and colleagues found that 42.9% were colonized with *M. catarrhalis* and that each patient was colonized with one to four different strains, each strain persisting for about 2 to 3 months.[367]The more frequent recovery of this organism from the respiratory tracts of children and elderly patients with chronic respiratory disease supports its role in certain childhood infections and in lower respiratory tract infections in older individuals.

Infections of the respiratory tract and adjacent anatomic areas account for the majority of clinical conditions involving *M. catarrhalis* as the etiologic agent. These infections include otitis media, sinusitis, bronchitis, and pneumonia.[347,469,672] Although otitis media caused by this organism may occur in any age group, most studies have centered on this organism's role in pediatric infection. *M. catarrhalis* causes about 15% to 20% of acute otitis media, based on cultural and molecular studies of carefully collected middle ear fluid specimens.[360,547] In a study by Van Hare and coworkers, *M.*

catarrhalis was the only bacterial pathogen isolated from middle ear fluid of 40 (11%) of 355 children with acute otitis media and was co-isolated with either *Haemophilus influenzae* or *Streptococcus pneumoniae* in 21 (6%) of the patients. In otitis-prone children with otitis media with effusion (presence of fluid in the middle ear without clinical signs of acute otitis media), *M. catarrhalis* has been detected by culture and molecular techniques in up to 6% of aspirated middle ear effusion specimens.[303,304] Serial cultural studies of otitis-prone children over the first 2 years of life have also shown that these children have consistently higher rates of colonization with *M. catarrhalis* than other children. The bacteriologic findings with acute otitis media are mirrored in studies of acute sinusitis in the same age groups. In carefully collected maxillary sinus aspirates from children with acute sinusitis, *M. catarrhalis* may be isolated in either pure or mixed cultures from 2% to 16% of patients.[94] Acute sinus infections in adults have also been associated with this organism but with less frequency than in children. Despite its involvement in otitis media and sinusitis in children, *M. catarrhalis* is an uncommon cause of community-acquired lower respiratory tract infection in this age group.[611] In children with pneumonia, *M. catarrhalis* may behave as a

primary pathogen or as a secondary pathogen superimposed on an antecedent viral infection (e.g., respiratory syncytial virus). *M. catarrhalis* pneumonia has been reported in immunocompromised children and those with chronic underlying lung disease.[381] On rare occasions, *M. catarrhalis* may cause fulminant tracheitis and lower respiratory tract disease in apparently healthy children.[580]

Lower respiratory tract infections due to *M. catarrhalis* in adults occur predominantly in elderly and immunocompromised patients, particularly individuals with chronic obstructive pulmonary disease (COPD), bronchiectasis, congestive heart failure, and predisposition to aspiration.[447,469] Immunologic abnormalities related to underlying diseases (e.g., diabetes, alcoholism, HIV infection, transplantation) are also important contributory factors.[13,432,570]Exacerbations of COPD due to *M. catarrhalis* are associated with dyspnea, increased production of purulent sputum, constitutional symptoms, and fever. The role of *M. catarrhalis* in these exacerbations is supported by the presence of *M. catarrhalis* in cultures of the distal airways (i.e., bronchoscopy, tracheal aspiration) and demonstration of new *M. catarrhalis* strain acquisition coincident with the acute increase in respiratory symptoms.[460,573,590] These patients also develop an antibody response against the newly acquired *M. catarrhalis* strain in the lower airways and will have elevated markers of airway inflammation associated with acquisition of the new strain.[50] In pediatric patients, *M. catarrhalis* bacteremia

can occur secondary to otitis media, sinusitis, or pneumonia and usually is seen in those infants with sickle cell disease, leukopenia, and HIV infection.[3,611,635] In a review of 17 children with *M. catarrhalis* bacteremia, most of the children were less than 2 years old, 82.3% of the children had no underlying disease, and 76.5% had concomitant lower respiratory tract infections.[9] In children, *M. catarrhalis* bacteremia may also result from periorbital and preseptal cellulitis, a presentation long associated with *H. influenzae* type b sepsis.[635] In adult patients, *M. catarrhalis* bacteremia usually occurs as a complication of respiratory tract infection in those who are immunocompromised due to acute lymphoblastic and myeloid leukemia, lymphoma, AIDS, hypogammaglobulinemia, sickle cell disease, and congenital neurologic abnormalities.[78,325,447,609,635] *M. catarrhalis* bacteremia associated with infection of a prosthetic vascular graft was reported in 2010 in a 53-year-old man diagnosed with aortic dissection and hypertension.[556] In a review of 53 cases of *M. catarrhalis* bacteremia, neutropenia and/or malignancy was present in 30.2% of patients and 24.5% had underlying respiratory tract disease.[325] However, 28.3% of the cases occurred in immunocompetent individuals. Bacteremia in adults may result from a primary focus, such as sinusitis or pneumonia, but in immunocompromised patients, the portal of entry may not be apparent.[635] Nosocomially-acquired pneumonia due to *M. catarrhalis* may occur in hospital respiratory units and pediatric intensive care units.[531]

M. catarrhalis has been involved in several other types of infections, including endocarditis, meningitis, ocular infections, urogenital tract infections, wound infections, septic arthritis, and continuous ambulatory peritoneal dialysis-associated peritonitis. Cases of endocarditis due to *M. catarrhalis* are rare, with only five cases reported through 2000. Endocarditis has been documented in individuals with and without prior valvular disease and has occurred as a complication of invasive procedures (e.g., balloon angioplasty).[557,603] Rare cases of *M. catarrhalis* meningitis and ventriculitis have occurred spontaneously in previously well patients, following surgical procedures involving the head and neck, or secondary to infected ventriculoperitoneal shunts or external ventricular drains.[337,472,546] Viagappan and colleagues reported an unusual case of *M. catarrhalis* brain abscess in a 36-year-old male following a penetrating orbital injury with a pool cue and fatal neonatal meningitis was described in a 26-day-old infant 2 weeks after undergoing abdominal surgery.[171,679] Conjunctival infections caused by *M. catarrhalis* have been documented during the neonatal period and later in childhood, and endophthalmitis secondary to cataract or glaucoma filtering surgery and periorbital cellulitis with sepsis have also been described in patients.[67,225,640] Ophthalmia neonatorum caused by *M. catarrhalis* results either from acquisition of the organism at birth from the mother's

colonized genital tract or from respiratory tract secretions of the child's caretakers. Recovery of this organism from the male or female genital tract is rare, but *M. catarrhalis* has been reported as a cause of a gonorrhea-like urethritis following oral–genital sexual contacts in a few cases.[2] Septic arthritis of both native and prosthetic joints caused by *M. catarrhalis* has been reported in patients with rheumatoid arthritis being treated with immunosuppressive therapies, including injected and oral steroids, methotrexate, and infliximab.[397,492] Peritonitis due to *M. catarrhalis* has also been described in patients who are undergoing continuous ambulatory peritoneal dialysis.[163,677]

Isolation of *Neisseria* Species

Neisseria gonorrhoeae

Direct Gram-Stained Smears. In sexually transmitted disease clinics, the diagnosis of gonococcal urethritis in adult males is frequently made by the observation of gram-negative diplococci within or closely associated with polymorphonuclear leukocytes (PMNs) on a smear prepared from the urethral discharge (see Image Plate 11-1A). When properly performed, the Gram stain has a sensitivity of 90% to 95% and a specificity of 95% to 100% for diagnosing genital gonorrhea in symptomatic men.[366] In females, Gram stains of endocervical specimens collected under direct visualization of the cervix (i.e., with a speculum in place) may also be very helpful in diagnosis (see section on specimen collection). Gram-stained smears of such specimens have a sensitivity of 50% to 70%, depending on the adequacy of the specimen and the patient population. An endocervical smear showing gram-negative intracellular diplococci, particularly from a woman with other signs and symptoms of gonococcal infection, is highly predictive. In asymptomatic women, however, the predictive value of the Gram stain is much lower. In patients with symptomatic proctitis, smears collected under direct visualization through an anoscope may provide a diagnosis in 70% to 80% of such patients, as opposed to blind collection, where Gram-stained smears have a sensitivity of only 40% to 60%.[700] Because of the presence of other gram-negative coccobacilli and bipolar staining bacilli

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in rectal and endocervical specimens contaminated with vaginal secretions, care must be taken not to overinterpret smears obtained from these sites. Gram-stained smears have no value in the diagnosis of pharyngeal gonococcal infection. Gram-stained smears should not be relied upon for diagnosis of gonorrhea and should be used

adjunctively along with more specific tests.

Direct smears for Gram's stain should be prepared from urethral and endocervical sites and should be collected with a separate swab. For smear preparation, the swab is rolled gently over the surface of a glass slide in one direction only. This technique minimizes distortion and breakage of PMNs and preserves the characteristic appearance of the microorganisms (Image Plate 11-1A). Smears prepared from specimens submitted in transport media may be more difficult to interpret due to distortion of the PMNs or to interfering substances (e.g., charcoal). Smears from normally sterile or minimally contaminated sites (e.g., joint fluid, skin lesions) should also be prepared.

Specimen Collection and Transport. As with other pathogenic microorganisms, successful isolation depends on the collection of proper specimens, and this is particularly important for the recovery of *N. gonorrhoeae*. Because this organism can cause infection at a variety of body sites, collection of appropriate specimens for culture and diagnosis is dependent on the sex and sexual practices of the patient and on the clinical presentation. In all cases, specimens from genital sites (male urethra, female endocervix) should be collected. If the patient has a history of orogenital or anogenital sexual contacts, collection of oropharyngeal or anal canal specimens is also appropriate. In suspected cases of DGI, blood cultures and specimens from genital and extragenital sites should be obtained. Appropriate sites for culture are summarized in Box 11-2.

Specimens should be collected with Dacron or Rayon swabs. The calcium alginate used to prepare some swab lots may be toxic to gonococci.[391] Cotton swabs may also be used; however, some brands of cotton contain fatty acids that may be inhibitory for gonococci. Therefore, calcium alginate and cotton swabs should be used only if the specimens are inoculated directly onto growth media or are transported in nonnutritive transport media. Some transport medium formulations contain charcoal to inactivate toxic materials present in the swab material or in the specimen itself. Instruments used to aid in the proper collection of specimens (e.g., vaginal specula) should be lubricated with warm water or saline because various water- and oil-based lubricants may also inhibit organism growth. Box 11-3 describes collection procedures for the recovery of *N. gonorrhoeae* from different anatomic sites.

The role of the clinical microbiology laboratory in diagnosing gonococcal infections in children is crucial and involves the proper handling of appropriately collected specimens and the accurate identification of isolated organisms.[58,291,292,423] Culture is the recommended method for detecting *N. gonorrhoeae* in urogenital, pharyngeal, or rectal swab specimens. Nonculture tests (e.g., probe-based direct detection methods, nucleic acid amplification tests) have not been recommended for testing either victims or alleged assailants implicated in sexual assaults.[126,128] In the case of prepubertal

females, specimens should be obtained from the vagina, the oropharynx, and the rectum and inoculated onto media as described below. Cervical specimens are not recommended for prepubertal girls. Vaginal specimens are collected by swabbing the vaginal wall for 10 to 15 seconds to absorb any secretions, or, if the hymen is intact, the specimen is collected from the vaginal orifice. For boys with a urethral discharge, a meatal discharge specimen is an adequate substitute for an intraurethral swab specimen. Specimens for diagnosis of rectal, urethral, or oropharyngeal gonococcal infections in children are collected as for adults. Because of the legal implications of a diagnosis of *N. gonorrhoeae* infection in a child, only standard culture procedures should be performed.[140] Gram's stains are inadequate to evaluate prepubertal children for gonorrhea and should not be used to diagnose or exclude gonorrhea. Specimens from the vagina, urethra, pharynx, or rectum should be inoculated onto selective media for isolation of *N. gonorrhoeae* as described below.

Although maximal recovery of gonococci is obtained when specimens are plated directly onto growth medium after collection, this technique might not always be possible or practical, particularly in busy clinics or hospital emergency rooms.[414] For these situations, various transport systems are available:

Nonnutritive Swab Transport Systems. Stuart's or Amie's buffered semisolid transport media are used for transport of swab specimens for *N. gonorrhoeae*. Some swab transport systems use sponges soaked with transport medium, while others use a semisolid medium with or without activated charcoal. Semisolid transport media are superior to devices that use medium-soaked sponges. Sponge material in some swab transport systems may contain substances (e.g., sulfur or quaternary ammonium compounds) that can inhibit or injure fastidious organisms such as gonococci. Some swab transport systems, such as the Copan Diagnostics M40

BOX 11-2

Body Sites to Culture for *Neisseria gonorrhoeae*

Patient	Primary Site(s)	Secondary Site(s)
Female	Endocervix	Rectum, urethra, pharynx
Male, heterosexual	Urethra	Pharynx
Male, homosexual/bisexual	Urethra, rectum, pharynx	
Female, disseminated infection	Blood, endocervix, rectum	Pharynx, skin lesions,[a] joint fluid[b]
		Pharynx, rectum, skin

lesions,[a] joint fluid[b]

[a] If present
[b] Culture if arthritis is present

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BOX 11-3

Specimen Collection Procedures for Diagnosis of Gonococcal Infections

Specimen	Collection Procedure
Male urethra	Purulent discharge may be expressed by stripping the penis anteriorly and collecting the material on a swab. Specimens from asymptomatic males are obtained by inserting a calcium alginate nasopharyngeal swab 2–4 cm into the urethra. The swab is gently rotated as it is withdrawn. For nonculture specimens, the swab supplied or specified by the manufacturer should be used.
Endocervix	After the speculum is in place, a sponge, swab, or gauze should be used to remove any cervical mucus or discharge. Insert the swab 1–2 cm into the cervical canal and rotated against the wall of the endocervical canal two or more times, with a gentle side-to-side motion. Allow time for the organisms to adsorb onto the swab surface. Sample any cervical discharge that may be present. The swab should be withdrawn without touching any vaginal surfaces and placed in the appropriate transport medium. Nonculture specimens should be obtained as directed by the test manufacturer in the package insert.
Rectum	Insert the swab 4–5 cm into the anal canal and gently move it from side to side to sample the anal crypts. Allow a few seconds for the organisms to adsorb onto the swab, and gently rotate the swab during withdrawal. If heavy fecal contamination is observed on the swab, collect another specimen with a fresh swab.
Oropharynx	With the aid of a tongue depressor, firmly swab the tonsillar areas and the posterior pharynx.
	After venipuncture, inoculate suitable blood culture media (tryptic soy broth,

Blood	After venipuncture, inoculate suitable blood culture media (tryptic soy broth, Columbia broth) containing SPS. If SPS Vacutainer tubes are used for blood collection, transfer the blood specimen from the tube into blood culture medium as soon as possible, because exposure to high concentrations of SPS may be inhibitory to gonococci.
Joint fluid	Joint fluid should be aspirated with a needle and syringe and inoculated into an aerobic blood culture bottle.
Skin lesions	Punch biopsy specimens are collected and placed in a sterile container with a small amount of broth or sterile saline and hand-carried to the laboratory.
Conjunctivae	Collect conjunctival discharge from the inner aspect of the lower eyelid with a small nasopharyngeal swab. Prepare smears for Gram's stain as described in the text.

Transystem, have consistently outperformed other swab transport systems (e.g., Medical Wire and Starplex Scientific) in studies addressing survival of gonococci over time.[212,282] Studies with these newer swab collection devices (Copan Transystems, Copan Diagnostics, Inc., Corona, CA; now marketed as BBL CultureSwab Plus, BD Diagnostics) suggest that semisolid Amie's transport medium with or without charcoal may preserve the viability of gonococci for as long as 48 hours, although the viability of many gonococcal isolates may decrease noticeably after 24 hours. The new Copan Eswab system with liquid Amie's transport medium (Copan Diagnostics, Inc., Corona, CA) has been shown to have equivalent or superior performance to other swab transport systems (e.g., CultureSwab MaxV [Becton–Dickinson], Remel BactiSwab [Remel]) in maintaining survival of *N. gonorrhoeae* during transport. These nylon-flocked Eswabs provide acceptable recovery of *N. gonorrhoeae* after 24 hours of storage at room temperature and they fulfill the acceptance criteria as described in the Clinical Laboratory Standards Institute (CLSI) M40-A quantitative elution method for validation.[158,673] The Eswab transport system has also been demonstrated to enhance detection of *N. gonorrhoeae* and *C. trachomatis* by nucleic acid amplification assays.[148] While some studies have shown that gonococci may survive refrigeration in some swab transport systems up to 48 hours, other studies have demonstrated that refrigeration beyond 6 hours may result in significant reductions in viable organisms, regardless of the transport system used.[37,212,319] In order to prevent loss of organism viability, swab specimens submitted in transport medium should not be refrigerated and should be inoculated onto growth medium within 6 hours after collection.

Culture Media Transport Systems. Transport of specimens on culture media presents certain advantages and several systems for this purpose are commercially

plates containing various formulations of selective media (Remel; BD Diagnostic Systems, Sparks, MD), the Gono-Pak system (BD Diagnostic Systems), and the InTray GC system (BioMed Diagnostics, Inc., White City, OR).[24,69,191,441] The JEMBEC and Gono-Pak systems are available with either MTM or Martin–Lewis (ML) selective medium from both BD Diagnostic Systems and Remel Laboratories. While the Gono-Pak and JEMBEC systems require refrigerated storage prior to use, the sealed InTray system permits storage of the medium at room temperature for up to 1 year. With these systems, media are inoculated with the specimen and placed in an impermeable plastic bag with a bicarbonate–citric acid pellet. Contact of the pellet with moisture (via evaporation from the medium [JEMBEC] or via crushing an ampoule of water adjacent to the pellet [Gono-Pak]) generates a CO_2-enriched environment within the bag. Incubation for at least 18 to 24 hours at 35°C prior to transport to a reference laboratory allows initial outgrowth of the organisms and minimizes the loss of viability that may be encountered with swab transport systems.

Selective Culture Media: Inoculation and Incubation. A variety of enriched selective media for culture of *N. gonorrhoeae* are available and include MTM medium, ML medium, GC–Lect medium (BD Diagnostic Systems), and New York City (NYC) medium. MTM, ML, and GC–Lect are chocolate agar-based media that are supplemented with

TABLE 11-1 Antimicrobial Agents in Selective *Neisseria* Media

Antimicrobial Agent	Media Formulation (μg/mL)			
	MTM	ML	NYC	GC–LECT
Vancomycin	3	4	2	2
Lincomycin	—	—	—	1
Colistin	7.5	7.5	5.5	7.5
Nystatin	12.5	—	—	—
Anisomycin	—	20	—	—
Amphotericin B	—	—	1.2	1.5
Trimethoprim	5	5	5	5

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	MTM	ML	NYC	GC–LECT
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Lincomycin	—	—	—	1
Colistin	7.5	7.5	5.5	7.5
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Anisomycin	—	20	—	—
Amphotericin B	—	—	1.2	1.5
Trimethoprim	5	5	5	5

growth factors for fastidious microorganisms, whereas NYC medium is a clear peptone–corn starch agar-based medium containing yeast dialysate, citrated horse plasma, and lysed horse erythrocytes.[237] These media contain antimicrobial agents that inhibit other microorganisms and allow the selective recovery of *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* (Table 11-1). Vancomycin and colistin, antimicrobials present in all four formulations, inhibit gram-positive and gram-negative bacteria (including saprophytic *Neisseria* species), respectively. GC–Lect agar (BD Diagnostic Systems) also contains lincomycin. Trimethoprim is added to inhibit the swarming of *Proteus* species present in rectal and, occasionally, in cervicovaginal specimens. Nystatin, amphotericin B, or anisomycin is added to inhibit yeasts and molds. These media allow selective recovery of *N. gonorrhoeae* from body sites harboring a large endogenous bacterial flora. NYC medium will also support the growth of genital mycoplasmas and ureaplasmas. These media are commercially available in either petri dishes or JEMBEC plates. The formulas for these media are available in general media references.

Commercially available selective media vary in their ability to support the growth of pathogenic *Neisseria* species and to inhibit growth of nonpathogenic *Neisseriae* and other contaminants. Failure of these various selective media to support gonococcal growth may also be due to the susceptibility of some *N. gonorrhoeae* strains to vancomycin. These strains account for a variable percentage of isolates depending on the geographic area. Because of this, it is prudent to inoculate male and female genital specimens onto both selective (e.g., MTM) and nonselective (chocolate agar) media. Inoculation of nonselective media enables the recovery of other possible genital tract

jars are used, candles should be made of white wax or bees' wax; scented or colored candles release volatile products during burning and extinction that may inhibit the growth of the organisms. Plates are inspected at 24, 48, and 72 hours before a final report of "no growth" is issued. Suspect colonies are subcultured to chocolate agar, incubated, and used as inoculum for identification procedures.

Neisseria meningitidis

Specimen Collection and Transport. Specimens helpful in the diagnosis of meningococcal disease include CSF, blood, aspirates and biopsy specimens, and nasopharyngeal and oropharyngeal swabs.[491] Occasionally, meningococci may be sought in sputum and tracheal aspirates. Procedures for collection and processing of specimens that may yield *N. meningitidis* are presented in Box 11-3. Genital, rectal, and oropharyngeal isolates of *N. meningitidis* may be recovered using the collection and inoculation procedures described for *N. gonorrhoeae* in Box 11-2. Incubation conditions for media inoculated with specimens are the same as those described for *N. gonorrhoeae* above. Meningococci grow well on all selective media for the pathogenic *Neisseriae* and vancomycin-susceptible strains have not been described. Contrary to widely held beliefs, most strains of *N. gonorrhoeae* will grow on commercially available sheep blood agar, albeit not as well as on chocolate agar. Recovery of both gonococci and meningococci from blood cultures may be adversely affected by the anticoagulant sodium polyanethol sulfonate (SPS) that is present in blood culture media.[224]This effect may be neutralized by addition of sterile gelatin (1% final concentration) to the media or by processing the blood specimen by lysis-centrifugation (i.e., Isolator).[502,569] Joint fluids from patients with suspected meningococcal arthritis should be inoculated into aerobic blood culture bottles and, if sufficient volume is obtained, an aliquot of joint fluid should be cytocentrifuged and examined by Gram stain.[491]

Laboratory Safety. Using postings on selected e-mail clinical microbiology discussion groups, Sejvar and colleagues obtained data regarding reports of laboratory-acquired meningococcal infections from 1985 through 2001 and identified 16 cases (9 cases due to serogroup B and 7 due to serogroup C *N. meningitidis*).[572] Based on these data, an attack rate of 13 cases per 100,000 microbiologists was calculated, as compared to an attack rate of 0.3 case per 100,000 in the general population. This represents more than a 40-fold increased risk of disease among clinical microbiology personnel.*N. meningitidis* is classified as a biosafety level 2 organism, which means that a biologic safety cabinet must be used for the manipulation of specimens that

meningococcal arthritis should be inoculated into aerobic blood culture bottles and, if sufficient volume is obtained, an aliquot of joint fluid should be cytocentrifuged and examined by Gram stain.[491]

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have a substantial risk for the generation of aerosols (e.g., grinding, centrifuging, vortex mixing).[653] Such manipulations include the preparation of heavy organism suspensions for inoculation of identification systems and for serogrouping of meningococcal isolates by slide agglutination.[25] Reports of laboratory-acquired meningococcal infections suggest that manipulation of cultures, rather than specimens, increases the risk of infection for microbiology laboratory technologists and technicians.[44,359,583] The use of a biologic safety cabinet when manipulating cultures for these purposes would ensure protection of the laboratorian from aerosolized organisms. Alternative measures for protection (e.g., safety glasses, splash guards, masks) may afford added protection. Education and adherence to established laboratory safety precautions should minimize the risk of meningococcal infections for clinical microbiology laboratory personnel. Laboratory policies should also be developed for situations that may require administration of prophylaxis to employees who are exposed to meningococci. Laboratory administrators currently offer the quadrivalent meningococcal vaccine to microbiology laboratory staff as this measure would decrease but not eliminate the potential risk of laboratory-acquired meningococcal infections.[127,131,141]

Direct Gram-Stained Smears and Direct Capsular Antigen Tests. A rapid presumptive diagnosis of meningococcal meningitis can be made by direct examination of CSF using the Gram stain. If sufficient (i.e., more than 1 to 2 mL) CSF is received, the specimen should be centrifuged to obtain a pellet of material for examination and culture. Cytocentrifugation of CSF specimens enhances the detection of small numbers of organisms and increases the sensitivity of the Gram stain in comparison

coagglutination tests, MLK Diagnostics AB, Sollentuna, Sweden). A negative test does not rule out meningitis caused by any of the organisms that commonly occur. In addition, false-positive latex agglutination tests may also occur, particularly with urine specimens, resulting in unnecessary treatment and prolonged hospitalization. These tests should always be performed in conjunction with a cytocentrifuged Gram's stain and culture on enriched agar media. Due to the enhanced sensitivity of the Gram's stain provided by specimen cytocentrifugation and the problems with the specificity of the antigen detection assays, most laboratories in the United States no longer perform these tests routinely and they are not recommended.

Isolation and Incubation. For recovery of *N. meningitidis,* CSF specimens should be cultured on nonselective chocolate agar and sheep blood agar as described in Box 11-4, whereas specimens that may harbor other organisms (e.g., oropharyngeal and nasopharyngeal swab specimens) should be inoculated onto both selective (e.g., MTM, ML, NYC, or GC–Lect agar) and nonselective media. Plates are incubated in 5% to 7% CO_2 at 35°C (CO_2 incubator or candle extinction jar) and inspected after 24, 48, and 72 hours before a final report of "no growth" is issued. Suspicious colonies are subcultured to blood and chocolate agar for further identification.

Identification of *Neisseria* Species

Colony Morphology

Gonococci produce several colony types in culture. In Kellogg's scheme, these types are termed T1 through T5 and are described in terms of colony size and other colonial characteristics (coloration, topography of the colonies, reflection of light, and so forth). On the individual cellular level, organisms comprising colony types P^+ and P^{++} (formerly T1 and T2, respectively) possess pili on the cell surface, whereas cells in colony type P^- (T3, T4, and T5) lack pili. Isolates obtained on primary cultures are predominantly of the P^+ and P^{++}colony types. These colonies tend to be small, glistening, and raised (Image Plate 11-1C and 11-1D). With subculture of individual piliated colonies, the culture can be maintained in this colonial type. Organism suspensions prepared from 18- to 24-hour cultures containing primarily P^+ and P^{++} colony types tend to be smooth and homogeneous. With nonselective subculture (i.e., a "sweep" of growth), the other colony types will become more evident, with all colonies eventually becoming the nonpiliated varieties. These types are larger, flatter, and do not have the characteristic glistening highlights of the piliated colony types. Cultures containing predominantly the large-colony types frequently do not form smooth suspensions, as the colonies become gummy owing to autolysis and release of

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BOX 11-4

Specimen Collection Procedures for Isolation of *Neisseria meningitidis*

Specimen	Collection Procedure
Cerebrospinal fluid	In cases of suspected meningococcal meningitis, as much spinal fluid as possible (at least 1 mL) should be sent to the laboratory for culture, because small numbers of organisms may be present. The CSF specimen should be hand-carried to the laboratory after collection and must not be refrigerated. An aliquot of the CSF should be cytocentrifuged for the Gram's stain. If sufficient CSF is available, the

Nasopharyngeal swabs	Nasopharyngeal swabs are particularly important for detecting colonization of individuals who are close contacts to cases of meningococcal disease and for carrier surveys. For these specimens, a fine swab on a flexible metal wire (e.g., a calcium alginate nasopharyngeal swab) is passed through to oropharynx and behind the uvula, where the nasopharynx is sampled. Usually, carefully collected throat swabs will provide the same information. These specimens are inoculated onto a selective medium such as MTM.
Biopsies	Biopsy specimens should be hand-carried to the laboratory in sterile containers. Specimens should be moistened with sterile saline or broth and should not be refrigerated. In the laboratory, the specimens should be aseptically teased apart and cultured on chocolate and blood agar.
Aspirates	Aspirates from closed spaces are collected with a needle and syringe. In the laboratory, these specimens are inoculated onto blood and chocolate agar media.

Colonies of *N. meningitidis* are larger than gonococcal colonies, usually attaining a diameter of about 1 mm or more after 18 to 24 hours' incubation. Colonies are low and convex, with a smooth, moist entire edge and a glistening surface. On sheep blood agar, colonies are usually gray, and heavily encapsulated strains may be mucoid (Image Plate 11-1F) The medium beneath and adjacent to the colonies may exhibit a gray-green cast, particularly in areas of confluent growth. Young cultures have a smooth consistency, while older cultures become gummy due to autolysis.

Gram Stain and Oxidase Test

Primary plates for isolation of *N. gonorrhoeae* should be examined after 24, 48, and 72 hours' incubation using a hand lens or, preferably, a dissecting microscope. Smears prepared from suspicious colonies should be examined with the Gram's stain, and an oxidase test should be performed. The Gram's stain of the colony should show uniform, characteristic gram-negative diplococci. Some of the organisms may appear as tetrads, particularly on smears prepared from young colonies. Organisms on smears prepared from older cultures may appear swollen and display a wide variation in counterstaining intensity. Smears prepared from partially autolyzed colonies may be uninterpretable. Examination by Gram's stain is essential for presumptive identification because other organisms may occasionally grow on selective media, particularly from oropharyngeal specimens (discussed later).

Oxidase test results are obtained with the tetramethyl derivative of the oxidase reagent (*N*,*N*,*N*,*N*-tetramethyl-1,4-phenylenediamine, 1% aqueous solution). This

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Oxidase test results are obtained with the tetramethyl derivative of the oxidase reagent (*N*,*N*,*N*,*N*-tetramethyl-1,4-phenylenediamine, 1% aqueous solution). This solution is placed on a piece of filter paper and a portion of the colonial growth is rubbed onto the reagent with a platinum loop, a cotton swab, or a wooden applicator stick. With fresh cultures, a dark purple color will appear within 10 seconds. Excellent results are obtained with the oxidase reagents that are packaged in crushable glass ampoules (e.g., BACTIDROP Oxidase, Remel Laboratories).

Superoxol Test

Superoxol is another helpful test for the rapid presumptive identification of *N. gonorrhoeae*.[553]Superoxol is 30% hydrogen peroxide (not the 3% solution routinely used for the catalase test).*N. gonorrhoeae* strains produce immediate, brisk bubbling when some of the colony material is emulsified with the reagent on a glass slide. Both *N. meningitidis* and *N. lactamica*, the other species that grow on selective media, produce weak, delayed bubbling. In a study using this test on organisms recovered on selective media, all 201 gonococci tested produced immediate, vigorous bubbling in superoxol, whereas 241 of 242 meningococci and 1 of 2 *N. lactamica* strains produced negative or delayed, weak positive reactions.[553] When testing isolates grown on MTM medium, Perez and colleagues found that the positive and negative predictive values of the superoxol tests for the identification of *N. gonorrhoeae* were 96.7% and 100%, respectively.[509]

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Differentiation of Other Organisms on Selective Media

appear swollen. Coccobacillary *Moraxella* species will form long filaments or spindle-shaped cells under the influence of subinhibitory concentrations of penicillin. *Acinetobacter* species can be differentiated by their negative oxidase reaction. *Capnocytophaga* species appear as pale-staining, gram-negative, slightly curved, fusiform bacteria and are both oxidase negative and catalase negative. On prolonged incubation (i.e., longer than 48 hours), these organisms tend to spread owing to their gliding motility and may impede recovery of gonococci from oropharyngeal specimens.

Presumptive Criteria for Identification of N. gonorrhoeae

All isolates of oxidase-positive, gram-negative diplococci that are recovered from urogenital sites and that grow on selective media may be presumptively identified as *N. gonorrhoeae*. The superoxol test described previously provides an additional presumptive test for identifying these isolates. However, confirmatory identification tests are recommended for all isolates and are required for identification of isolates from extragenital sites (i.e., throat, rectum, blood, joint fluid, CSF) should be identified definitively by at least two tests. Furthermore, presumptive isolates of *N. gonorrhoeae* recovered from children should also be confirmed by at least two tests that involve different principles. These may include carbohydrate utilization tests, immunologic methods (e.g., monoclonal antibody coagglutination tests), enzymatic procedures (e.g., chromogenic detection of specific enzyme activities) or the DNA probe culture confirmation test.[404] Isolates should be preserved to enable additional or repeated testing.[128,140,392,696] This is extremely important because certain social and medical–legal issues are raised following release of the results. At the present time, nucleic acid amplification tests are not recommended for testing of specimens from children (see below).

Identification Tests for Neisseria Species

Confirmatory tests for *gonococci, meningococci*, and other *Neisseria* species include carbohydrate utilization tests, chromogenic enzyme substrate tests, immunologic tests (e.g., coagglutination, other tests), multitest identification systems, and DNA probe tests. Carbohydrate utilization tests and the multitest identification systems can be used to identify *N. gonorrhoeae, N. meningitidis*, and the other *Neisseria* species. Chromogenic substrate identification procedures are limited to identifying those isolates that are able to grow on selective media (i.e., *N. gonorrhoeae, N. meningitidis, N. lactamica*, and some strains of *M. catarrhalis*). Coagglutination, other immunologic tests, and the DNA probe culture confirmation test are available for identification of *N. gonorrhoeae* only. The newer nucleic acid hybridization and nucleic acid amplification procedures are approved for direct detection of *N.*

legal issues are raised following release of the results. At the present time, nucleic acid amplification tests are not recommended for testing of specimens from children (see below).

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Carbohydrate Utilization Tests

Conventional CTA Carbohydrates. The conventional technique for the identification of *Neisseria* species employs cystine-tryptic digest semisolid agar base (CTA) medium containing 1% carbohydrate and a phenol red pH indicator (Image Plate 11-1E). The usual test battery includes CTA-glucose, -maltose, -sucrose, and -lactose, plus a carbohydrate-free CTA control. The lactose structural analog, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), may be substituted for the lactose tube, and the addition of fructose to the test battery is helpful for identifying the various *N. subflava* biovars. Some commercial CTA formulations may be supplemented with ascitic fluid to support the growth of more fastidious organisms. CTA media are inoculated with a dense suspension of the organism to be identified from a pure 18- to 24-hour culture on chocolate agar. The inoculum is either prepared in 0.5 mL of saline and divided among the tubes, or each tube is individually inoculated with a loopful of the organism. The inoculum is restricted to the top 1/2 inch of the agar-deep tubes. The tubes are incubated in a non-CO_2incubator at 35°C with the caps tightened firmly. With a heavy inoculum, most isolates produce a detectable change in the color of the phenol red indicator within 24 hours. If the inoculum is heavy enough, many strains will change the indicator within 4 hours. However, some fastidious gonococcal strains may require 24 to 72 hours to produce sufficient acid to change the indicator. Because CTA media

Species	OX	CAT	Growth			Acid produced from:					Polysaccharide from sucrose	Reduction of:		DNase	Tributyrin hydrolysis	Habitat
			Selective media, 35°C	Chocolate agar, 22°C	Nutrient agar, 35°C	GLU	MAL	FRU	SUC	LAC		NO_3	NO_2[1]			
N. gonorrhoeae	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	Humans
N. meningitides	+	+	+	–	–	+	+	–	–	–	–	–	V	–	–	Humans
N. lactamica	+	+	+	–	–	+	+	–	–	+	–	–	V	–	–	Humans
N. cinerea	+	+	–[2]	–	–	–	–	–	–	–	–	–	V	–	–	Humans
N. polysaccharea	+	+	V	–	+	+	+	–	–	–	+	–	V	–	–	Humans
N. sicca	+	+	–	+	+	+	+	+	+	–	+	–	+	–	–	Humans
N. subflava																Humans
Bv. *subflava*	+	+	–	+	+	+	+	–	–	–	–	–	+	–	–	Humans
Bv. *flava*	+	+	–	+	+	+	+	+	–	–	–	–	+	–	–	Humans
Bv. *perflava*	+	+	–[3]	+	+	+	+	+	+	–	+	–	+	–	–	Humans
N. mucosa	+	+	–	+	+	+	+	+	+	–	+	+	+	–	–	Humans
N. flavescens	+	+	–	–	+	–	–	–	–	–	+	–	+	–	–	Humans
N. elongata																
Subsp. *elongata*	+	–	–	+	+	–	–	–	–	–	–	–	+	–	–	Humans
Subsp. *glycolytica*	+	+	–	+	+	+[w]	–	–	–	–	–	–	+	–	–	Humans
Subsp. *nitroreducens*	+	–	–	+	+	–/+[w]	–	–	–	–	–	+	+	–	–	Humans
N. bacilliformis	+	V	–	–	–	–	–	–	–	–	–	V	NA	NA	V	Humans
N. weaveri	+	+	–	+	+	–	–	–	–	–	–	–	+	–	–	Dogs
N. animaloris[4]	+	+	–	+	+	+	–	–	–	–	–	+	–	–	NA	Dogs

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N. zoodegmatis[4]	+	+	–	+	+	+	–	–	–	–	–	+	+	–	NA	Dogs
N. wadsworthii[5]	+	+	–	+	+	+	–	–	–	–	NA	+	NA	–	NA	Humans
N. shayeganii[5]	+	+	–	+	+	+	–	–	–	–	NA	+	NA	–	NA	Humans
N. canis	+	+	NA	NA	NA	–	–	–	–	–	–	+	–	NA	–	Dogs
N. macacae	+	+	–	NA	NA	+	+	+	+	NA	+	–	+	NA	NA	Nonhuman primates
N. iguanae	+	+	NA	NA	NA	V	–	NA	V	NA	+	+	V	NA	NA	Iguanas
Moraxella catarrhalis	+	+	V	+	+	–	–	–	–	–	–	+	+	+	+	Humans

+, positive reaction; –, negative reaction; V, variable reaction; +w, weak positive reaction; –/+w, negative or weak positive reaction; NA, data not available; OX, oxidase; CAT, Catalase; GLU, Glucose; MAL, Maltose ; FRU, Fructose ; SUC, Sucrose ; LAC, Lactose

[1]Reactions shown are for media with 0.1% nitrite; *N. gonorrhoeae* will reduce 0.01% nitrite

[2]Some strains recovered on selective media

[3]Some strains grow on selective media

[4]*N. animaloris* is arginine dihydrolase (ADH)-positive, while *N. zoodegmatis* is ADH-negative.

[5]Carbohydrate reactions shown are from the API NH system.

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volumes of a balanced salts solution (BSS; pH 7.0) with phenol red indicator are dispensed in nonsterile tubes to which single drops of 20% filter-sterilized carbohydrates are added (Image Plates 11-1G). A dense suspension of the organism is prepared in the BSS with a bacteriologic loop; this suspension may be mixed on a vortex mixer to disperse clumps. One drop of this suspension is added to each of the carbohydrate-containing tubes. The tubes are incubated for 4 hours at 35°C in a non-CO_2 incubator or a water bath. This method is very economical, the reagents are easy to prepare and inoculate, and the results are clear-cut. Details for this method are presented in Chart 11-1. The key to this technique is the use of "reagent grade" carbohydrates. Maltose obtained from some bacteriologic media companies may produce positive or equivocal results for *N. gonorrhoeae* in the rapid carbohydrate

volumes of a balanced salts solution (BSS; pH 7.0) with phenol red indicator are dispensed in nonsterile tubes to which single drops of 20% filter-sterilized carbohydrates are added (Image Plates 11-1G). A dense suspension of the organism is prepared in the BSS with a bacteriologic loop; this suspension may be mixed on a vortex mixer to disperse clumps. One drop of this suspension is added to each of the carbohydrate-containing tubes. The tubes are incubated for 4 hours at 35°C in a non-CO_2 incubator or a water bath. This method is very economical, the reagents are easy to prepare and inoculate, and the results are clear-cut. Details for this method are presented in Chart 11-1. The key to this technique is the use of "reagent grade" carbohydrates. Maltose obtained from some bacteriologic media companies may produce positive or equivocal results for *N. gonorrhoeae* in the rapid carbohydrate degradation test, presumably owing to the presence of contaminant glucose. Inocula for this procedure may be obtained from the primary culture if sufficient colonies are present and if the growth is less than 24 hours old. Because bacterial growth does not occur in the test medium, small numbers of contaminants that may be present do not interfere with results. However, incubation cannot be continued overnight.

CarboFerm *Neisseria* Test. The CarboFerm Neisseria kit (Hardy Diagnostics, Santa Maria, CA) consists of an 8-well reagent cuvette that comes packaged as 12 strips snapped into a plastic frame (Image Plate 11-1H). The frame is labeled with row designations A through H. The wells of the cuvette are sealed with a plastic-cap strip, which is removed prior to inoculation. The wells in row A contain dehydrated peptone medium base with phenol red indicator and no added carbohydrates (control wells). The wells in rows C, D, E, and F contain the same medium base with added carbohydrates; these wells contain glucose (wells in row C), maltose (wells in row D), lactose (wells in row E), and sucrose (wells in row F) along with the dehydrated peptone medium and phenol red indicator. The wells in row H contain bromo-chloro-indoyl butyrate (butyrate esterase substrate) impregnated on a filter paper disk at the bottom of the well. The wells in rows B and G are empty. The kit also includes screw-capped test tubes containing inoculation fluid. The kit is designed to identify the gram-negative, oxidase-positive diplococci belonging to the genus *Neisseria* and *Moraxella catarrhalis*.

To inoculate the CarboFerm *Neisseria*, an organism suspension equal to or exceeding the turbidity of a No. 4 McFarland standard is prepared in the inoculation buffer. This inoculum is prepared from an 18- to 24-hour culture of the organism on chocolate, blood, or selective media (e.g., MTM medium). Four to five drops of the inoculum are placed in wells A (control), C (glucose), D (maltose), E (lactose), F (sucrose), and H (butyrate esterase substrate). The cuvette is placed in an ambient air incubator at 35°C. After 15 minutes, well H (the butyrate esterase substrate) is examined for the presence of a blue color. If the filter paper disk is teal-blue in color, the organism is identified as *M. catarrhalis*, and testing is completed. If the disk in

Other Carbohydrate Utilization Methods. *Neisseria*-Kwik test kit (Micro-Biologics, St. Cloud, MN), and the Gonobio Test (I.A.F. Production, Inc., Laval, Quebec, Canada) are also commercial modifications of the rapid carbohydrate test procedure. The *Neisseria*-Kwik uses a tray containing dehydrated carbohydrates in separate wells. Each well is inoculated with a heavy suspension of the organism prepared in buffer, and results are read after 3 to 4 hours by noting changes in the colors of indicators. The Gonobio Test is a 2-hour method that requires heavy inoculation of microtubes containing carbohydrate substrates. Both of these systems have been evaluated and compare well with conventional methods.[198]

Chromogenic Enzyme Substrate Tests

The enzymatic identification systems use specific biochemical substrates that, after hydrolysis by bacterial enzymes, yield a colored end product that is detected directly (e.g., a yellow nitrophenol or nitroaniline product) or after the addition of a diazo dye-coupling reagent (i.e., cinnamaldehyde reagent for detection of free β-naphthylamide). The use of these systems is restricted to those species that are able to grow on selective media—*N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica*. Because some strains of *M. catarrhalis* grow on selective media, these systems will also provide a presumptive identification of this organism. Chromogenic substrate identification tests should not be used to identify suspected meningococci recovered on blood and chocolate agars without prior subculture of the isolate to selective media. The enzymatic activities that are detected in these systems include β-galactosidase (BGAL), γ-glutamyl transpeptidase (GGT) aminopeptidase, and prolyliminopeptidase (PIP) (Table 11-3). β-galactosidase and γ-glutamylaminopeptidase are specific for *N. lactamica* and *N. meningitidis*, respectively. Occasional isolates of *N. meningitidis* may lack γ-glutamyl aminopeptidase activity; a meningococcal strain that was isolated from a carrier was found to lack this enzyme because of the presence of an insertion sequence disruption in the *ggt* gene.[619]Reexamination of the GGT activity of 245 *N meningitidis* isolates identified the presence of two other GGT-defective isolates.[618] Absence of GGT and presence of PIP identifies an organism as *N. gonorrhoeae. M. catarrhalis* lacks all three of these enzymatic activities. The commercial systems that use this approach are the Gonochek II, the BactiCard Neisseria, and the Neisstrip.

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TABLE 11-3 Enzymatic Activities Used for Identification of the Pathogenic *Neisseria* Species

Absence of GGT and presence of PIP identifies an organism as *N. gonorrhoeae. M. catarrhalis* lacks all three of these enzymatic activities. The commercial systems that use this approach are the Gonochek II, the BactiCard Neisseria, and the Neisstrip.

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TABLE 11-3 Enzymatic Activities Used for Identification of the Pathogenic *Neisseria* Species

Species	β-Galactosidase	γ-Glutamyl Aminopeptidase	Prolyl Iminopeptidase	Butyrate Esterase
N. lactamica	+	–	+	–
N. meningitidis	–	+	V	–
N. gonorrhoeae	–	–	+	–
M. catarrhalis	–	–	–	+

+, positive reaction; –, negative reaction; V, variable reaction.

The utility of preformed enzyme tests for identification of *N. gonorrhoeae* may be limited in the future due to the recognition and spread of gonococcal strains that lack PIP activity. This enzyme is encoded by the *pip* gene, and translation of this gene results in the functional 310-amino-acid PIP protein. Failure to produce functional PIP was determined to result from either 1 of 2 mutations in the *pip* gene—a thymidine deletion mutation resulting in a frame-shift in the codon sequence and a nonfunctional 123-amino acid truncated protein—or a thymidine insertion resulting in a truncated, nonfunctional protein 55 amino acids in length.[75] From 2000 through 2002, a clone of *N. gonorrhoeae* that lacked this enzyme activity became widely disseminated throughout the United Kingdom.[505] This strain typed as serovar IB-4 by coagglutination serology. Phenotypic and genotypic characterization of gonococcal isolates in Denmark during 2002 and 2003 identified the same PIP-negative gonococcal clone in that country.[250] In both the United Kingdom and Denmark, these isolates were recovered primarily from MSM. Subsequent studies by Alexander and colleagues determined that the overall prevalence of PIP-negative *N. gonorrhoeae* strains in England and Wales was 4.33%, and variation in the prevalence of these strains in various clinics in London reflected a higher frequency of these isolates among MSM.[17,18] A 2006 study conducted in Sydney, Australia documented the appearance, spread, and subsequent decrease in subtypes of PIP-negative gonococci among 3,926 strains collected from July 2002 to September 2005.[43] During the third quarter of 2002, PIP-negative *N. gonorrhoeae* comprised only 3% of isolates and, by the third quarter of 2003, these strains comprised 22% of all isolates. These strains subsequently decreased in prevalence and by the third quarter of 2005, 6% of referred

2007, one PIP-negative strain was detected among 250 isolates at the Quebec Public Health Laboratory.[96] A subsequent study in Canada surveyed 5,675 gonococcal isolates submitted to the public health laboratories revealed 19 PIP-negative strains distributed among several Canadian provinces, including British Columbia, Saskatchewan, Manitoba, Quebec, and Prince Edward Island.[438] Another study conducted in Spain found 10 (6.9%) PIP-negative, serovar IB-4 *N. gonorrhoeae* among 143 isolates received by the Regional Laboratory for Gonococci in Oviedo, Spain from 2003 through 2006.[500]

Because of the increasing prevalence of these PIP-negative isolates throughout the world, the performance of test systems (i.e., BactiCard *Neisseria*, Gonochek II, RapID NH; *Neisseria* PET, and API NH) that rely on this enzyme activity to help identify gonococcal isolates and to differentiate them from other neisseriae may be compromised. PIP-negative *N. gonorrhoeae* isolates may generate ambiguous identifications, including "doubtful *N. gonorrhoeae*" and "presumptive *Kingella kingae*," and these types of spurious identification should prompt the performance of additional identification approaches.[17] PIP is a hydrolase enzyme that selectively removes the *N*-terminal proline residue from peptides, and the name of the enzyme varies depending upon the manufacturer of the kit system being used (e.g., PIP, proline aminopeptidase, hydroxyproline aminopeptidase, prolyl arylamidase). Manufacturers of these commercial systems need to standardize the format and names of these tests to avoid confusion for the end users and include warnings in the package inserts concerning proper use and interpretation of the tests in light of the increasing prevalence of PIP-negative strains. Emergence of these gonococcal strains has made it necessary and imperative to use at least two different identification methods based on different criteria (i.e., biochemical, immunologic, or molecular) for confirmation of *N. gonorrhoeae*.[438,652]

Gonochek II. The Gonochek II (EY Laboratories, San Mateo, CA) is a single tube that contains the three dehydrated chromogenic substrates (Image Plate 11-2A). After rehydration with four drops of phosphate-buffered saline (pH 7.4), 5 to 10 colonies of oxidase-positive, gram-negative diplococci from a pure culture growing on selective medium, or a suitable subculture, are emulsified in the tube with a wooden applicator stick. The tube is capped with the stopper and incubated at 35°C for 30 minutes. Specific color reactions in the bacterial suspension confirm the isolate as

N. meningitidis (hydrolysis of γ-glutamyl-*p*-nitroanilide; yellow) or *N. lactamica* (hydrolysis of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; blue). If the suspension is colorless at the end of the incubation period, the stopper is split apart

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N. meningitidis (hydrolysis of γ-glutamyl-*p*-nitroanilide; yellow) or *N. lactamica* (hydrolysis of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; blue). If the suspension is colorless at the end of the incubation period, the stopper is split apart and the top part of the stopper is inserted into the tube. The tube is inverted so that the bacterial suspension comes in contact with the diazo dye-coupler (o-aminoazotoluene diazonium salt [Fast Garnet]) present on the stopper. The detection of β-naphthylamine released by bacterial hydroxyprolyl aminopeptidase activity (red) identifies the isolate as *N. gonorrhoeae*. The absence of a colored product at the completion of the testing and reading steps provides a presumptive identification of *M. catarrhalis*. In a large evaluation of this system, the Gonochek II identified 99% of 176 gonococci, 97% of 173 meningococci, and 100% of 48 *N. lactamica* and 10 *M. catarrhalis*, respectively.[332] Other investigators have also evaluated Gonochek II and have found it reliable for identifying these organisms.[198] Isolates that lack the PIP or GGT enzymes will not be correctly identified by Gonochek II.[17]

BactiCard *Neisseria*. The BactiCard *Neisseria* (Remel Laboratories, Lenexa, KA) uses four chromogenic substrates that are impregnated on four individual test circles within a cardboard holder (Image Plate 11-2B). After moistening each of the four circles with a single drop of rehydrating fluid, several colonies of the organism taken from selective media (or from a subculture from selective media) are rubbed onto each of the four test areas. After incubation on the bench top for 2 minutes, the indoxyl butyrate esterase substrate circle (IB; 5-bromo-4-chloro-3-indolyl butyrate) is inspected for the appearance of a blue to blue–green color. If this test is positive, the organism can be identified as *M. catarrhalis*, and no further testing is necessary. If the IB test is negative, the card is incubated on the bench for an additional 13 minutes (total test time: 15 minutes). After this time, the β-galactosidase substrate circle (BGAL; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is inspected for the presence of a blue–green color. If this test is positive, the organism can be identified as *N. lactamica,* and no further testing is necessary. If the BGAL test is negative, a single drop of color developer reagent is placed on the PIP (PRO; l-proline-β-naphthylamide) and the GGT (GLUT; γ-glutamyl-β-naphthylamide) test circles. These two tests are read as positive if a definite pink or red color develops within 30 seconds of reagent addition. A positive PRO reaction identifies the isolate as *N. gonorrhoeae,* whereas a positive GLUT reaction identifies the organism as *N. meningitidis*. The PRO reaction

Immunologic Methods for Culture Confirmation of N. gonorrhoeae

Coagglutination Test. Coagglutination tests make use of the ability of protein A on *Staphylococcus aureus* cells to bind IgG molecules by their Fc region. Binding of antigonococcal antibody to killed *S. aureus* cells, and subsequent mixture with a suspension of gonococci causes visible agglutination of the suspension.[25] Two coagglutination tests for the identification of *N. gonorrhoeae* are currently available—the Phadebact Monoclonal GC Test and the GonoGen I test. The Phadebact monoclonal GC coagglutination test (MKL Diagnostics AB, Sollentuna, Sweden; Remel [USA distributor]) employs monoclonal antibodies to gonococcal Por (protein I). Unlike the GC OMNI test previously marketed by Phadebact, the monoclonal GC test contains one reagent that reacts with serogroup WI *N. gonorrhoeae* strains, and a second reagent that reacts with serogroup WII/WIII strains. Because a negative control reagent is not included, gonococcal isolates will react with either the WI or the WII/WIII reagent, depending on the Por antigen composition of the individual isolate. Failure to agglutinate in either reagent constitutes a negative test. To perform the test, a suspension (0.5 McFarland turbidity standard) is prepared in buffered saline (pH 7.2 to 7.4) and is boiled for 5 minutes. After cooling, the suspension is mixed with test and control reagents on a cardboard slide. Agglutination within 1 minute is a positive test. A blue dye is incorporated in the reagents to help visualize agglutination against the white background of the card. Careful attention to procedural details is necessary in order to prevent false-positive and false-negative results. Evaluations of the previously available GC OMNI test reported sensitivities of 98% to 100% and specificities of 99% to 100%.[198,206,332]Organism suspensions heavier than the specified McFarland density, however, may yield false-positive results. In addition, the use of saline with a pH less than or greater than 7.4 has also been reported to produce false-positive results with some strains of *N. lactamica*, *N. cinerea*, and *M. catarrhalis*.[332,333] The GonoGen I coagglutination culture confirmation test for *N. gonorrhoeae* (New Horizons Diagnostics, Columbia, MD) is no longer commercially available.[350,394] In addition the direct fluorescent antibody culture confirmation test, as previously described,[588,687,692] originally marketed by Syva and subsequently by several other commercial vendors (lastly by Trinity Biotech, Wicklow, Ireland) has also been discontinued.

GonoGen II Test. The GonoGen II (New Horizons Diagnostics, Columbia, MD) uses anti-Por monoclonal antibodies that are conjugated to colloidal gold as the detection reagent. A suspension (McFarland no. 1) is prepared in 0.5 mL of an organism lysing solution, and 1 drop of the antibody reagent is added. After 5 minutes, 2 drops of the suspension are passed through a membrane filter that retains antigen-antibody complexes. Retention and concentration of the complex on the filter turns the filter

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N. lactamica isolates repeatedly produced false-positive test results. Kellogg and Orwig found that the GonoGen II identified 99.6% of 248 gonococcal strains, but 7 of 22 *N. meningitidis* strains produced false-positive test results, leading these investigators to suggest that positive GonoGen II test results need to be confirmed by another method.[350]

Multitest Identification Systems

Five kit systems are available that can be used not only for identifying *Neisseria* species but also for identifying other fastidious gram-negative organisms. These systems are the RapID NH (*Neisseria-Haemophilus*) system (Remel Laboratories, Image Plate 11-2C), the *Haemophilus-Neisseria* identification (HNID) panel (Dade/American Microscan, Sacramento, CA; Image Plate 11-2D), the API NH system (bioMerieux, Inc., La Balme-les-Grottes, France;Image Plate 11-2E, 11-2F, 11-2H), the BD Crystal *Neisseria-Haemophilus* ID kit (BD Diagnostic Systems, Sparks, MD) and the Vitek 2 *Neisseria-Haemophilus* (NH) identification card (bioMerieux, Inc., Hazelwood, MO).[53,206,328,529,658] All of these systems use modified conventional tests (e.g., acid production from carbohydrates, urease, indole, ornithine decarboxylase) and chromogenic substrates to provide either 2- to 6-hour identifications of *Neisseria*, *Haemophilus*, and other fastidious gram-negative bacteria encountered in clinical specimens. The MicroScan HNID panel does not include *N. cinerea* in its database, and these organisms are misidentified as either *N. gonorrhoeae*

identification.[53] The Crystal *Neisseria-Haemophilus* ID kit uses fluorescent substrates along with rapid conventional test to identify *Neisseria* and other organisms. The Vitek 2 NH card provides automated identification of *Haemophilus* and *Neisseria* species, along with several other fastidious gram-negative bacteria. Evaluations have shown that the NH card is able to identify the pathogenic *Neisseria* species and *M. catarrhalis* within 6 hours.[529,658]

DNA Probe Test for Culture Confirmation of N. gonorrhoeae

The Accuprobe *Neisseria gonorrhoeae* Culture Confirmation Test (Gen-Probe) identifies the *N. gonorrhoeae* by the detection of species-specific rRNA sequences. In the test, organisms from growth on agar media are lysed and mixed with a chemiluminescent-labeled single-stranded DNA probe that is specifically complementary to gonococcal rRNA. After hybridization occurs, the DNA probe/rRNA double-stranded complex is selected by a chemical process, and the presence of the probe in the double-stranded material is detected by addition of detection reagents that hydrolyze the chemiluminescent tag on the probe, thereby releasing light energy. This energy is detected in a chemiluminometer instrument, and the result is reported as positive or negative. In an evaluation conducted at the University of Illinois examining monoclonal antibody coagglutination procedures, the direct fluorescent antibody test, and the AccuProbe for *N. gonorrhoeae* identification, the latter test was the only procedure that was 100% accurate in identifying *N. gonorrhoeae*.[333] Similar results for the AccuProbe test have been reported by others.[715] This test may be particularly useful for confirming those isolates that may not be easily identified by other confirmatory test procedures.

Nucleic Acid Hybridization Tests for N. gonorrhoeae

Probe hybridization technology is also used in the currently available tests for direct detection of *N. gonorrhoeae* in urogenital specimens. One of the two direct hybridization assays, the PACE 2 (Hologics, San Diego, CA), has been discontinued. The remaining nucleic acid hybridization assay for direct use on clinical specimens is the Digene Hybrid Capture 2 CT/GC assay (Qiagen Gaithersburg, MD).

The Digene Hybrid Capture 2 assay uses signal amplification instead of target amplification to increase its sensitivity of detection. In this assay, target DNA becomes hybridized to sequence-specific RNA probes provided with the assay. Hybrid products are transferred to wells of a microtiter tray, where they become immobilized by antibodies in a microtiter well. A detection reagent, consisting of an antibody conjugate that is specific for RNA/DNA hybrids, is then added to each microwell. Multiple enzymes are conjugated to each of these antibody molecules, and many antibody molecules bind to each RNA/DNA hybrid, resulting in marked signal amplification

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Nucleic Acid Amplification Tests for N. gonorrhoeae

Several nucleic acid amplification assays (NAATs) are available for the direct detection of *N. gonorrhoeae* in clinical specimens (Box 11-5).[695] FDA-cleared tests employ various amplification approaches, including PCR (AMPLICOR *Neisseria gonorrhoeae* PCR, COBAS AMPLICOR *Neisseria gonorrhoeae* PCR, COBAS 4800 CT/NG test, Roche Molecular Diagnostics, Indianapolis, IN), strand displacement amplification (SDA) (BD ProbeTecET, BD Diagnostics, Sparks, MD) and TMA (APTIMA Combo 2, Gen-Probe, San Diego, CA), and real-time PCR (Abbott RealTime CT/NG,

BOX 11-5

Nucleic Acid Amplification Assays (NAATs) for *N. gonorrhoeae*

Parameter	Roche COBAS AMPLICOR Assay	Roche COBAS 4800	Gen-Probe APTIMA 2 COMBO (AC2) Assay	Gen-Probe APTIMA GC Assay	BD ProbeTec Assay	Abbott RealTime CT/NG Assay
Manufacturer	Roche Molecular Systems, Branchburg, NJ	Roche Molecular Systems, Branchburg, NJ	Gen-Probe, Inc., San Diego, CA	Gen-Probe, Inc., San Diego, CA	Becton-Dickinson, Sparks, MD	Abbott Molecular Diagnostics, Des Plaines, IL
Amplification method	PCR	Real-time PCR	TMA	TMA	SDA	Real-time PCR
Amplification target	Cytosine DNA methyltransferase gene (single copy)	DR9 (multicopy direct repeat region)	16S subunit of ribosomal RNA gene (multicopy)	16S subunit ribosomal RNA Gene (multicopy); different target than AC2	Multicopy pilin gene-inverting protein homologue	*opa* gene (multicopy)
Reported sensitivity	65%–100%	92.9%–100%	91.3%–98.5%	98.7%–100%	85%–100%	96.8%–96.9%
Reported specificity	93.8%–100%	100%	98.7%–99.3%	100%	98.4%–100%	99.7%–100%
Positive reactivity with other *Neisseria* species	*N. meningitidis, N. lactamica, N. subflava/sicca, N. cinerea, N. flavescens, N. polysaccharea, M. catarrhalis*	None reported	None reported	*N. meningitidis*	*N. meningitidis, N. lactamica, N. cinerea, N. mucosa, N. flavescens*	Low level reactivity with some *N. meningitidis, N. mucosa*

Abbott Molecular Diagnostics, DesPlaines, IL). All of these NAATs detect both *N. gonorrhoeae* and *C. trachomatis*. None of the currently available NAATs are FDA-cleared for use with extragenital (i.e., oropharyngeal, rectal), pediatric, or ocular specimens, although studies have been performed addressing some of these specimen types (see below).

The AMPLICOR *N. gonorrhoeae* PCR assay and the automated COBAS AMPLICOR systems use conventional PCR-based amplification. For both AMPLICOR assays, the amplification target sequence in *N. gonorrhoeae* is a 201 base pair fragment of the cytosine DNA methyltransferase gene. The original nonautomated assay uses the Perkin–Elmer 9600 thermal cycler with subsequent amplicon detection by EIA using microwell plates that are coated with an oligonucleotide to capture the biotinylated amplicon products. The COBAS AMPLICOR is a benchtop automated system in which target DNA is amplified by a conventional PCR reaction using built-in thermal cyclers. Biotinylated amplification products are captured with magnetic particles that are coated with amplicon-specific oligonucleotide probes, and the products are detected colorimetrically using an avidin–

horseradish peroxidase conjugate.

Several studies have examined the performance of the AMPLICOR gonorrhea assay and most studies have documented sensitivities in excess of 90%.[144,399] While this assay has demonstrated greater than 90% sensitivity for the detection of *N. gonorrhoeae* in endocervical specimens, the clinical trial data showed that the test was less sensitive with urine specimens from females (i.e., 64.8%).[504] This has been seen in other evaluations of this system as well.[144,666] In a study of 344 urine and urethral swab specimens from males and 192 urine and endocervical swab specimens from females, Crotchfeldt and colleagues found that the sensitivity of the AMPLICOR PCR assay was 94.4% for male urine specimens and 97.3% for male urethral swab specimens, while the sensitivities of the assay for urine and endocervical swab specimens from women were 90% and 100%, respectively.[168] Assay specificities generally range from 95.9% to 98.5% for urine specimens and from 97.0% to 99.4% for swab specimens. A large multicenter evaluation of both the semiautomated AMPLICOR microwell assay and automated COBAS AMPLICOR assay examined 2,192 matched endocervical swab and urine specimens from women and matched urine and urethral swab specimens from 1,981 men.[437] The AMPLICOR microwell assay and the COBAS AMPLICOR yielded concordant results on 98.8% of the specimens and exhibited virtually identical sensitivities of 99.5% and 99.8% for endocervical swab and urine specimens from women, respectively, and 98.9% and 99.9% for male urethral swab and urine specimens, respectively. This assay has been reported to have false positives caused by nonpathogenic *Neisseria* species.[504] In a study examining the specificity of

the AMPLICOR microwell assay, Farrell found that 6 of 15 *N. subflava* strains produced positive results, including one strain that was isolated from a vaginal specimen.[236] Other studies, as well as the manufacturer, have also acknowledged that saprophytic neisseriae (e.g., *N. cinerea*, *N. subflava/sicca*, and *N. flavescens*) may produce positive, cross-reactive results in the AMPLICOR *N. gonorrhoeae* PCR assay.[504,538,663] This cross-reactivity may contribute to lower positive predictive values for this assay with urogenital specimens from certain populations. Because of these specificity problems, the CDC issued guidelines suggesting that additional testing for gonococcal infection be performed in situations where the positive predictive value of a test was expected to be lower than 90%.[128] Confirmatory testing with a more specific method such as 16S rRNA PCR should be considered in low-prevalence populations.[194]

Roche Molecular Systems markets the COBAS 4800 CT/NG test. This is an

automated multiplex PCR assay that detects plasmids of *Chlamydia trachomatis* and the DR-9 direct repeat sequence that is specific to *N. gonorrhoeae.* A randomized internal target sequence of nucleic acid is added to each specimen as an internal control to minimize false-negative results due to amplification inhibition. An evaluation performed in Australia tested 419 specimens (318 urine and 180 swab specimens) on the COBAS 4800 system and compared the results with those obtained with the Roche AMPLICOR PCR assay.[539]Specimens that were positive for *N. gonorrhoeae* were confirmed with an in-house real-time PCR assay. For urine specimen, the sensitivity, specificity, and negative and positive predictive values for *N. gonorrhoeae* were 92.9%, 100%, 99.7%, and 100%, respectively. For swab specimens, the corresponding values were 100%, 99.4%, 100%, and 90%, respectively. An assessment of test specificity was performed using a panel of 223 other *Neisseria* isolates, and no nongonococcal *Neisseria* strains were positive with the 4800 assay. A second study evaluating the specificity of NAATs tests also found no false-positive reactions with other *Neisseria* species for the COBAS 4800 assay.[612]

The BD ProbeTec ET system uses SDA rather than PCR for amplification, so all testing is performed isothermally without the need for a thermal cycler.[413] The system has a higher throughput than other amplification methods, with a capacity to perform 300 assays in an 8-hour shift. The system also has options for test performance and interpretation both with and without an amplification control, and uses reagents that can be stored at room temperature. In this assay, the amplification target is in the chromosomal pilin gene-inverting homologue, which is already present in multiple copies in the gonococcal cell.[112] The ProbeTec ET is approved for the detection of *Neisseria gonorrhoeae* in female endocervical, male urethral, and female and male urine specimens. Studies have documented sensitivities of greater than 96% for endocervical, male urethral, and male urine specimens, but lower sensitivity for urine specimens from females. Van Der Pol and associates[662] evaluated the ProbeTec ET with both swab and urine specimens from men and women. For swab and urine specimens from women, the sensitivities of the ProbeTec ET system were 96.6% and 84.9%, respectively. For swab and urine specimens from men, the sensitivities of the ProbeTec ET were 98.5% and 97.9%, respectively. Specificities ranged from 94.4% to 100% for urine specimens and from 94.8% to 99.6% for swab specimens. In another evaluation of the ProbeTec ET with 733 endocervical swab specimens from commercial sex workers in Belgium, the SDA procedure performed with a sensitivity of 90% and a specificity of 100% compared with culture.[667] In a study of 3,544 urine specimens submitted for *N. gonorrhoeae* testing, Akduman and colleagues found that the sensitivity and specificity of the ProbeTec ET were 99.2% and 99.3%, respectively, in a population with a prevalence of 3.6% by culture.[10]Another evaluation performed in Canada with urine specimens from 825 males and 399

females compared the ProbeTec ET with the Roche COBAS AMPLICOR PCR and found that the SDA procedure was 100% sensitive and 99.7% specific, while the PCR assay was 96.7% sensitive and 98.9% specific.[144] Like the AMPLICOR assays for *N. gonorrhoeae*, the BD ProbeTec ET also can yield false-positive reactions with several nongonococcal *Neisseria* species.[504] Subsequently, BD Diagnostics marketed the BD Viper System, which automates previously manual and semiautomated steps of the BD ProbeTec ET assays for *N. gonorrhoeae* and *C. trachomatis* testing.[516,630] With this automated approach, incubations, amplification, and detection all occur on the BD Viper System, with repetitive pipetting labor relegated to a robotics system.

The APTIMA Combo 2 assay uses isothermic TMA for detection of gonococci and chlamydiae in female endocervical and male urethral swab specimens and first-voided male and female urine specimens. This NAAT amplifies gonococcal 16S rRNA, and uses a chemiluminescent single-stranded DNA probe for detection of the amplified product. This test is perhaps the most sensitive NAAT for detection of *N. gonorrhoeae,* with sensitivities above 97% for endocervical specimens, male urethral specimens and urine specimens from males, and above 91% for urine specimens from females. Gaydos and colleagues evaluated this assay system with both endocervical swabs and urine specimens submitted for detection of *N. gonorrhoeae*.[262] The sensitivity and specificity of the APTIMA system were 99.2% and 98.7%, respectively, for endocervical swab specimens, and 91.3% and 99.3%, respectively, for first-collected urine specimens. This assay has undergone several evaluations, including the use of physician- and self-collected vaginal swabs for organism detection.[149,150] The APTIMA test also appears to be less susceptible than other amplification assays to inhibitors of amplification that are usually found in urine specimens. In addition, false-positive reactions with saprophytic *Neisseria* species have not been observed with the APTIMA assay.[275] Gen-Probe also markets a *N. gonorrhoeae* stand-alone NAAT (Gen-Probe AG) that detects a slightly different gene target of the 16S rRNA subunit; this assay can be used to confirm positive gonorrhea results obtained with the combo assay.[275] The AG assay has demonstrated a sensitivity of 99.4% to 100% for urethral swab specimens from both symptomatic and asymptomatic males, with corresponding specificities of 97.1% to 97.5%. Sensitivities and specificities for the AG assay with first-voided urine specimens were 90.9% to 99.4% and 99.0% to 99.5%, respectively.[150]

The Abbott RealTime CT/GC assay is a PCR assay for direct qualitative detection of the opacity (*opa*) gene of *N. gonorrhoeae* and plasmid DNA of *C. trachomatis.* The Abbott CT/NG uses an automated sample preparation platform (m2000sp) that enables magnetic microparticle DNA

capture/extraction. An internal control is added to each sample, and postextraction, the template DNA and master mix is dispensed into an optical reaction plate. This plate is transferred to a real-time PCR instrument (m2000rt) for amplification. The test is approved for female endocervical swab, clinician-collected vaginal swab, and self-collected vaginal swab specimens, male urethral swab specimens, and male and female urine specimens from symptomatic patients. This test has also been cleared for testing clinician-collected and patient-collected vaginal swab specimens and female and male urine specimens from asymptomatic patients. In a side-by side evaluation of the Abbott RealTime PCR, Gen-Probe TMA, and BD ProbeTec ET SDA assays on 500 first-void urine specimens, the sensitivity of the RealTime assay was 96.9%, compared with 99% for TMA and 98% for SDA. RealTime PCR and TMA demonstrated 100% specificity while SDA showed a specificity of 99.5%.[400] In a large, multicenter study, real-time PCR was evaluated and compared to TMA, SDA, and culture as reference assays to determine the infection status of the patient.[261] The evaluation included all approved specimens types. The overall sensitivity and specificity of the Abbott RealTime PCR assay for *N. gonorrhoeae* were 96.9% and 99.7%, respectively. In comparison, the sensitivity and specificity for the TMA assay were 96.1% and 99.5%, respectively, while those for the SDA assay were 92% and 97.3%, respectively. Comparison of this real-time system with an in-house real-time *porA* pseudogene assay showed 100% concordance for male urine specimens with corresponding urethral swab specimens and some male rectal specimens as well.[686] Cheng and colleagues compared the Abbott RealTime assay with the Roche COBAS AMPLICOR PCR assay with endocervical and male urethral swab specimens and male and female urine specimens from 1,384 patients.[146] In this study all positive AMPLICOR PCR assays were confirmed by an in-house real-time PCR assay for the *por* A pseudogene. After resolution of results obtained with the Roche test there was complete agreement between the positive and negative test results. These workers also pointed out an enhanced ability of the RealTime CT/NG assay to detect dual infections. Specificity studies on the Abbott RealTime assay showed low-level cross-reactivity with single strains of *N. meningitidis* and *N. mucosa.*[612]

One of the newest NAAT for *N. gonorrhoeae* and *C. trachomatis* detection is the Versant CT/GC DNA 1.0 assay (Siemens Healthcare Diagnostics, Berkeley, CA). This is a "kinetic PCR" system that consists of a sample preparation module for automated specimen preparation and processing, and a real-time PCR amplification/detection module. The assay is performed on the Versant kPCR system. This assay was compared to the APTIMA Combo 2 TMA assay using 1,129 patient specimens (589 urine and 540 swab specimens).[357] For detection of *N. gonorrhoeae*, the agreement between the Versant assay and TMA was 100% for the positive urine and swab specimens, and the agreement for negative specimen was 99.6% for urine specimens and 99.0% for swab specimens. The Versant assay did not demonstrate any cross-

reactivity with nongonococcal *Neisseria* species, other bacteria, or yeasts. This assay is not yet FDA-cleared for use in the United States.

Even though NAATs are not currently FDA-cleared for diagnosis of gonorrhea and *Chlamydia* infections at extragenital body sites, several studies evaluating the utility of NAATs in the diagnosis of rectal and pharyngeal gonococcal infections have been done, particularly in populations of MSM.[54,528] In many studies, NAATs were compared to culture and were found to be equivalent to or far more sensitive than culture methods. Some studies performed culture plus all three commercially available NAAT systems (i.e., PCR, SDA, and TMA) and defined a "true positive" using a rotating standard where 3 of 4 comparator tests were positive. Bachmann and colleagues evaluated culture and NAATs for diagnosis of oropharyngeal gonorrhea with 961 evaluable specimens from patients with a history of oral–genital sex in the previous two months.[46] Using the defined standard described above, the sensitivity of culture, PCR, SDA, and TMA were 65.4%, 91.9%, 97.1%, and 100%, respectively. The specificity of culture was 99%, and the specificities of SDA and TMA were 94.2% and 96.2%, respectively. The specificity of the AMPLICOR PCR was only 71.8%, which may be related to the detection of saprophytic *Neisseria* species in the oropharynx described previously for the AMPLICOR assay. Bachmann and coworkers[47] also evaluated NAATs for the diagnosis of rectal gonococcal infection and used the same rotating standard to evaluate the results. For rectal (n = 441) specimens, the sensitivity of culture, PCR, SDA, and TMA were 71.9%, 95.8%, 100%, and 100%, respectively. The specificity of culture was 99.7% and ranged from 95.5% to 96% for the three NAATs. These workers concluded that the currently available NAATs were more sensitive for the detection of gonococcal infections than culture at rectal and oropharyngeal sites, that PCR, TMA, or SDA could be valuable for diagnosis of rectal gonococcal infections, and that TMA and SDA but not PCR could reliably detect oropharyngeal gonococcal infections. Other studies have found similar results.[399,499,501] Because of high specificity, TMA appears particularly useful for detection of gonococcal oropharyngeal and rectal infections. PCR, on the other hand, appears to have a lower sensitivity than either TMA or SDA for detection of extragenital gonococcal infections. Because these test systems are not FDA-cleared for testing oropharyngeal or rectal specimens, this use is considered "off-label" and CLIA verification of NAAT performance is required prior to their use for diagnosis of gonococcal infections at extragenital sites. Many workers in the STD field feel that NAATs may play an important role in the diagnosis of extragenital gonococcal infections that may be missed by traditional methods and that these tests may be critical for effective public health intervention in sexually active populations.[455,528,564]

The use of nucleic acid-based methods for detection of *N. gonorrhoeae* in clinical specimens has both advantages and disadvantages. The advantages include the demonstrated superior sensitivity of these methods compared with culture, the ability

to test easily collected specimens (i.e., urine, self-collected vaginal swabs), and more rapid turnaround times. While gonococcal culture ranges from 85% to 95% in sensitivity, the increased sensitivity of NAAT approaches make them highly suitable for patient screening.[259]Testing of urine in place of endocervical or urethral swab specimens makes collection easy and does not require special collection equipment or entailed collection instructions. Lastly, organisms do not need to be viable in order to detect the nucleic acid, so less stringent collection and transport conditions can be used in comparison with culture-based testing. Disadvantages

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include the cost of the assays (including equipment and expendables), the need for separate, dedicated space for specimen processing/test performance and modifications of laboratory workflow to prevent amplicon contamination of the instrumentation and the environment. A very real "down side" to the widespread use of these non–culture-based assays is the unavailability of gonococcal isolates for surveillance of emergent resistance to antimicrobial agents. Lastly, different gonococcal strains may have altered target gene sequences, or nongonococcal *Neisseria* species may have genes that are also present in certain gonococcal strains, leading to both false-negative and false-positive results. Despite the high specificities of these assays, their performance in low-prevalence populations requires additional study. As mentioned previously, the CDC has suggested that positive NAAT results should be confirmed in populations where the test has a positive predictive value of less than 90%.[128] Confirmation may involve repeating the original NAAT on the same specimen, repeating the original test on the original specimen with quenching by a competitive probe, testing the original specimen with an NAAT that detects a different gonococcal gene target, or testing a second specimen with a second NAAT that is different from the initial NAAT and that detects a different gonococcal gene sequence.[128] Some workers have recommended confirmatory testing with more specific molecular methods targeting gonococcal 16S rRNA or genes that are "specific" for *N. gonorrhoeae* (e.g., the *cppB* gene, the *porA* pseudogene, multicopy *opa* genes) for positive amplified test results obtained in populations with a low prevalence of gonorrhea.[194,271,306] However, even some of these gene targets, such as the *cppB* gene, show considerable differences in prevalence in gonococcal strains in different parts of the world.[98,422] Dual-target molecular assays targeting more than a single gonococcal gene (e.g., the *porA* pseudogene and conserved multicopy *opa* genes) may decrease the potential for sequence-related, false-negative results and may provide greater confirmatory power.[271,306,433] An evaluation of the CDC confirmatory approaches conducted in San Francisco found that 89% to 96% of samples positive by SDA, PCR, and TMA were confirmed by

repeat testing of the same specimen and that 85% to 98% of SDA, PCR, and TMA results were confirmed by using different NAATs on the original specimen. For first-void urine specimens from men, any NAAT could be used for confirmation, but for other specimen types some NAATs could not be used to confirm positive results with other NAATs.[459] Other workers have also found that some NAATs cannot be used to confirms the results of other NAATs due to different specimen extraction steps and other procedural details.[16,294] Because of specificity problems with NAATs in low-prevalence populations, none of these assays can be used to document gonococcal or chlamydial infections in children, since a false-positive result for a sexually transmitted infection can lead to erroneous reports of sexual abuse, with far-reaching legal consequences.[291,292] However, a 2009 multicenter study found that NAAT testing of urine specimens from prepubertal children undergoing evaluation for sexual abuse had high sensitivity and specificity, and that a positive NAAT confirmed with an alternate gene target NAAT may be the best method and approach for detection of *N. gonorrhoeae* in children suspected of being sexually abused.[74]

MALDI-TOF for Identification of Neisseria Species

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is an emerging method for the identification of bacterial agents and has been applied to a wide variety of organisms, including *Neisseria* species.[470] Ilina and colleagues examined 29 *N. gonorrhoeae*, 13 *N. meningitidis*, and 15 saprophytic *Neisseria* species isolates using MALDI-TOF.[321] Mass spectra profiles of *N. gonorrhoeae* and *N. meningitidis* showed limited intra-species variability and comparisons of the spectra between species revealed numerous significant differences. Alignment of the main spectra clearly separated isolates of *N. gonorrhoeae*, *N. meningitidis*, and the nonpathogenic species that were tested (*N. lactamica*,*N. mucosa*, *N. subflava* biovars, *N. elongata*, *N. polysaccharea*, and *N. sicca*). MALDI-TOF has also been used to characterize and differentiate meningococcal isolates belonging to specific enzyme (ET) and ST complexes.[420] MALDI-TOF shows promise as a rapid, inexpensive method for identification of *Neisseria* species.

Sexual Abuse/Assault and N. gonorrhoeae

Because of the medical–legal issues that are raised after isolating *N. gonorrhoeae* from a child, and the recovery of gonococci from both genital and nongenital body sites, proper identification of isolates is imperative. Whittington and associates[696] reported on 40 bacterial isolates from children younger than 15 years of age that had been identified as *N. gonorrhoeae* and that were referred to the CDC for confirmation. Fourteen (35%) of these strains had been misidentified by the originating laboratory,

including 4 *N. cinerea*, 3 *N.lactamica*, 2 *N. meningitidis*, 3 *M. catarrhalis*, 1 *K. denitrificans*, and an unidentified nongonococcal *Neisseria* species. In 10 of the 14 cases, the organisms were isolated from children for whom there was no supporting evidence of sexual abuse. In many instances, the laboratories reporting these isolates were using the commercial kits described earlier for identification of *N. gonorrhoeae*. As mentioned previously, some commercial carbohydrate utilization test systems may produce false-positive glucose reactions for *N. cinerea*, which could lead to the misidentification of this species as *N. gonorrhoeae*.[91] Coagglutination tests, although generally quite reliable, may produce false-negative results with some gonococcal strains and may also show false-positive results with some isolates of other *Neisseria* or related species, including *N. lactamica*, *N. cinerea*, and *M. catarrhalis*. Procedural details for these tests differ among manufacturers, and package insert instructions must be followed closely. Enzymatic tests for detection of gonococcal prolyl iminopeptidase (PIP) must be used only for those neisserial isolates that are able to grow well on selective media. Some *N. cinerea* strains and occasional isolates of *N. subflava* bv. *perflava* may be recovered on selective media and may also be positive for prolyl -iminopeptidase in commercial systems.[329] Isolates recovered from oropharyngeal sites may be particularly troublesome. *N. lactamica*, a species that grows well on selective media and that may be misidentified as *N. gonorrhoeae*, colonizes the oropharynges of almost 60% of children between the ages of 1 and 4 years.[272]

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As mentioned previously, the CDC recommends that presumptive isolates of *N. gonorrhoeae* recovered from children, regardless of the site of isolation, should be confirmed by at least two tests that involve different principles. These may include carbohydrate utilization tests, immunologic methods (e.g., monoclonal antibody coagglutination tests), enzymatic procedures (e.g., chromogenic detection of specific enzyme activities), or the DNA probe culture confirmation test (AccuProbe *Neisseria gonorrhoeae*, Gen-Probe). Nonculture techniques, such as nucleic acid hybridization and NAATs, are investigational tests in children, so positive results with these assays must be confirmed with a culture. If a laboratory is unable to establish a definitive identification, the isolate should be sent to a reference laboratory for further testing. If possible, the isolate should also be saved in the event additional testing is needed. This can be accomplished by removing the growth from a few chocolate agar plates, suspending the growth in 1 to 2 mL of decomplemented horse serum:brain–heart infusion broth (1:1), and storing at −70°C.

Typing Methods for N. gonorrhoeae

Although the identification systems discussed in the previous section of this chapter and the biochemical characteristics in Table 11-2 are reliable for the routine identification of *N. gonorrhoeae* in the clinical laboratory, other techniques have contributed greatly to our understanding of the biology and epidemiology of the gonococcus. One of the first methods used for typing of gonococcal strains was auxotyping. Auxotyping of *N. gonorrhoeae* is based on the requirements of different *N. gonorrhoeae* strains for various nutritional requirements such as amino acids, purines, pyrimidines, and vitamins.[110,118] This method uses a completely chemically defined medium. Comparison of growth on the complete medium compared with media having individual deletions of these growth constituents define the auxotype. For example, if a strain failed to grow on the defined medium without proline, that strain was defined as a proline-requiring auxotype. Auxotyping was still being regularly used when serovar typing methods were described. Serovar typing is based on the antigenic differences of the PorB (also called PorI) OMPs encoded by the *porB* gene of *N. gonorrhoeae*.[373,656]Serovars are determined by coagglutination using a panel of monoclonal antibodies. With the serovar typing system used at the CDC *Neisseria* Reference Laboratory, two coagglutination reagents initially divided all gonococcal strains into two groups that expressed either the PorIA or the PorIB epitope. Strains that expressed PorIA were subdivided into 18 serovars, while strains that expressed PorIB were subdivided into 28 serovars.[373] Serovar determinations by coagglutination have higher discriminatory power than auxotyping and both methods were often used together (i.e., A/S, auxotype/serovar typing) to augment the auxotyping data and provide even more discriminatory power. Serovar typing was fast and easy to do, but reproducibility, subjectivity of reading coagglutination reactions, and the increasing prevalence of nontypeable strains due to changes in PorB proteins over time were distinct disadvantages of serovar typing. In addition, reagents for serovar typing are now either unavailable or too expensive. Both auxotyping and serotyping have been largely supplanted by molecular-based typing approaches.

Molecular typing methods for *N. gonorrhoeae* are generally more reproducible and provide better discrimination between and among gonococcal strains than auxotyping or serotyping. Adaptation of several gel analysis-based methods used to characterize other organisms have been applied to typing of gonococcal isolates These techniques included plasmid content analysis, and restriction fragment length polymorphism (RFLP) fingerprinting using pulsed-field gel electrophoresis (PFGE), ribotyping, and Opa typing. Plasmid analysis initially defined the characterization of some strains and included molecular weight determinations, and plasmid sequence analysis. This technique resulted in the discovery of cryptic plasmids, plasmids carrying genes for β-lactamase production and tetracycline resistance, and gonococcal plasmids that contained genetic information enabling gene transfer between strains (i.e., conjugative plasmids).[199,200,467] However, the ability of this method to differentiate gonococcal

strains was insufficient due to acquisition and loss of plasmids over time. RFLP/PFGE methods involve digestion of cellular DNA with rare-cutting restriction enzymes and subsequent separation of DNA fragment by gel electrophoresis. This method is highly discriminatory and can be used to define populations of gonococci, identify clusters in defined geographical areas, and in forensic evaluations.[184,649,709] The disadvantages of RFLP/PFGE approaches include cost, long turnaround times for results, and the need for considerable expertise in performing and interpreting the results. Ribotyping is a typing technique in which rRNA nucleic acid probes are used to detect ribosomal DNA sequences in gonococcal chromosomal DNA following restriction endonuclease digestion and DNA fragment separation by RFLP. This method has its greatest utility when coupled with other typing methods, but it is expensive and labor intensive to perform.[405,480] Opa typing involves amplification of the eleven *Opa* genes followed by restriction endonuclease digestion and gel elecrophoresis.[496] This method, like RFLP/PFGE methods is labor intensive, has a long turnaround time, and is not highly standardized. Nevertheless, *Opa* typing has been used to define gonococcal populations and clusters, to trace organism transmission, and to discriminate reinfection from treatment failures.[378,503] Opa typing may provide greater discriminatory ability when coupled with other sequence-based typing methods.[466,682] Typing of the *lip* gene has been used similarly to *Opa* typing.[639] This method involves amplification and sequencing of the *lip* genes, which encode an outer membrane lipoprotein of 18- to 30-kDa molecular weight that is found in all gonococci. Three *lip* genes have been identified and sequenced. The nucleotide sequences of these genes predict peptides that consist of an amino acid pentamer that is repeated 13 to 19 times. Variations in the numbers of lip repeats in a sequence, and the nature of the sequences themselves, can be used along with auxotype, serovar, antimicrobial susceptibilities, and other characteristics, to construct a highly discriminatory system for typing of individual isolates. Lip typing has been used along with PFGE in investigations of sexual abuse of children.[184]

Several gonococcal typing methods are based on sequence analysis of specific genes, and include *porB* sequencing, NG-MAST, and MLST. Full or partial sequence analysis of the *porB* gene provides a reproducible and highly discriminatory method for strain typing. *porB* sequencing has been used

to define regional gonococcal strains, identify disease contacts, and for study of gonococcal population genetics.[409,651] Sequencing of the *porB* gene can be accomplished by a variety of methods, including automated DNA sequencing instrumentation and pyrosequencing. The *porB* ST, based on sequencing of a specific

490 base pair region of the *porB* gene, is also a part of another typing method called *N. gonorrhoeae* multiantigen sequence typing (NG-MAST).[440] The NG-MAST approach uses sequence analysis of the variable internal fragments of the *porB* gene (490 base-pairs) and the *thpB* gene (390 base-pairs), which encodes the B-subunit of the transferrin-binding protein. NG-MAST is reproducible and provides robust discrimination between and among gonococcal strains, thereby providing unambiguous data that can be standardized for global comparisons of gonococcal strains. Because of the reproducibility of this method and the ease of performance, an online database of numbered STs is available to researchers (http://www.ng-mast.net). Strains with identical ST or NG-MAST types can be further differentiated by any of the methods already described (e.g., Opa typing, PFGE, etc.).[406,466] NG-MAST has been used for definition and identification of populations and clusters of *N. gonorrhoeae*, contact tracing, investigation of treatment failures, and forensic investigations.[72,182,406] MLST involves sequencing of seven or more conserved housekeeping genes.[510,681] MLST is a reproducible typing method, although the use of more than seven genetic loci may be necessary to provide greater discriminatory power when examining large numbers of strains. MLST has also been used to study the evolution of the gonococcus as a species over time.[65]

Molecular Methods for Detection of N. meningitidis

Molecular methods for direct detection of *N. meningitidis* in clinical specimens have also been described, but none are commercially available.[197] Lansac and colleagues described genus- and species-specific PCR identification assays using primer pairs derived from the aspartate β-semialdehyde gene (*asd*) of *Neisseria* species and a conserved OMP gene (*ctrA*) of *N. meningitidis*, respectively.[387] The first assay was 100% specific for identification of 321 of 322 strains representing 13 *Neisseria* species, while the second assay amplified DNA from 256 *N. meningitidis* isolates belonging to nine different serogroups. It is important that molecular assays detect the known serotypes as these are not predictable by location, as evidenced by Ibarz-Pavón and the SIREVA working group, which denoted that serogroup distribution is highly variable among Latin American and Caribbean countries.[255] Several PCR assays have also been described for direct detection of meningococci in clinical specimens. During an ongoing epidemic of serogroup B disease that began in Chile in 1987, Saunders and his research group developed a nested PCR assay that amplified the *porA* gene encoding the subserotype-specific protein.[561] Use of this assay on CSF specimens during the epidemic resulted in a test sensitivity of 96.7% with a specificity of 100%.[560] Molecular assays have been designed that detect the meningococcal insertion element IS*1106*, but, due to the mobility of these sequences and the

possibility of transfer to other neiserriae, molecular detection of IS*1106* may result in false-positive tests.[85] PCR methods that amplify multiple capsular polysaccharide genes (e.g., *ctrA* PCR assay) or that amplify serogroup-specific genes (e.g., the *siaD* PCR assay) have also been described.[83,84,401,402,519] Taha described a PCR assay performed directly on CSF for simultaneous meningococcal detection and serogrouping. The detection assay amplified a conserved gene involved in the regulation of meningococcal adherence to target cells (*crgA*), while a simultaneous multiplex PCR assay was performed with oligonucleotides in the *siaD* genes for serogroup B, C, Y, and W135 detection and in the *orf-2* gene cassette required for serogroup A capsular biosynthesis for serogroup A detection.[614]Orvelid and colleagues described a PCR-based method for detection of serogroup A meningococci in CSFs.[497] This assay used primers and probes specific for the gene cassette encoding the (α1-6)-linked *N*-acetyl-D-mannosamine comprising the serogroup A capsular polysaccharide. LightCycler-based PCR assays also have been described that are able to detect, identify, and perform *porA* gene serosubtyping of *N. meningitidis* directly from clinical specimens.[458]

Serogrouping and Typing of N. meningitidis

Identification procedures for meningococci (and gonococci as well) produce the best results when inoculated from fresh 18- to 24-hour subcultures on chocolate or blood agar. In carbohydrate utilization confirmatory tests, the acidic reaction in the maltose tube will frequently be stronger than that in the glucose tube because maltose is degraded by the organism to two glucose molecules, which are then metabolized. Glucose-negative, maltose-negative, and asaccharolytic *N. meningitidis* strains may also be occasionally isolated. An unusual maltose-positive, glucose-negative strain of *N. meningitidis* has also been recovered from a patient with septicemia.[690] If such biochemically aberrant strains are recovered, chromogenic substrate confirmatory tests or serogrouping of the isolates should be performed.

Slide agglutination is the most commonly used technique for serogrouping meningococci. A dense suspension of the organism is prepared in 0.5 to 1.0 mL of phosphate-buffered saline (PBS), pH 7.2, from a 12- to 18-hour subculture on trypticase–soy blood agar. One drop of this suspension is mixed with one drop of meningococcal antisera on a sectored slide, and the slide is rotated for 2 to 4 minutes. Groupable strains will generally agglutinate strongly within this time. Although isolates from systemic infections will usually agglutinate rapidly, those from carriers may fail to agglutinate (nongroupable strains) or may autoagglutinate in PBS. Use of younger cultures from blood agar (6 to 8 hours) or use of a serum-enriched media, such as trypticase–soy agar containing 10% decomplemented horse serum, may resolve these problems. Antisera for the major meningococcal serogroups are available from BD

Diagnostic Systems. Some of these nongroupable strains may actually be *N. polysaccharea*; testing for production of polysaccharide from sucrose will help identify this species (see later discussion).[88] Rare strains of *N. meningitidis* may also produce polysaccharide from sucrose due to acquisition of the gene for amylopectin production from *N. polysaccharea* by horizontal gene exchange.[723]

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In addition to serogroup determinations, *N. meningitidis* isolates may also be serotyped and serosubtyped on the basis of their OMP and LOS antigens. These techniques are used mainly for investigations of epidemics and sporadic outbreaks of disease and are not amenable for use in routine clinical microbiology laboratories. In addition to serologic techniques, several molecular techniques have been applied to investigations of meningococcal disease and to the epidemiology of *N. meningitidis* strains. These techniques were described in an earlier section of this chapter (see **Meningococcal Epidemiology: Serogroups, Serotypes, and Serosubtypes**).

Cultural Characteristics of Other *Neisseria* Species

The following sections present helpful features for the laboratory identification and characterization of the gram-negative cocci. Suggestions for the performance of differential and confirmatory tests described in detail in the previous section plus additional test procedures are also outlined. Characteristics for identification of *Neisseria* species and *M. catarrhalis* are shown in Table 11-2.

N. lactamica

N. lactamica resides in the upper respiratory tract and is found more frequently in children and adolescents than in adults.[49] *N. lactamica* grows on selective media and produces acid from glucose, maltose, and lactose. ONPG is also hydrolyzed and can be used as a substitute for lactose in the test battery. Some strains of this organism have been reported to cause false-positive reactions with some commercial coagglutination tests.[334,335] Penicillin-resistant strains of *N. lactamica* contain an altered PBP 2 that is similar to the PBP 2 found in relatively penicillin-resistant strains of *N. meningitidis*, suggesting that commensal species like *N. lactamica* (and *N. polysaccharea*) are the source of genetic resistance determinants currently being found in meningococci and gonococci.[421,551] A 2002 study of 286 *N. lactamica* isolates

found that all strains were considered to have intermediate susceptibility to penicillin (MIC 0.25 μg/mL), that 1.7% were resistant to ampicillin, and that 2.1% had decreased susceptibility to ciprofloxacin.[39] All isolates were susceptible to cefotaxime and ceftriaxone.

N. cinerea

Colonies of *N. cinerea* resemble the large-colony types of *N. gonorrhoeae* and may yield results consistent with *N. gonorrhoeae* in some kit identification systems.[91] *N. cinerea* grows on both blood and chocolate agar. On chocolate agar after 24 hours' incubation, colonies are about 1 mm in diameter and are smooth with entire edges. The organism does not produce acid from carbohydrates in either CTA-based media or the rapid carbohydrate degradation test. Weak positive reactions with glucose after overnight incubation have been reported in some identification systems and the positive hydroxyprolyl aminopeptidase reaction may also result in misidentifications of *N. cinerea* as *N. gonorrhoeae*. Most *N. cinerea* isolates, however, do not grow well on MTM or other selective media, which precludes testing of this organism on chromogenic substrate tests such as the Gonochek and the BactiCard *Neisseria*. *N. cinerea* can be differentiated from the asaccharolytic species *N. flavescens* by its inability to produce polysaccharide from sucrose (see later discussion) and the lack of a discernible yellow pigment. This species can also be separated from *M. catarrhalis*, another asaccharolytic species, by its negative reactions for nitrate reduction, DNase, and tributyrin hydrolysis (Table 11-2). A helpful test for differentiating *N. cinerea* from *N. gonorrhoeae* is the **colistin susceptibility test**. A suspension of the organism (0.5 McFarland turbidity standard) is prepared in broth and is swabbed onto a chocolate or blood agar plate as for a disk diffusion susceptibility test. A 10-mg colistin disk is placed on the inoculum, and the plate is incubated in CO_2 for 18 to 24 hours. N. cinerea is colistin-susceptible and will have a zone that is larger than or equal to 10 mm around the disk. Generally, *N. gonorrhoeae* will grow up to the edge of the disk.

Neisseria subflava *biovars*, N. mucosa, *and* N. sicca

Identification of the "nonpathogenic" *Neisseria* species is not generally necessary unless the organism is determined to be clinically significant, or if the organism is isolated from a systemic site (e.g., blood, CSF) or in pure culture. Identification is based on colony morphology, growth on simple nutrient medium, inability to grow on selective media, acid production from carbohydrates, reduction of nitrate and nitrite, and synthesis of a starch-like, iodine-staining polysaccharide from sucrose. **Nitrate reduction** and **nitrite reduction** are determined in medium (tryptic soy or heart infusion broth) containing 0.1% (w/v) KNO_3 and 0.01% (w/v) KNO_2, respectively. **Polysaccharide synthesis** is determined by inoculating the organism onto brain–heart

infusion agar containing 5% sucrose. Medium lacking sucrose is inoculated as a negative control. After incubation at 35°C for 48 hours, the plates are flooded with Gram's or Lugol's iodine (1:4 dilution). A positive test is indicated by the development of a deep blue color in and around the colonies synthesizing the polysaccharide. We have also obtained excellent results by adding regular Gram's iodine (1 to 2 drops) to the sucrose-containing tube in the rapid carbohydrate degradation technique after 4 hours incubation. If positive, a deep blue color appears in the tube. This is compared with the tan color seen in the other carbohydrate tubes (e.g., the maltose tube) after addition of Gram's iodine.

Strains of *N. subflava* can be subdivided into three biovars (*subflava*, *flava*, and *perflava*) on the basis of acid production from fructose and sucrose and synthesis of iodine-positive polysaccharide from sucrose (Table 11-2). All three biovars reduce nitrite, but not nitrate. *N. mucosa* has a carbohydrate utilization pattern similar to *N. subflava* bv. *perflava* and also produces the iodine-positive polysaccharide, but *N. mucosa* is able to reduce both nitrate and nitrite to nitrogen ($N_{2)}$ gas. All of these organisms also display varying degrees of yellow pigmentation. The *N. sicca* strains are biochemically identical with *N. subflava* bv. *perflava*, but they characteristically form dry (desiccated), adherent, leathery colonies on agar media that cannot be emulsified readily.

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N. polysaccharea

N. polysaccharea is found in the human oropharynx. This organism is an oxidase-positive, catalase-positive, gram-negative diplococcus that forms smooth yellow colonies.[520] In the original description of this organism, the ability to grow on selective media (e.g., MTM agar) was a key characteristic. Subsequent studies indicate, however, that growth on selective media for the pathogenic *Neisseria* is a variable characteristic of *N. polysaccharea* because of the colistin susceptibility of some strains.[23] Strains that are able to grow on selective media have colistin MICs of 64 µg/mL or greater, whereas strains that are inhibited have colistin MICs of 1 µg/mL or less. After 24 hours growth, *N. polysaccharea* forms colonies of about 2 mm in diameter on chocolate or blood agar. Acid is produced from glucose and maltose but not from fructose or lactose. Acid production from sucrose is variable and appears to depend on the types of media used to determine this characteristic. *N. polysaccharea* also possesses an amylosucrase that synthesizes an acidic extracellular polysaccharide from sucrose.[105,520] The polymer is composed mainly of α1,4-linked D-glucopyranosyl residues along with about 6% O-substituted α4,6 D-glucopyranosyl branch points.[539] Production of various amounts of the material by different strains

may explain the variable nature of the sucrose reaction.[533,534] Nitrate is not reduced, whereas nitrite frequently is reduced. *N. polysaccharea* can be differentiated from *N. meningitidis* by polysaccharide synthesis and the γ-glutamylaminopeptidase tests. *N. polysaccharea* produces iodine-positive polysaccharide from sucrose and is γ-glutamylaminopeptidase-negative, whereas *N. meningitidis* does not produce iodine-positive polysaccharide from sucrose and is γ-glutamyl aminopeptidase-positive.[23,88] Like *N. gonorrhoeae*, *N. lactamica*, and some *N. meningitidis* strains, *N. polysaccharea* is prolyl aminopeptidase-positive. The organism requires cysteine for growth and does not grow on nutrient agar or on chocolate agar at 22°C.

N. flavescens

N. flavescens is found in the respiratory tract and is rarely associated with infectious processes. This organism grows as smooth, yellowish colonies on both blood and chocolate agar. In addition to growth on nutrient agar at 35°C, most strains will also grow at room temperature on chocolate or blood agar. This organism is able to synthesize iodine-positive polysaccharides from sucrose (see later discussion) and can be differentiated from *M. catarrhalis* by its inability to reduce nitrate and its negative DNase and tributyrin hydrolysis reactions.

N. elongata Subspecies

N. elongata subspecies *elongata, glycolytica*, and *nitroreducens* are rod-shaped members of the genus *Neisseria*.[89,282,704] All subspecies are members of the human upper respiratory tract flora and all have been isolated from infectious processes.[473,704] These subspecies can be differentiated on the basis of catalase reactivity, acid production from glucose, and reduction of nitrate (Table 11-2).

N. weaveri

N. weaveri is an oxidase- and catalase-positive rod that is found in the oral cavity of dogs. It does not produce acid from carbohydrates and does not reduce nitrate, but does reduce nitrite to nitrogen gas. DNase is not produced and tributyrin hydrolysis is negative.

N. bacilliformis

N. bacilliformis is a rod-shaped *Neisseria* species that is oxidase-positive and catalase-variable.[293] The organism grows on blood and chocolate agar, forming light-yellow pigmented, smooth colonies. No growth is seen on selective media such as MTM medium. *N. bacilliformis* is assacharolytic in carbohydrate utilization tests, and is both nitrate reductase- and tributyrin hydrolysis-variable. This organism may be

misidentified as a *Moraxella* species with the API 20NE system.

N. animaloris *and* N. zoodegmatis

N. animaloris and *N. zoodegmatis* were formerly called CDC EF-4a and EF-4b, respectively.[660]These gram-negative coccobacilli are found in the mouths of dogs and have been isolated from dog bite wounds. Both of these organisms are gram-negative coccobacilli that are weakly hemolytic on blood agar, are oxidase- and catalase-positive, and produce acid from glucose only. Both species grow on MacConkey agar and neither produce indole, acetoin (VP test), urease, ornithine decarboxylase, lysine decarboxylase, or β-galactosidase. Both species reduce nitrate to nitrite, but only *N. animaloris* reduces nitrite to nitrogen gas. In addition, *N. animaloris* is arginine dihydrolase (ADH)-positive, while *N. zoodegmatis* is ADH-negative. Genotypically, these species are most closely related to *N. canis* and *N. dentiae*.

N. wadsworthii

This species was described in 2011 and is based on two isolates that were recovered from a hand wound and a peritoneal fluid specimen in 2005 in New York.[702] This organism is gram-negative, coccoid, is oxidase- and catalase-positive, and reduces nitrate to nitrite. On Gram's staining, pairs and short chains may be seen. Colonies are small, are nonhemolytic, and have a yellow-to-orange pigment. On the API NH system, *N. wadsworthii* produces acid from glucose, but not from fructose, maltose, or sucrose. Reactions for ornithine decarboxylase, urease, alkaline-phosphatase, indole, lipase, and β-galactosidase are negative. On this identification system, the proline arylamidase (prolyl iminopeptidase) is positive and γ-glutamyl aminopeptidase is variable.

N. shayeganii

This species is based on two isolates recovered from a sputum specimen and an arm wound.[702] This rod-shaped organism forms small, nonhemolytic, yellow-to-gray colonies on agar media. Like *N. wadsworthii*, the organism is oxidase- and catalase-positive and reduces nitrate to nitrite. On the API NH, this organism produces acid from glucose and is γ-glutamyl aminopeptidase-positive. No acid is produced from maltose, fructose, or sucrose, and reactions for urease,

ornithine decarboxylase, β-galactosidase, alkaline-phosphatase, lipase, and indole production are negative. The prolyl iminopeptidase reaction is variable and strain-dependent.

Cultural Characteristics and Identification of *Moraxella catarrhalis*

M. catarrhalis grows well on both blood and chocolate agars, and some strains will also grow well on MTM and other selective media. Colonies are generally gray-to-white, opaque, and smooth. The organism is asaccharolytic in carbohydrate degradation tests and may actually turn peptone-based identification media alkaline. Most strains reduce nitrate and nitrite and produce DNase. The DNase activity is detected by heavily spot-inoculating a plate of DNase test medium containing toluidine blue O on an area the size of a dime. After overnight incubation, hydrolysis of the DNA is detected by a change in the color of the media around and under the inoculum from blue to pink. *S. aureus* and *S. epidermidis* strains are also inoculated onto the plate as positive and negative test controls, respectively.

M. catarrhalis may also be distinguished from *Neisseria* species by its ability to hydrolyze ester-linked butyrate groups (butyrate esterase).[594] This enzyme activity is detected with a substrate called tributyrin. A rapid fluorescent tributyrin hydrolysis test that uses 4-methylumbelliferyl butyrate as a substrate was reported by Vaneechoutte and coworkers.[669] In this study, all 62 *M. catarrhalis* strains were positive with this test within 5 minutes, whereas all other *Neisseria* species tested were negative. A very rapid (2.5 minute) and reliable indoxyl butyrate hydrolysis spot test has also been described and is commercially available (Remel Laboratories; Carr-Scarborough, Stone Mountain, GA; Image Plate 11-2G).[174,416] This same test is also included on the BactiCard *Neisseria* along with the three other chromogenic substrates for *Neisseria* identification (see Image Plate 11-2B).[329] The RapID NH system also contains a fatty acid ester hydrolysis test to assist in the identification of *M. catarrhalis* (see Image Plate 11-2C). Indoxyl acetate, which is used for the identification of *Campylobacter* species, can also be used as a substrate for the esterase enzyme of *M. catarrhalis*.[329,594] In addition, Most clinically significant *M. catarrhalis* strains also produce an inducible, cell-associated β-lactamase (see below).[205] Because of its inducible nature, rapid acidometric β-lactamase tests (i.e., those that rely on conversion of hydrolysis of penicillin to penicilloic acid) may yield false-negative results. Best results are obtained with the iodometric method or with the chromogenic cephalosporin test.

Antimicrobial Susceptibility of *Neisseria* Species

N. gonorrhoeae

Prior to the mid-1970s, penicillin was the drug of choice for the treatment of gonococcal infections, with tetracycline and macrolides being used for treatment of patients with penicillin allergy.[403,706] Incremental increases in the MICs of penicillin for *N. gonorrhoeae* isolates lead to several modifications of the US Public Health Service treatment schedules over the years. In 1976, strains of *N. gonorrhoeae* with high-level resistance to penicillin were imported into the United States from West Africa, and the Far East. These isolates contained plasmids that carried genes for a β-lactamase enzyme and were given the moniker "PPNG" for penicillinase-producing *N. gonorrhoeae*. During the mid- to late 1970s and into the 1980s, the number of cases of gonorrhea caused by PPNG strains increased 15-fold, with large outbreaks occurring in NYC, Los Angeles, and Miami, and these strains became endemic in several metropolitan areas of the United States. Plasmid-mediated resistance to tetracycline also appeared during this time.[706] At that time, spectinomycin was recommended for the treatment of these infections. By 1981, however, several spectinomycin-resistant *N. gonorrhoeae* isolates had been reported, and subsequently, spectinomycin-resistant PPNG isolates also appeared.[40,721] At the same time, strains of *N. gonorrhoeae* that were penicillin-resistant but β-lactamase-negative were also documented. Because of the increasing resistance of *N. gonorrhoeae* to previously recommended antimicrobial agents and based on several controlled clinical efficacy trials, the US Public Health Service recommended that all patients with uncomplicated gonococcal infection receive 1 of 5 single-dose treatment regimens.[126] These include ceftriaxone (125 mg IM), cefixime (400 mg PO), ciprofloxacin (500 mg PO), ofloxacin (400 mg PO), or levofloxacin (250 mg PO). Each of these therapies also included azithromycin (1 g PO) or doxycycline (100 mg PO, twice a day for 7 days) for treatment of co-infecting *Chlamydia trachomatis*.

In Europe, fluoroquinolone agents were being used for the treatment of gonococcal infections in the mid-1980s, and, beginning in 1986, treatment failures with enoxacin and ciprofloxacin were being reported from the Netherlands and the United Kingdom.[280,684] In Japan, treatment failures with the fluoroquinolones were first reported in 1994, and by the late 1990s, more than 80% of gonococcal isolates in Japan were resistant or intermediately susceptible to fluoroquinolones.[181] Subsequently, other treatment failures were documented in patients treated with fluoroquinolones in the Far East, Canada, and Australia.[178,345,625,627] In the United States, fluoroquinolone resistance was first noted in Hawaii among *N. gonorrhoeae* strains from heterosexuals, likely due to importation from Asia. During the next few years, fluoroquinolone-resistant *N. gonorrhoeae* strains appeared in California among MSM, necessitating revision of treatment recommendations for MSM populations.[123,125,129,130,372]Gonococcal strains that have decreased susceptibility or

that are resistant to the fluoroquinolones have been now been documented worldwide.[157,375,620,627] Fluoroquinolone-resistant *N. gonorrhoeae* strains were also resistant to penicillin and many were resistant to tetracycline as well; all strains were susceptible to ceftriaxone and cefixime.[180]Fluoroquinolone-resistant isolates have gene mutations that result in amino acid substitutions in the A and B subunits (*GyrA* and *GyrB*, respectively) of DNA gyrase, and in the topoisomerase IV *parC*-encoded subunit.[61,179] In the United States, the prevalence of fluoroquinolone-resistant gonococci was less

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than 1% prior to 2001, but increased to 2.2% in 2002, 4.1% in 2003, 6.8% in 2004, and 9.4% in 2005.[132] In a collection of over 3,000 strains isolated between January and June, 2006, 13.35% were resistant to the fluoroquinolones. Consequently, in 2007, the CDC discontinued recommending fluoroquinolone regimens for the treatment of gonorrhea.[133] In the 2010 CDC guidelines, treatment for uncomplicated gonococcal infection of the genital tract includes ceftriaxone (250 mg single dose, IM), cefixime (400 mg single dose, PO), or a single-dose injectable cephalosporin, plus either azithromycin (1 g single dose, PO) or doxycycline (100 mg, bid, 7 days).[140] DGI due to a ciprofloxacin-resistant gonococcal strain has also been reported.[170] The isolate in this case was susceptible to ceftriaxone (MIC 0.002 μg/mL), but resistant to penicillin (MIC > 32 μg/mL) and tetracycline (MIC 24 μg/mL).[170]

During 2000 through 2010, the Gonococcal Isolates Surveillance Project (GISP) documented the emergence of gonococcal strains with elevated MICs to both ceftriaxone (MIC ≥ 0.125 μg/mL) and cefixime (MIC ≥ 0.25 μg/mL). Strains with elevated MICs for ceftriaxone comprised only 0.1% of isolates in 2000 and 0.3% of isolates in 2010. For cefixime, such strains comprised only 0.2% of isolates in 2000 and 1.4% of isolates in 2010.[142] During the same 2000 to 2010 time period, the emergence of similar strains occurred in Canada.[439]As a corollary to increasing in vitro resistance to these agents, gonococcal treatment failures were documented in patients treated with either cefixime or ceftriaxone in Asia, Norway, and Japan.[489,624,626,650] Initially, reports from Japan described treatment failures in several men who were treated with cefixime, an oral cephalosporin.[181,714] Isolates from these patients had elevated MICs to cefixime (0.5 to 1.0 μg/mL) and ceftriaxone (0.125 to 0.5 μg/mL) by Etest methods. In Japan the proportion of gonococcal isolates with elevated MICs to cefixime (≥0.5 μg/mL) increased from 0% in 1999/2000 to 30% by 2002.[326] In China, the proportion of *N. gonorrhoeae* strains with elevated MICs of ceftriaxone was 18% in 1999 and doubled to 38% by 2006.[608] In Australia, such strains

comprised about 2% of the isolates recovered in 2008.[45] Increases in MICs to the cephalosporins have not been as dramatic in Europe as in other places. In the Netherlands, about 6% of isolates obtained from STD clinic patients between 2006 and 2008 had elevated cefotaxime MICs (0.125 to <0.5 μg/mL) by Etest.[192] These investigators also found that MSM were more likely to be infected with gonococcal strains having elevated MICs to the cephalosporins than heterosexual males. Confirmed cefixime treatment failures have been documented in both heterosexuals and MSM in Norway.[650] These cases were the first confirmed oral cephalosporin treatment failures reported outside of Japan. Multidrug-resistant gonococcal isolates with decreased susceptibilities to cefixime were isolated in 2001 from three patients in Hawaii.[689] According to data from GISP, US isolates with elevated MICs to ceftriaxone (MIC ≥ 0.06 μg/mL) increased from 0.6% in 2006 to 2% in 2008.[139] At the present time, CLSI defines decreased susceptibility to cefixime and ceftriaxone as those isolates with MICs ≥ 0.5 μg/mL.[140]

Strains of *N. gonorrhoeae* that have reduced susceptibility to the cephalosporins generally have mutations in the *penA* gene that affect PBP 2. Mutations of the *penA* gene are termed "mosaic" mutations because multiple mutations occurring at this genetic site incrementally affect the binding of cephalosporin molecules. Isolates of *N. gonorrhoeae* that have mutations of *penA* with mosaic arrangements display decreased susceptibility to both parenteral and oral cephalosporins.[20,364,487,498] Mutations in other chromosomal genes (e.g., the *ponA* gene encoding PBP 1, the *mtr* gene encoding a gonococcal efflux pump, and *penB*, which encodes proteins in the PorB1b outer membrane porin) may also result in or can contribute significantly to reduced cephalosporin susceptibility.[395,409,621,694] Mutations in the *ponA* gene results in decreased cephalosporin binding by PBP 1, while mutations in the *mtrR* gene decrease intracellular antimicrobial concentrations via increased expression and activity of antimicrobial efflux mechanisms. Mutations in *penB* result in an altered PorB1b porin structure and reduced permeability of the cells to antimicrobial agents, including cephalosporins. Isolates with mosaic *penA* mutations may display a range of cephalosporin susceptibilities, with mutations in the other genes incrementally resulting in higher MICs.[490]The *penA* genes that express mosaic mutations are also found in certain commensal *Neisseria* species that are found in the oropharynx, including *N. cinerea*, *N. subflava* bv *perflava*, *N. polysaccharea*, and some strains of *N. meningitidis*. Resistance determinants found in *N. gonorrhoeae* may have been acquired from these commensal species by genetic transformation.

In January of 2009, the first high-level ceftriaxone-resistant gonococcal strain was isolated from the pharynx of a female commercial sex worker in Kyoto, Japan.[488,489] This isolate had a ceftriaxone MIC of 2 μg/mL. Prior to this time, only one isolate with a ceftriaxone MIC of 0.5 μg/mL had been reported previously.[621] Further

examination of this isolate revealed resistance to all β-lactam antimicrobial agents (except for the carbapenems [meropenem, ertapenem] and piperacillin-tazobactam, which are not recommended treatments) and was negative for β-lactamase.[488] The MICs of ceftriaxone were 2 to 4 μg/mL (by Etest, CLSI, and WHO methods), and the MIC of cefixime was 8 μg/mL. This resistant strain possessed a unique resistance determinant ($penA_{H041}$ for strain H041) along with several previously described resistance mutations. The $penA_{H041}$ locus was similar in sequence to loci of cefixime-resistant isolates recovered in Japan from cefixime treatment failures that had only modest increases in the MIC of ceftriaxone (i.e., 0.064 to 0.125 μg/mL).[488,489] Transformation tests confirmed that transfer of this unique allele resulted in the transfer of high-level resistance to ceftriaxone and other extended-spectrum cephalosporins to previously susceptible isolates. The presence of the additional resistance determinants provided sufficient alterations of penicillin-binding proteins to boost the MICs of ceftriaxone by 16-fold to 500-fold. This strain was also resistant to tetracycline, the macrolides, the fluoroquinolones, trimethoprim–sulfamethoxazole, and chloramphenicol and was susceptible to spectinomycin and rifampin.[488] The potential for the development of resistance to these agents is of great concern, since no other well-studied and effective antimicrobial treatment options or combination therapies are available at this time. CDC and PHS have stated that clinicians seeing patients with gonococcal infections must be vigilant for possible treatment failures among their patients who are being treated with the CDC/PHS-recommended regimens. Clinical specimens from these patients must be

cultured and gonococcal isolates recovered from these specimens should be forwarded to a reference laboratory so that antimicrobial susceptibility testing can be performed. The continued emergence of *N. gonorrhoeae* strains that are resistant to the last remaining chemotherapeutic agents is an incredible challenge to the healthcare community and the world.[403] New, multifaceted strategies are needed for gonorrhea prevention and control that include surveillance for antimicrobial resistance, enhanced primary (e.g., sexually active women, MSM, culture of genital/nongenital sites) and secondary (e.g., routine testing among high-risk groups, expedited partner notification/therapy) screening, and development of new approaches/agents for treatment (e.g., combination therapies, provider/public/private partnerships and collaborations, legislative/regulatory industry incentives for drug development).[403,623,624,626,706]

Azithromycin is also effective for the treatment of uncomplicated gonococcal infections, but emergent resistance to macrolides in *N. gonorrhoeae* is a real

concern.[71,406,629] In the past, a 2-g oral dose of azithromycin has been used to treat gonococcal infections, and a 1-g oral dose is currently given to treat chlamydial infections following treatment for gonorrhea. Clinical treatment failures using a 1-g single-dose azithromycin treatment regimen have been documented in patients infected with gonococcal strains with azithromycin MICs of 0.125 to 0.5 μg/mL.[634] Such strains with decreased susceptibility to azithromycin have been documented in the United States and other countries since the mid-1990s.[706] According to the 2007 GISP data, 0.4% of isolates collected in the United States had azithromycin MICs of≥2 μg/mL, and 30% of isolates had azithromycin MICs of ≥0.5 μg/mL.[135] In Japan, over 60% of *N. gonorrhoeae* strains isolated in 2007 to 2008 had azithromycin MIC of ≥0.5 μg/mL. In 2004, gonococcal strains with high-level azithromycin resistance (MIC ≥ 256 μg/mL) were isolated in Scotland and, over the next 3 to 4 years, the same strains appeared in England, Wales, and Italy.[154,506,601] In Scotland, the percentage of isolates with high-level azithromycin resistance increased from 0.3% in 2004 to 3.9% in 2007. Given the increasing prevalence of gonococci with elevated azithromycin MICs and the emergence of strains with high-level resistance to this agent, it is doubtful that a single 2-g dose of azithromycin will be effective for treatment of gonococcal infections caused by these particular isolates, and widespread use of azithromycin may lead to the further development of resistance.[706] Strains with decreased susceptibility to azithromycin and other macrolides usually have mutations, deletions, or insertions in genes encoding efflux pump mechanisms, mutations in one or more of the 23S rRNA methylase *erm* genes, or ribosomal mutations in 23S rRNA.[481,537,718] CLSI has not established breakpoints for susceptibility testing of *N. gonorrhoeae*, although GISP places isolates with azithromycin MICs of ≥2 μg/mL as having decreased susceptibility to this agent.[135]

The CLSI currently recommends either agar dilution or disk diffusion for antimicrobial susceptibility testing of *N. gonorrhoeae*.[160] The recommended medium is GC agar base plus 1% defined growth supplements (e.g., Isovitalex). Cysteine-free growth medium must be used for testing the carbapenems or drugs containing clavulanate. The inoculum, equivalent to a 0.5 McFarland turbidity standard, is prepared in Müeller–Hinton broth or 0.9% PBS, pH 7.0, from a direct colony suspension using growth from a 20- to 24-hour chocolate agar plate incubated in 5% CO_2. The agar dilution plates are incubated at 36°C ± 1°C in 5% CO_2 for 20 to 24 hours. The disk diffusion procedure uses GC agar base with 1% defined growth supplements (cysteine-free medium is not required for testing carbapenems and clavulanate-containing drugs by the disk diffusion method). Breakpoints are available for interpretation of both agar dilution and disk diffusion procedures. Gonococcal isolates with ceftriaxone MICs of ≤0.25 μg/mL are considered susceptible. In addition to CLSI procedures for disk diffusion and agar dilution, Etest (bioMérieux, Inc.)

methods for antimicrobial susceptibility testing of *N. gonorrhoeae* have also been published.[668,712]

N. meningitidis

Despite the occasional recovery of *N. meningitidis* strains with decreased susceptibility to penicillin, penicillin G remains the drug of choice in the United States for treatment of meningococcal meningitis because the vast majority of strains are susceptible to penicillin and ampicillin. For treatment of serious meningococcal infections, parenteral penicillin G (300,000 units/kg/day) is administered for 10 to 14 days. Chloramphenicol (100/mg/kg/day up to 4 g/day) is an alternative in penicillin-allergic patients. The third-generation cephalosporins (ceftriaxone, cefotaxime, and ceftazidime) achieve levels in CSF several orders of magnitude greater than the susceptibility of the meningococcus to these agents. The duration of antibiotic therapy may vary somewhat with the manifestation of the disease and with the response of the patient. At present, when the meningococcus is sensitive to the agents just mentioned, 10 to 14 days of therapy is usually sufficient. Ceftriaxone is currently the third-generation cephalosporin of choice for the treatment of meningococcal disease. For children, ceftriaxone is given at a dosage of 25 mg/kg every 12 hours, IV, up to 1 g. For adults, 1 g ceftriaxone is administered IV every 12 hours. In addition to antimicrobial therapy, patients with serious meningococcal disease require intensive supportive care, including management of the shock state, and careful monitoring of vital signs for detection of complications (e.g., ARDS, neurologic sequelae, pericarditis) and disease progression (e.g., coagulation studies for DIC).[665] New adjunctive therapies, such as recombinant human bactericidal/permeability-increasing protein show promise in the prevention of multiorgan system failure due to endotoxemia.[269] Other biologic agents, such as monoclonal antibodies directed against endotoxin or against cytokines involved in meningococcal septic shock (e.g., IL-1, IL-6, TNF-α), may also play a role as adjunctive therapeutic interventions.[187,665,683]

Despite the fact that the vast majority of strains, particularly in the United States, remain susceptible to penicillin, evidence is accumulating that the antimicrobial susceptibility profiles of *N. meningitidis* strains are evolving. Historically, penicillin-susceptible strains of *N. meningitidis* have penicillin MICs of ≤0.06 μg/mL. In 1983, Dillon and associates[199] in Canada isolated the first β-lactamase-producing meningococcal strain from a urogenital specimen. This isolate harbored the gonococcal 4.5 Mdal β-lactamase plasmid and the 24.5 MDa conjugative plasmid. Subsequently, only three additional β-lactamase-producing *N. meningitidis* isolates

have been reported in the literature: two strains were isolated in 1988 from two patients with meningitis in South Africa, and the fourth isolate was recovered in 1989 from a patient in Spain.[87,252] β-Lactamase-positive strains have penicillin MICs of >256 μg/mL. In 1987, an *N. meningitidis* isolate was recovered in Spain that had diminished susceptibility to penicillin (MIC > 0.06 μg/mL) but was β-lactamase-negative.[549] These strains are referred to as relatively resistant, moderately susceptible, or as having diminished susceptibility to penicillin. Most of the relatively resistant meningococci that have been reported have belonged to either serogroup B or C. Since that time, additional isolates with similar characteristics have been reported in Canada, the United Kingdom, Europe, Greece, South Africa, Belgium, and the United States.[68,76,95,99,214,647,654,671,705] In 1997, active surveillance by the CDC accumulated 90 isolates from 121 cases of reported meningococcal disease. Of these, 3 isolates (<3%) were moderately susceptible to penicillin, with MICs of 0.12 μg/mL.[545] Of the remaining 87 penicillin-susceptible strains, 49 had MICs of 0.06 μg/mL. While penicillin-susceptible strains have MICs of ≤0.06 μg/mL, strains with decreased susceptibility have penicillin MICs ranging from 0.10 μg/mL to 1.0 μg/mL.[545,550] In studies that have examined the prevalence of these strains, decreased susceptibility to penicillin has been defined as those strains with penicillin MICs of ≥0.125 μg/mL. *N. meningitidis* with decreased susceptibility to penicillin are now found worldwide.

Diminished susceptibility to penicillin in moderately resistant *N. meningitidis* strains is apparently due to decreased binding of penicillin by altered meningococcal cell wall PBP 2, which is encoded by the meningococcal *penA* gene.[33,364,617,636] Similar low-affinity forms of PBP 2 are found in penicillin-resistant strains of other *Neisseria* species, including *N. lactamica*, *N. flavescens*, *N. polysaccharea*, and *N. gonorrhoeae*. The altered, low-affinity forms of PBP 2 in *N. meningitidis* strains apparently arose from recombinational events that resulted in the replacement of sequences in the native meningococcal *penA* gene with corresponding genetic material from the commensal *Neisseria* species.[90,551,598] These sequence alterations in the native meningococcal *penA* gene result in *penA* genes with mosaic structures containing commensal *Neisseria* gene sequences. Only a few amino acid changes in the *penA* gene are necessary to decrease the affinity of PBP 2 for penicillin.[597] The clinical significance of diminished penicillin susceptibility in *N. meningitidis* is unclear at present. Although both treatment failures and higher rates of complications have been observed in patients infected with relatively resistant strains, the administration of higher doses of penicillin has been clinically effective.[646] Third-generation cephalosporins (i.e., ceftriaxone, cefotaxime) are active against both penicillin-susceptible and moderately penicillin-resistant *N. meningitidis* strains, but the MICs of some agents for moderately susceptible strains—particularly cefuroxime, aztreonam,

and imipenem—may be significantly higher than those of susceptible strains. In 2001, a serogroup B strain with a penicillin MIC of 2 μg/mL was reported from Poland, and in 2005, a serogroup C meningococcal isolate with a penicillin MIC of 1.5 μg/mL by Etest was isolated from a case of meningitis in Belgium.[234,286] With the increasing prevalence of *N. meningitidis* strains with decreased susceptibility to penicillin and the appearance of resistant strains with high penicillin MICs, the role of penicillin in the treatment of meningococcal disease will most definitely undergo dramatic changes in the next several years.

Recently, reports have appeared documenting fluoroquinolone resistance among *N. meningitidis* isolates. In 2000, an *N. meningitidis* strain with decreased susceptibility to ciprofloxacin (MIC of 0.25 μg/mL) was isolated from a 19-year-old female with invasive meningococcal disease in Australia.[567] Susceptible strains usually have ciprofloxacin MICs of ≤0.03 μg/mL. PCR amplification and sequencing of the *gyrA* gene of this isolate revealed a three-nucleotide difference from wild type, ciprofloxacin-susceptible strains. Decreased susceptibility to fluoroquinolones, particularly ciprofloxacin, has been reported in serogroup B *N. meningitidis* in France and Spain, serogroup C meningococci from Australia, serogroup Y strains from Argentina, and serogroup A meningococci from India.[15,152,166,324,567,584] A case of meningitis due to a ciprofloxacin-resistant serogroup A strain was also documented in a traveler from Italy who acquired the infection in New Delhi, India.[388] As mentioned previously, three cases of meningococcal disease due to ciprofloxacin-resistant serogroup B strains were reported among residents of North Dakota and Minnesota during 2007 and 2008.[136,708] The first case was epidemiologically linked to a case that occurred in the same region in 2006, except that the particular strain lacked the *gyrA* mutation that confers resistance to fluoroquinolones. Mutations in *gyrA* appear to be the genetic events that result in fluoroquinolone resistance, as efflux pump mechanisms in these strains appear to be intact.[117] Consequently, ciprofloxacin is no longer recommended for meningococcal chemoprophylaxis in regions of the United States where resistant strains have been isolated.*N. meningitidis* strains have also demonstrated resistance to other antimicrobial agents. High-level chloramphenicol resistance has been reported in isolates from France and Vietnam.[256]Fear of the spread of chloramphenicol resistance is justified since the drug is a mainstay of therapy for meningitis in sub-Saharan Africa.[592] High-level resistance to sulfonamides, the drug formerly used for prophylaxis, is now widespread and may be found commonly among certain epidemic clones of serogroup A *N. meningitidis*.[592] In a study conducted in Spain, 43.6% of 55 strains from both cases and carriers were resistant to trimethoprim–sulfamethoxazole.[251] The emergence of rifampin resistance has also been observed even during the administration of rifampin prophylaxis.[165] Rifampin resistance emerges either due to alterations in cell membrane permeability or to

mutations in the *rpoB* gene that codes for the β-subunit of the meningococcal RNA polymerase.[1,602] Tetracycline resistance among meningococci is due to acquisition of the *tetM* tetracycline resistance determinant.[645]

The CLSI has published microbroth dilution, agar dilution, and disk diffusion procedures for standardized antimicrobial susceptibility testing of *N. meningitidis*.[161,675] The microbroth dilution method uses cation-supplemented Müeller–Hinton broth with lysed horse blood (2.5% to 5%, v/v), while both agar dilution and disk diffusion methods use Müeller–Hinton agar supplemented with 5% sheep's blood. The inoculum for all three procedures is prepared from a direct colony suspension adjusted to a 0.5 McFarland turbidity standard from a chocolate agar subculture incubated at 35°C

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in 5% CO_2. The tests are incubated for 20 to 24 hours at 35°C ± 2°C in a 5% CO_2 atmosphere. MIC breakpoints and MIC correlates for the disk diffusion procedure are available for penicillin and ampicillin, cefotaxime, ceftriaxone, meropenem, azithromycin, minocycline, fluoroquinolones (i.e., levofloxacin, ciprofloxacin), trimethoprim–sulfamethoxazole, sulfisoxazole, chloramphenicol, and rifampin. The Etest may also prove valuable in determining the antimicrobial susceptibility of individual meningococcal isolates.[169,436,676]

Antimicrobial Susceptibility of *Moraxella catarrhalis*

The appearance and spread of resistance to antimicrobial agents among the pathogenic *Neisseria* are also reflected in the antimicrobial susceptibility of *M. catarrhalis* isolates. Prior to the mid-1970s, this organism was broadly susceptible to antimicrobial agents. β-Lactamase-positive *M. catarrhalis* were first isolated in 1976; by the end of the decade, about 75% of strains produced β-lactamase enzymes. Worldwide, the percent of β-lactamase-positive *M. catarrhalis* strains ranges from 80% to 100%.[60] A study by Doern and Tubert demonstrated that the chromogenic cephalosporin (nitrocefin) disk and tube tests had superior sensitivity for detection of β-lactamase and the nitrocefin disk chromogenic cephalosporin test was adopted by many laboratories.[205] Studies of *M. catarrhalis* β-lactamase enzymes have shown that three types of enzymes are found in strains of this organism. These enzymes are called BRO-1 (or Ravisio-type) and BRO-2 (or 1908-type), and BRO-3.[81,228,566]BRO-3 is a

membrane-bound precursor of the other two BRO enzymes. The "BRO" moniker is a contraction of "*BR*anhamella" and "*mOraxella*," since similar β-lactamases are found in rod-shaped moraxellae as well. Strains of *M. catarrhalis* produce either BRO-1 or BRO-2, and these enzymes can be differentiated by isoelectric focusing. Strains that produce BRO-1 enzymes account for about 90% of the β-lactamase-producing *M. catarrhalis* isolated from clinical specimens, while BRO-2-producing strains account for the remaining 10%.[213]Molecular analysis of β-lactamase production indicates that the BRO-1 enzyme is encoded by a single chromosomal gene (*bla*) that encodes a 314-amino acid polypeptide.[82] Sequence analysis of the *bla* genes from BRO-1- and BRO-2-producing isolates differ by five nucleotide bases that result in only a single amino acid residue difference in the amino acid sequences of the BRO-1 and BRO-2 enzymes. However, a 21 base pair deletion was found in the promoter region of the BRO-2 gene. Differences in MICs of BRO-1- and BRO-2-producing *M. catarrhalis* strains may be explained by lower levels of BRO-2 production due to the deletion in the promoter region, while the structure and enzymatic activities of the two enzymes are similar.[82] Patients who have been infected with BRO-2 β-lactamase-producing *M. catarrhalis* strains have also responded clinically to ampicillin and penicillin. The BRO-1 has a molecular weight of about 32.5 kDa. Variations in the reported molecular weight of this enzyme, which range from 28 kDa to 41 kDa, are explained by the presence of free and lipid-associated forms of BRO-1.[80] The *bla* gene may be transposon-associated and transferable by either transformational or conjugative mechanism.[80] In vitro studies indicate that the *M. catarrhalis* β-lactamases may indirectly function in virulence by inactivating penicillin or ampicillin given for other respiratory tract infections, such as pneumococcal pneumonia.[308]

Studies published during the early 1990s on isolates from the United States and abroad indicated that *M. catarrhalis* strains are generally resistant to penicillin, ampicillin, and amoxicillin, and susceptible to amoxicillin-clavulanate, second- and third-generation parenteral and oral cephalosporins (i.e., cefixime and cefaclor), macrolides, tetracyclines, and rifampin.[77,204,241,554,634] This susceptibility pattern has remained stable as demonstrated in more recent studies of *M. catarrhalis* from the US Sentry Antimicrobial Surveillance Program and other countries (e.g., Italy, Turkey).[189,228,340,434] While most isolates are susceptible to the fluoroquinolones, resistance has emerged in isolates recovered from patients who were on long-term therapy with such agents.[188,201] Other than variations in β-lactamase production, no notable differences in the antimicrobial susceptibilities of *M. catarrhalis* strains isolated from several countries have been noted.[188,189,228] Rare strains of *M. catarrhalis* may be resistant to tetracyclines, macrolides, or trimethoprim–sulfamethoxazole.[227]

In 2010, the CLSI published guidelines for antimicrobial susceptibility testing of *M.*

catarrhalis.[159] Microbroth dilution testing is performed with cation-adjusted Müeller–Hinton broth with a direct colony suspension inoculum that is equivalent to a 0.5 McFarland turbidity standard. The tests are incubated at 35°C in ambient air for 20 to 24 hours. Breakpoints are available for amoxicillin-clavulanate, parenteral (i.e., cefuroxime, cefotaxime, ceftazidime, ceftriaxone) and oral (i.e., cefaclor) third-generation cephalosporins, macrolides (i.e., azithromycin, clarithromycin, erythromycin), quinolones (i.e., ciprofloxacin, levofloxacin), tetracycline, clindamycin, trimethoprim–sulfamethoxazole, chloramphenicol, and rifampin. The absence of resistant strains of *M. catarrhalis* to several of the agents precludes defining any results categories other than "susceptible." Prior to having a standardized method for antimicrobial susceptibility testing of this organism, it had been noted that broth dilution tests for ampicillin, penicillin G, cephalothin, cefamandole, cefuroxime, and cefaclor were highly inoculum-dependent due to the production of varying amounts of β-lactamases.

More detailed information on antimicrobial susceptibility testing of these organisms is presented in Chapter 17.

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CHAPTER 12

Gram-Positive Cocci

Part I: Staphylococci and Related Gram-Positive Cocci

thePoint For additional ancillary materials related to this chapter, please visit the point.

Taxonomy of Staphylococci and Related Gram-Positive Cocci

Clinical Significance of Staphylococci and Related Gram-Positive Cocci

Staphylococcus aureus subsp. *aureus*

Staphylococcus aureus subsp. *anaerobius*

Coagulase-Negative Staphylococci

Staphylococcus epidermidis

Staphylococcus saprophyticus subsp. *saprophyticus*

Other Coagulase-Negative Staphylococci

Micrococcus Species and Related Genera

Rothia mucilaginosa

Isolation and Preliminary Differentiation of Staphylococci and Related Gram-Positive Cocci

Direct Gram-Stained Smears

Isolation From Clinical Specimens
Colony Morphology
The Catalase Test
Methods for Differentiating Micrococci and Staphylococci
Fermentation of Glucose
Susceptibility to Lysostaphin
Production of Acid From Glycerol in the Presence of Erythromycin
Susceptibility to Furazolidone
Modified Oxidase Test
Susceptibility to Bacitracin
Coverslip Growth Method
Methods for Direct Detection of MRSA in Clinical Specimens
Chromogenic Media for Surveillance of MRSA Colonization
Molecular Methods for Surveillance of MRSA Colonization
Molecular Methods for Detection of MRSA and MSSA in Blood Cultures and Skin/Soft Tissue Infections
Identification of *Staphylococcus aureus*
Slide Coagulase Test
Tube Coagulase Test
Alternative Coagulase Test Procedures
Agglutination Tests
Additional Confirmatory/Identification Tests for *Staphylococcus aureus*
Deoxyribonuclease Test
Thermostable Endonuclease Test
Mannitol Fermentation
AccuProbe *Staphylococcus aureus*
Rapid Tests for Detection of Methicillin Resistance
Genotype MRSA
Identification of Coagulase-Negative Staphylococci
Conventional Identification Methods
Pyrrolidonyl Arylamidase Activity
Susceptibility to Polymyxin B
Ornithine Decarboxylase Test
Urease Production
Acetoin Production
Susceptibility to Novobiocin for Presumptive Identification of *Staphylococcus*

saprophyticus
Commercial Identification Systems
Automated Identification Systems
Manual Identification Systems
Staphylococcus aureus/Coagulase-Negative Staphylococci PNA FISH Culture Confirmation Kit
Molecular Identification and Typing Methods for Staphylococci
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
Identification of *Micrococcus* and Related Species
Identification of *Rothia mucilaginosa*
Laboratory Approach to the Identification of Staphylococci

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With the exception of the *Enterobacteriaceae*, gram-positive bacteria, particularly the cocci, are the microorganisms most frequently isolated from clinical specimens. These bacteria are widespread in nature and can be recovered from the environment or as commensal inhabitants of the skin, mucous membranes, and other body sites in humans and animals. The ubiquity of these gram-positive bacteria in nature makes the interpretation of their recovery from patient specimens occasionally difficult unless clinical manifestations of an infectious disease process are apparent. Recovery of these organisms from specimens should always be correlated with the clinical condition of the patient before their role in an infectious process can be established.

Although gram-positive bacteria may cause infection by multiplication both locally and systemically, some organisms may multiply at a localized site and exert their pathogenic effects by producing exotoxins or enzymes that act at distant sites. Staphylococcal toxins are responsible for food poisoning, scalded skin syndrome, and toxic shock syndrome.[303,304] Streptococcal toxic shock syndrome (see Chapter 13) is a clinical entity where signs and symptoms of infection and pathologic features of disease are largely due to the effects of exotoxins. Gram-negative bacteria, on the other hand, possess endotoxin, which is the lipid portion of the outer membrane lipopolysaccharide. Systemic infections with gram-negative bacteria may lead to endotoxic shock, which is characterized by hypotension, vascular collapse, and sometimes death. This occurs most frequently when gram-negative organisms gain entry to the bloodstream.

With the increasing numbers of staphylococcal species being recognized in human infections and the finding of resistance to multiple antimicrobial agents in both the common and the uncommon isolates, it is imperative that the clinical microbiologist be

familiar with current methods for characterizing these organisms. In this chapter, the clinical significance and laboratory procedures for isolation and identification of the staphylococci and related organisms will be presented. In Chapter 13, the streptococci and *streptococcus*-like bacteria will be discussed. Antimicrobial susceptibility testing of these bacterial groups will be addressed in Chapter 17.

Taxonomy of Staphylococci and Related Gram-Positive Cocci

The taxonomy of staphylococci and related gram-positive cocci has undergone significant modifications with the application of molecular and chemotaxonomic methods, which have documented the diversity of organisms in the genera *Staphylococcus*,*Micrococcus*, *Macrococcus*, and *Rothia*. In volume 3 of the revised *Bergey's Manual of Systematic Microbiology*, the genera *Staphylococcus* and *Macrococcus* are classified in the family *Staphylococcaceae*, order *Bacillales*, in the phylum *Firmicutes*, while the genera *Micrococcus, Rothia*, and related cocci are distributed between the family *Dermacoccaceae* and family *Micrococcaceae* in the phylum *Actinobacteria* (Box 12-1). All of the former members of genus *Stomatococcus* are now placed in the genus *Rothia*.[47,109]

The genus *Micrococcus* has undergone extensive taxonomic dissection based on 16S ribosomal DNA sequence analysis.[512] Genus *Micrococcus* originally included nine species: *M. luteus*, *M. lylae*, *M. varians*, *M. roseus*, *M. agilis*, *M. kristinae*, *M.nishinomyaensis*, *M. sedentarius*, and *M. halobius*. In 1995, *M. roseus*, *M. varians*, and *M. kristinae* were transferred into the genus *Kocuria* as *K. rosea*, *K. varians*, and *K. kristinae*, respectively, while *M. halobius*, *M. nishinomiyaensis,* and *M. sedentarius*were placed into three separate genera as *Nesterenkonia halobia*, *Kytococcus sedentarius*, and *Dermacoccus nishinomiyaensis*, respectively. *M. agilis* was reclassified in the genus *Arthrobacter* as *A. agilis*. Since the dissection of genus *Micrococcus* into these genera began in 1995, several additional species in the genus *Micrococcus*, *Dermacoccus*, *Kocuria*, *Kytococcus*, and *Nesterekonia* have been described. Most species belonging to these genera have been isolated from air, soil, various extreme environments (e.g., marine sediment, deep sea mud, activated sludge), and food (usually fermented seafood). The various genera were initially delineated from each other based on chemotaxonomic (i.e., menaquinone analysis, fatty acid analysis, polar lipid characterization, peptidoglycan composition) and phylogenetic (i.e., 16S ribosomal DNA sequencing) data. Species within genera have been characterized further by additional genotypic analyses and phenotypic test results (i.e., carbohydrate assimilation and acidification tests, urease, acetoin production,

enzymatic activities, etc.). In general, these "*Micrococcus*-like" bacteria are cocci in various cellular arrangements or short rods. Micrococci and related species are gram-positive cocci that are slightly larger than staphylococcal cells. Micrococci are 1 to 1.8 µm in diameter, while staphylococci are 0.5 to 1.5 µm in diameter. The cells are arranged mostly as pairs, tetrads, and irregular clusters. In the rest of this chapter, these organisms will be referred to as micrococci and related species. Colonies of most species are pigmented, and all are catalase-positive; results for the oxidase test vary among the species.

Rothia mucilaginosa, a former member of genus *Stomatococcus*, is an encapsulated gram-positive coccus that is a part of the normal human respiratory microbial flora.[47] Based on 16S rDNA sequencing and chemotaxonomic data, this organism is most closely related to organisms in the genus *Rothia,* which also includes *R. dentocariosa, R. nasimurium* (an organism found in the nares of mice), *R. aeria*, *R. amarae*, and *R. terrae*.[109] While *R. mucilaginosa* and *R. nasimurium* are gram-positive cocci, the other *Rothia* species are gram-positive coryneform bacilli that sometimes show rudimentary branching. Because of their coccal morphology, *R. mucilaginosa* will be described in this chapter, whereas the other *Rothia* species will be discussed in Chapter 14.

Members of the genus *Macrococcus* are animal isolates that appear as large, gram-positive cocci, with individual cells measuring 1.0 to 2.5 µm in diameter. The macrococci have fewer 16S rRNA sequences in common with the staphylococci, have a greater G+C content in their DNA, lack cell wall teichoic acids, and generate unique ribotyping patterns. Members of the genus *Macrococcus* are related to the "*S. sciuri* group" of staphylococci, which are the only staphylococcal species that are oxidase-positive. The genus *Macrococcus* includes seven animal species: *M. equipercicus* and *M. caroselicus* (horses), *M. bovicus* (cattle), *M. caseolyticus*

BOX 12-1

Classification of the Staphylococci and *Staphylococcus*-Like Bacteria

Class	Order	Suborder	Family	Genera
"Bacilli"	*Bacillales*		*Staphylococcaceae*	*Gemella, Jeotgalicoccus, Macrococcus, Nosocomicoccus, Salinicoccus, Staphylococcus*
Actinobacteria	*Actinomycetales*	*Micrococcineae*	*Dermacoccaceae*	*Demetria, Dermacoccus, Kytococcus, Yamilla*
			Micrococcaceae	*Arthrobacter, Kocuria, Micrococcus, Nesterenkonia, Renibacterium, Rothia* (includes former *Stomatococcus* species)

(formerly *Staphylococcus caseolyticus,* dairy products, meats, pilot whales), and *M. brunensis*, *M. hajekii*, and *M. lamae* (skin of llamas).[281,344] These organisms have not been implicated in human infections.

Clinical Significance of Staphylococci and Related Gram-Positive Cocci

The genus *Staphylococcus* is currently composed of several species, many of which may be encountered in human clinical specimens (Box 12-2). Staphylococci are nonmotile, non–spore-forming, catalase-positive, gram-positive cocci. These organisms are arranged as single cells, pairs, tetrads, and short chains, but appear predominantly in grapelike clusters. Most species are facultative anaerobes except for *S. aureus* subsp. *anaerobius* and *S. saccharolyticus*. These two species grow anaerobically and, unlike the facultative species, are often catalase-negative. Staphylococci are generally found on the skin and mucous membranes of humans and other animals. In some cases, this association is highly specific. For example, *S. capitis* subsp. *capitis* is a part of the normal microbiome of human skin and sebaceous glands of the scalp, forehead, and neck, while *S. auricularis* is found primarily in the external auditory canal.[285,286] Some species are found only in animals and are recognized as veterinary pathogens. For example, *S. hyicus* causes an infectious dermatitis in swine, and *S. intermedius* has been isolated from several types of infections in dogs, including cutaneous infections, mastitis, wounds, and reproductive tract infections.[134,204,205] The latter species has also been isolated from dog bite wounds in humans.[529] *S. delphini, S. felis, S. schleiferi* subsp. *coagulans,* and *S. lutrae* cause infectious processes in dolphins, domesticated cats, dogs, and sea otters, respectively.[162,241,242,569] Humans may become colonized or infected with these organisms by frequent or close contact with animals (e.g., veterinarians, zoo workers, farmers). Some of the pathogenic staphylococci in both humans and animals produce an enzyme called coagulase, and detection of this enzyme is used in the

laboratory to identify these organisms.[204,569] Among the staphylococci, the coagulase-positive species, *S. aureus*, and two coagulase-negative species, *S. epidermidis* and *S. saprophyticus*, are seen frequently in human infections.

Staphylococcus aureus *subsp.* aureus

S. aureus is by far the most important human pathogen among the staphylococci. It is found in the external environment and in the anterior nares of 20% to 40% of adults. Other sites of colonization include intertriginous skin folds, the perineum, the axillae, and the vagina. Although this organism is frequently a part of the normal human microflora, it can cause significant opportunistic infections under the appropriate conditions. Factors which may predispose an individual to serious *S. aureus*infections include the following:

- Defects in leucocyte chemotaxis, either congenital (e.g., Wiskott–Aldrich syndrome, Down's syndrome, Job's syndrome, and Chediak–Higashi syndrome) or acquired (e.g., diabetes mellitus, rheumatoid arthritis);
- Defects in opsonization by antibodies secondary to congenital or acquired hypogammaglobulinemias or complement component (especially C3 and C5) deficiencies and/or defects;
- Defects in intracellular killing of bacteria following phagocytosis due to inability to activate the membrane-bound oxidase system, resulting in the absence of peroxides and superoxide from phagocytic vacuoles (e.g., chronic granulomatous disease, lymphoblastic leukemia, and both acute and chronic myelogenous leukemia);
- Skin injuries (e.g., burns, surgical incisions, eczema);
- Presence of foreign bodies (e.g., sutures, intravenous lines, prosthetic devices)[393];
- Infection with other agents, particularly viruses (e.g., influenza);
- Chronic underlying diseases such as malignancy, alcoholism, and heart disease;
- Therapeutic or prophylactic antimicrobial administration.

Under these circumstances, *S. aureus* may cause a variety of infectious processes ranging from relatively benign skin infections to life-threatening systemic illnesses (Box 12-3). Skin infections include simple folliculitis and impetigo, as well as cellulitis, furuncles and carbuncles involving subcutaneous tissues and causing systemic symptoms, such as fever (Image Plate 12-1A and 12-1B). *S. aureus* is frequently isolated from postsurgical wound infections, which may serve as a nidus for the development of systemic infections. Community-acquired staphylococcal bronchopneumonia is usually seen in elderly individuals and has been associated with viral pneumonia as a

BOX 12-2

Human, Animal, and Environmental *Staphylococcus* Species

Species	Comments
Staphylococci Found in Humans and Nonhuman Primates	
S. aureus	See text
S. epidermidis	See text
S. saprophyticus	See text
S. auricularis[286]	This species is found in the human external auditory canal and is rarely implicated in infections. *S. capitis* has been isolated from blood cultures during an episode of sepsis in a low-birth-weight infant and from peritoneal fluid of a patient on peritoneal dialysis.[233,322]
S. capitis[285]	*S. capitis* is part of the normal human flora and is found surrounding the sebaceous glands on the scalp and forehead. In 1991, this species was divided into two subspecies, *S. capitis* subsp. *capitis* and *S. capitis* subsp. *ureolyticus*. *S. capitis* subsp. *ureolyticus* can be differentiated from subsp. *capitis* by its positive urease activity, the ability to produce acid from maltose under aerobic conditions, and cellular fatty acid profiles. This species has been reported as a cause of native valve and prosthetic valve endocarditis, endocarditis associated with upper endoscopy, and pacemaker implantation.[110,127,258,379,384,528] *S. capitis* has been documented as a cause of sepsis in critically ill premature infants, including one case of meningitis.[442,589] In 2010, a case of community-acquired meningitis caused by *S. capitis* was reported in a 65-year-old woman.[402] In these cases, all of the isolates were resistant to oxacillin, erythromycin, and clindamycin. Strains of *S. capitis* have also displayed heteroresistance to vancomycin.[140] An outbreak of *S. capitis* colonization and infection in an intensive care unit was traced to almond oil used for neonatal skin care.[196]
S. caprae[135,265]	*S. caprae* was originally thought to be solely an animal species isolated from goats and goats' milk. In 1991, *S. caprae* was cultured from an exudate from a patient with dermatitis and from the urine of a second patient.[259] In a study of the distribution of *Staphylococcus* species among human clinical specimens, *S. caprae* comprised 10.7% of the 1,230 strains examined.[265] *S. caprae* was the cause of an outbreak of bacteremia in a neonatal intensive care unit, where it was recovered from the nares of 6 of 32 neonates during an MRSA surveillance study.[460] In this outbreak, all *S. caprae* isolates were methicillin-resistant and positive for the *mecA* gene. *S. caprae* was reported isolated from an intra-articular empyema following arthroscopic knee surgery and as a cause of iatrogenic osteomyelitis following surgical implantation of orthopedic materials.[7,151,494] *S. caprae* has been implicated in cases of endocarditis, catheter-related bacteremia, urinary tract infection, sepsis in a neonate with congenital heart disease, and iatrogenic meningitis.[42,262,280,509] *S. caprae* is most closely related to *S. epidermidis* and *S. capitis* subspecies.[177]
S. cohnii[288,480]	*S. cohnii* was first described in 1975, and in 1991, was divided into two subspecies: *S. cohnii* subsp. *cohnii* and *S. cohnii* subsp. *urealyticum*.[288] Subspecies *cohnii* has only been isolated from humans, while the latter urease-positive subspecies has been isolated from both human and nonhuman primates. Both are normal flora on the skin. *S. cohnii* is an emergent opportunistic agent, having been reported as a cause of community-acquired pneumonia, primary septic arthritis, infection of spinal fixation materials, brain abscesses, and catheter-related sepsis in immunocompromised patients.[33,152,160,352,353,612] Chorioamnionitis and neonatal sepsis with meningitis due to *S. cohnii* have also been reported.[266,506] Linezolid resistance has been described in this species, and methicillin-resistant strains of *S. cohnii* appear similar to MRSA isolates on certain brands of chromogenic agar for detection of MRSA.[420,428,578]
S. haemolyticus[480]	*S. haemolyticus* is part of the human normal skin microflora and is also found in nonhuman primates. This organism has been documented as a cause of primary and nosocomial bacteremia, wound/soft tissue infections, central venous catheter-associated septic pulmonary emboli, peritonitis, endocarditis, urinary tract infections, and nosocomial neonatal/pediatric infections.[45,154,156,200,297,422,473,527] Strains of *S. haemolyticus* that have relatively high MICs (i.e., from 2 to $\geq$8 μg/mL) for vancomycin have been reported in clinical settings of prolonged vancomycin administration, suggesting selection of resistant clones over time.[228,484,572] In this regard, strains of *S. haemolyticus* that are resistant to both glycopeptides (i.e., vancomycin and teicoplanin) and/or that are susceptible to vancomycin and resistant to teicoplanin have been described.[52,98,297] Glycopeptide resistance in *S. haemolyticus* and other coagulase-negative staphylococci is usually expressed heterogeneously, with both susceptible and resistant subpopulations existing within a single culture.[52] The emergence of glycopeptide resistance in *S. haemolyticus* has underscored the importance of correct identification and susceptibility testing of these isolates and monitoring their spread within the hospital environment. The causes of glycopeptides resistance are multifactorial and relate principally to alterations in the cross-linkages within the cell wall peptidoglycan and, therefore, with the successful interaction of the

	antibiotic with its target.[54] Vancomycin-resistant strains of *S. haemolyticus* demonstrate a reduced growth rate, reduced peptidoglycan hydrolase activities, and possess thicker cell walls than vancomycin-susceptible strains.[277] Methicillin-, fluoroquinolone-, and linezolid-resistant isolates of *S. haemolyticus* have also been reported.[30,355,453,527,615] The presence of multi-antibiotic-resistant *S. haemolyticus* in the hospital environment and transmission of resistant clones via hands of healthcare workers has been documented using several chemotaxonomic and molecular methods (e.g., immunoblotting of staphylococcal polypeptides, plasmid analysis, restriction-length polymorphisms of chromosomal DNA, PFGE of total DNA).[527]
S. hominis[283,285]	*S. hominis* is found on the skin of humans and has been isolated from infections as a low-grade pathogen causing catheter-related sepsis in immunocompromised hosts. *S. hominis* has also been reported as a cause of endocarditis and endophthalmitis.[247,274,523] In 1998, the species was divided into two subspecies: *S. hominis* subsp. *hominis*, and *S. hominis* subsp. *novobiosepticus*.[283] *S. hominis* subsp. *hominis* is predominantly skin-associated is an infrequent isolate from infections, while *S. hominis* subsp. *novobiosepticus* is recovered more frequently from blood cultures and *bona fide* infections.[94,123,406] Subspecies *novobiosepticus* is novobiocin-resistant and does not produce acid from D-trehalose or *N*-acetyl-D-glucosamine, while the subspecies *hominis* is novobiocin-susceptible and produces acid aerobically from both of these carbohydrates. 2010, an intensive care unit isolate of *S. hominis* subsp. *hominis* was shown to possess mutations resulting in elevated MICs for both teicoplanin and linezolid.[507]
S. lugdunensis[170]	This species, first described in 1988, has rapidly established itself as a significant human pathogen.[167] *S. lugdunensis* colonizes the human inguinal area as established by inguinal fold culture sampling and the predominance of *S. lugdunensis* isolates obtained from abscesses in the pelvic girdle region.[39,560] In recent years, several reports have described *S. lugdunensis* as an important cause of native valve- , prosthetic valve-, and pacemaker-associated endocarditis. These infections have been complicated by the development of embolisms and myxomas.[51,91,105,327,405] CNS infections have included meningitis, brain abscess, and ventriculoperitoneal shunt infections.[148,183,256,472] *S. lugdunensis* osteoarticular infections include infected hip prostheses, osteomyelitis, vertebral discitis, and septic arthritis following arthroscopic surgical procedures.[72,296,359,361,487] Bacteremia, vascular line infections, urinary tract infections, skin and soft tissue infections, endophthalmitis, and peritonitis may also involve this pathogen.[23,99,101,203,289,410,417,481,619] A septic shock presentation similar to that seen with gram-negative bacteria has also been associated with *S. lugdunensis* bacteremia.[81] Oxacillin-resistant strains of *S. lugdunensis* carrying the *mecA* gene have also been characterized.[538] According to the CLSI, *S. lugdunensis* antimicrobial susceptibilities are interpreted using the *S. aureus* breakpoints rather than those used for other coagulase-negative staphylococci.[107] *S. lugdunensis* and *S. pseudolugdunensis* are ornithine decarboxylase- and PYR-positive.
S. massiliensis[8]	This species, described in 2010, was isolated from a brain abscess in a 52-year-old man.[8] Newer information indicates that *S. massiliensis* is probably part of the normal flora of human skin.[620]
S. pasteuri[96]	*S. pasteuri* is a newly described species that is found in human and animal clinical specimens and in food. *S. pasteuri* has been isolated from blood cultures of a patient with leukemia and from contaminated apheresis platelet units.[474,475]
S. petrasii[409]	This new species, described in 2013, was isolated from blood, wound pus, nasal swabs, and several ear swabs from patients with acute otitis externa. This coagulase-negative species was distinct from known *Staphylococcus* species by 16S rRNA, *hsp60, rpoB, dnaj, tuf*, and *gap* gene sequencing. The two subspecies—*petrasii* and *croceilyticus*—can be differentiated by phenotypic and genotypic techniques.[409]
S. pettenkoferi[547,548]	*S. pettenkoferi* is a new human staphylococcal species that has been isolated from bloodstream infections, wound infections, and a case of osteomyelitis in a diabetic foot infection.[13,329,505,547,548] This species has also demonstrated resistance to linezolid.[365]
S. pseudolugdunensis[532]	This new PYR- and ornithine decarboxylase-positive species has been isolated from blood cultures, where it has been misidentified as *S. lugdunensis*.[532] This species was not recognized by a *S. lugdunensis*-specific primer/probe set targeting the *tuf* gene (for elongation factor Tu) and cannot be differentiated from other commonly isolated staphylococci based on phenotypic reactions.
S. saccharolyticus[276]	This anaerobic species was previously called *Peptococcus saccharolyticus* and was transferred to the genus *Staphylococcus* based on DNA sequence analysis of 16S ribosomal RNA. The organism is found on human mucous membranes. Two cases of endocarditis (one involving a native heart valve, and the other a prosthetic heart valve), a case of spondylodiscitis, and one patient with fatal pneumonia caused by *S. saccharolyticus* have been reported.[189,299,599,610] Over a 6-month period, *S. saccharolyticus* was isolated from blood cultures of 16 different patients in 12 different wards in a hospital in Germany. Despite intensive investigations, no common links among the patients or no "outbreak" source could be identified.[517]

S. schleiferi[170]	*S. schleiferi* was first described in 1988 and has been isolated from several human infections, including brain empyema, wound infections, bacteremia complicating vertebral osteitis, infection of a hip prosthesis, indwelling catheter infection, nosocomial urinary tract infections, prosthetic valve endocarditis, osteomyelitis, and pacemaker infections.[71,82,227,290,301,403,542] In 1990, the species was divided into two subspecies; human and canine isolates (associated with pyoderma) have been designated *S. schleiferi* subsp. *schleiferi,* and isolates causing external otitis in dogs have been designated *S. schleiferi* subsp. *coagulans.*[243,354] When the subspecies were first described, it was thought that subspecies *schleiferi* isolates were clumping factor-positive and tube coagulase-negative, while subspecies *coagulans* isolates were clumping factor-negative and tube coagulase-positive.[243] Vandenesch and colleagues[556] subsequently isolated three strains of *S. schleiferi* subsp. *schleiferi* that were both clumping factor- and tube coagulase-positive.
S. simulans[285]	*S. simulans* is found on the skin and in the urethras of healthy women. *S. simulans* has been isolated as a cause of septicemia, osteomyelitis, native valve endocarditis, septic arthritis following open reduction of a fractured fibula, vertebral osteomyelitis, and prosthetic joint infection.[133,339,343,445,554] As with certain other coagulase-negative staphylococci (i.e., *S. epidermidis*, *S. haemolyticus*, and *S. hominis*), methicillin-resistant strains of *S. simulans* possesses an additional PBP2a (PBP2') that exhibits a low affinity for methicillin, cephalothin, and cefamandole and has amino acid sequences analogous to the PBP2a (PBP2') found in MRSA isolates.[431]
S. warneri[285]	This species represents about 1% of the staphylococci normally found on human skin. *S. warneri* is now a well-recognized cause of catheter-related bacteremia, native valve and prosthetic valve endocarditis, hematogenous vertebral osteomyelitis and discitis, and ventriculoperitoneal shunt-associated meningitis.[18,26,66,70,279,350,520] Incani and colleagues[244] described a patient with meningitis caused by *S. warneri*; the patient also had lymphoma and *S. sterccoralis* hyperinfection syndrome. Isolates recovered from humans are now called *S. warneri* subsp. 1, while isolates from nonhuman primates are called *S. warneri* subsp. 2.
S. xylosus[480]	*S. xylosus* is found in both human and nonhuman primates and has been reported as a cause of upper and lower urinary tract infections, septicemia, and endocarditis associated with intravenous drug use.[113] This organism was isolated from a pancreatic pseudocyst in an HIV-infected patient, from a otogenic brain abscess in an adolescent female, and from a root canal infection.[5,351,497] *S. xylosus* has also been isolated from goats' milk and cheese.[363]
Staphylococci Found in Other Animals	
S. agnetis[536]	This newly described species was isolated from milk samples from cows with subclinical and mild clinical mastitis.
S. arlettae[479]	This species is found on mammals and birds and is of undetermined clinical significance and taxonomic status.
S. chromogenes[134,205]	This species, formerly a subspecies of *S. hyicus*, causes cutaneous infections in cattle, swine, and goats.[15]
S. delphini[569]	This coagulase-positive species causes purulent skin lesions in dolphins.
S. devriesei[524]	This new species has been isolated from the teats milk of dairy cows.
S. equorum[479]	*S. equorum* is a rare species of undetermined pathogenic significance. *S. equorum* has been recovered primarily from horses as well as from goat milk and cheese, and has also been isolated from human clinical specimens.[363,391] *S. equorum* subsp. *linens* serves as a starter culture component for various surface-ripened cheeses.[434]
S. felis[241,242]	*S. felis* is the most commonly isolated *Staphylococcus* species in cats, causing otitis, cystitis, abscesses, wounds, and other cutaneous infections.
S. gallinarum[135]	This species is found in poultry and is nonpathogenic.
S. hyicus[134]	*S. hyicus* is found in cattle and cows' milk and is associated with exudative epidermitis ("greasy pig syndrome"), an acute disease of suckling and weaned pigs. Strains of *S. hyicus* causing this skin infection produce three types of exfoliative toxins and a calcium-dependent extracellular lipase/phospholipase enzyme that contribute to the pathology of this syndrome.[14,459] Although not considered a pathogen of humans, a case of human wound infection caused by *S. sciuri* following a donkey bite was reported in 1997.[399]
S. intermedius[204]	*S. intermedius* is part of the flora of dogs, minks, horses, and cats and may cause cutaneous, urinary tract, bone, and central nervous system infections in several animal species. *S. intermedius* is the predominant coagulase-positive *Staphylococcus* recovered from normal and infected canine skin and from serious canine infections.[187] Human carriage of *S. intermedius* is rare even among individuals with frequent exposure to animals.[336] This organism has been isolated from humans with infected dog bite wounds and has also been

	recovered from individuals with non–canine-inflicted injuries, including infections of varicose vein ulcers and suture incisions.[529,530] Minor infections (e.g., otitis externa) with this organism may also occur as a consequence of close, nontraumatic contact with the family canine.[534] *S. intermedius* catheter-related sepsis in a 63-year-old cancer patient and *S. intermedius* pneumonia following coronary artery bypass surgery have also been reported.[180,555] *S. intermedius* may also cause cutaneous infections in persons with no exposure to dogs or other animals.[270] *S. intermedius* is characterized by being tube coagulase-positive, but clumping factor-variable.
S. kloosii[479]	This species is found in mammals and is of uncertain clinical significance and taxonomic status. In 2011, *S. kloosii* was isolated from several blood cultures from a 60-year-old patient following a intracranial bleed.[420]
S. lentus[478]	*S. lentus*, formerly a subspecies of *S. sciuri* (i.e., *S. sciuri* subsp. *lentus)* is part of the normal skin flora of sheep and goats.
S. lutrae[162]	This species, described in 1997, was isolated during postmortem examinations of three sea otters (*Lutra lutra*) from the Inner Hebrides Islands. Isolates were obtained from liver tissue and spleen tissues and from a mammary gland and supramammary lymph node. *S. lutrae* is free coagulase-positive and clumping factor-negative, and is most closely related to *S. delphini*, *S. felis*, *S. intermedius*, *S. schleiferi*, and *S. muscae*.
S. microti[389]	This species, described in 2010, was isolated from the viscera of common voles.[389]
S. muscae[206]	This species is found as a transient part of the body surface flora of flies that inhabit cow barns, but not found on flies inhabiting human dwellings.[206]
S. nepalensis[511]	This recently described species was isolated from nares swabs and lung tissue of goats with respiratory tract symptoms in areas of Nepal.[511] This species has also been isolated from rectal swabs of healthy South American squirrel monkeys, a urine specimen from a human with cystitis, pig skin, and the environment.[390]
S. pseudintermedius[136]	This coagulase-positive species was isolated from clinical and necropsy specimens obtained from a cat, a dog, a horse, and a parrot and was found to constitute a distinct taxon related to *S. intermedius* and *S. delphini*.[136] This organism has been isolated from infections in dogs, including necrotizing fasciitis and infection after joint prosthesis implantation.[364,594] *S. pseudintermedius* has also been isolated from nares specimens of humans with frequent exposure to dogs and cats (e.g., dog and cat owners, veterinary staff).[503,561]
S. rostri[450]	This relatively newly described species was isolated from nares specimens of pigs in Switzerland.[450,516]
S. sciuri[287]	*S. sciuri* is widely distributed in nature and has been isolated from foods, farm animals, rodents, marsupials, marine mammals, and occasionally from humans and their pets. Three subspecies are currently recognized.[282,346] *S. sciuri* subsp. *sciuri* is part of the transient skin flora in various mammalian and avian species; it is seldom found on humans and nonhuman primates. *S. sciuri* subsp. *carnaticus* is found mainly on bovine hosts and beef products. *S. sciuri* subsp. *rodentium* is found mainly on rodents. Human isolates have been recovered from wounds, skin, and soft tissue infections, and, in 1998, a case of *S. sciuri* endocarditis was reported.[218,346,519] *S. sciuri* has also been isolated as a cause of peritonitis in a dialysis patient. An examination of 30 human clinical isolates of *S. sciuri* found that 70% were subsp. *sciuri*, 23% were subsp. *rodentium*, and 7% were subsp. *carnaticus*. All subspecies of *S. sciuri* have been found to harbor a homolog of the *S. aureus* methicillin resistance gene *mecA* that is found in MRSA strains.[114,608] These two *mecA* homologs have about 80% base-pair similarities. *S. sciuri* strains that carry their native *mecA* homolog express borderline resistance to methicillin, while those strains that carry the MRSA *mecA* gene express heterogenous resistance to methicillin.
S. simiae[408]	This species was isolated from rectal swab specimens obtained from squirrel monkeys.
S. stepanovicii[216]	*S. stepanovicii* is a novobiocin-resistant staphylococcal species that was isolated from the skin, fur, and intestinal tracts of wild, small mammals, including rodents, voles, field mice, and shrews.
S. vitulinus[593]	This species was described in 1994 and is found as a part of the flora of horses, voles, and pilot whales. It has also been isolated from meat products, including lamb, chicken, ground beef, and veal. DNA hybridization studies show that this species is most closely related to *S. lentus* and *S. sciuri*.
Other Staphylococci (Mostly Environmental)	
S. carnosus[477]	This species is used as a starter culture in the processing of meats such as salami and sausage. At present, two subspecies are recognized: *S. carnosus* subsp. *carnosus* and *S. carnosus* subsp. *utilis*.[438]
S. condimenti[438]	This species is found in soy sauce mash (as is *S. piscifermentans*) and is closely related to *S. carnosus* subspecies.

S. fleurettii[577]	This species has been isolated from cheeses prepared from goats' milk.
S. piscifermentans[531]	This species is found in fish, fermented fish products, and soy mash and is closely related to *S. carnosus* and *S. condomenti*.[407]
S. succinus[310]	This species, first described in 1998, was isolated from a fragment of 25- to 35-million-year-old Dominican amber.[310] This species phenotypically resembles *S. xylosus* and is closely related to *S. xylosus*, *S. saprophyticus*, *S. equorum*, and other novobiocin-resistant staphylococci. A subspecies of *S. succinus*, *S. succinus* subsp. *casei*, has been proposed for isolates from Swiss surface-ripened cheese.[434] In 2006, several isolates from human clinical specimens were identified as *S. succinus*.[391]

BOX 12-3

Infections Associated With *Staphylococcus* aureus

Infection	Comments
Folliculitis	Folliculitis is a benign infection of the superficial dermis that is restricted to the ostia of the hair follicles and is characterized by the presence of small, reddish, painful lesions and the absence of systemic symptoms.
Impetigo	Impetigo is a superficial staphylococcal infection of the dermis that is seen mostly in children and usually presents on exposed areas, especially the face. Impetigo occurs in two forms: nonbullous and bullous.[179] Nonbullous impetigo begins as a single red macule or papule that quickly becomes a vesicle. The vesicle ruptures to form an erosion, and the lesion and its contents dry to form characteristic honey-colored crusts with erythematous margins that may be pruritic. Impetigo may be spread to surrounding areas by autoinoculation. Bullous impetigo is a localized form of staphylococcal scalded skin syndrome that most commonly affects neonates but also can occur in older children and adults. Superficial vesicles progress to enlarging bullae with sharp margins and no surrounding erythema. When the bullae rupture, yellow crusts with oozing result. Bullous impetigo is usually found in moist, intertriginous areas (i.e., the diaper area, axillae, neck folds). Systemic symptoms are not common but may include fever and sometimes gastroenteritis. Most cases are self-limited and resolve without scarring in several weeks. *S. aureus* accounts for 80%–90% of cases of impetigo, with the remaining being associated with group A β-hemolytic streptococci. Impetigo caused by these two organisms are indistinguishable clinically.
Furuncles and carbuncles	Furuncles are deeper-seated infections of the hair follicles in which suppuration extends through the dermis into the subcutaneous tissues. These lesions present as painful, firm, raised lesions with necrotic centers containing purulent material. Generalized systemic symptoms are usually absent. Carbuncles refer to even more deep-seated lesions that involve the subcutaneous tissues and several adjacent hair follicles. Several lesions may be present and may coalesce via the formation of subcutaneous sinus tracts. Carbuncles tend to occur on the back of the neck and are often associated with systemic signs of chills and fever. Carbuncles require prompt surgical drainage and management includes parenteral antimicrobial therapy.
Hidradinitis supperativa	Hidradenitis suppurativa is a chronic or relapsing inflammatory disease of the skin, involving the apocrine gland-bearing areas including axillae, groin, perineal and perianal regions This condition is characterized by the presence of multiple furuncle-like lesions associated with blocked and infected apocrine sweat glands. While local pain, swelling, and erythema are present, systemic symptoms such as fever are usually absent.
Mastitis	Mastitis refers to breast infections associated with childbirth and lactation and is characterized by edema, swelling, firmness and, occasionally, erythema of the breast tissues. Superficial abscesses may be drained by needle aspiration, while deeper, persistent lesions require incision and drainage for cure, often along with antibiotic therapy.
Wound infection	Most staphylococcal wound infections occur in the postsurgical setting and are recognized by redness, swelling, and pain around the surgical site, and the presence of cloudy, serosanguinous drainage. The approach to wound care in patients with staphylococcal wound infection is dependent on the depth of the infection, the condition of the host, the presence and/or severity of clinical signs and symptoms, and the presence/absence of foreign bodies within the wound. These factors determine the extent of debridement necessary and the requirement for parenteral antimicrobial chemotherapy. These infections are often caused by the same strain of *S. aureus* carried in the anterior nares of the infected patient.[579]
Cellulitis and complicated soft tissue infections	Cellulitis refers to rapidly spreading inflammation and infection of the soft subcutaneous tissues and may go on to involve the superficial fascia and the subcutaneous fat. While group A β-hemolytic streptococci are the most frequent causes of this infection, *S. aureus* is occasionally responsible, especially in the presence of an abscess

or cutaneous ulcer. Cellulitis may involve *S. aureus*, other gram-positive organisms, and gram-negative bacteria as well, so specimen collection should include aspirates from the leading edge of the spreading cellulitis and collection of blood cultures, and broad-spectrum empiric therapy should be initiated. **Erysipelas** is a type of cellulitis with a well-demarcated raised border that is usually caused by group A β-hemolytic streptococci but is occasionally caused by *S. aureus*. This infection is painful and lymphatic involvement is prominent. As with cellulitis, collection of localized specimens and blood cultures are helpful, and antimicrobial coverage should be broad-spectrum and promptly administered. Necrotizing fasciitis can result from localized spread of staphylococcal infection or may arise hematogenously. This infection evolves rapidly so prompt diagnosis, surgical debridement, and fasciotomy are absolutely necessary for good clinical outcomes. Necrotizing fasciitis may involve *S. aureus*, β-hemolytic streptococci, and gram-negative bacteria (e.g., *P. aeruginosa*), so high-dose broad-spectrum antimicrobial therapy is warranted.

Bloodstream infections

S. aureus is responsible for between 20 and 30 episodes of bloodstream infection per 100,000 persons per year worldwide and is associated with a mortality rate of 20%–30%.[43,454] Operationally, bloodstream infections are hospital-acquired or community-acquired. The former usually occur 2 or more days after hospitalization, while community-acquired infections arise 2 or more days before hospitalization. Community-acquired (CA) bacteremia may be associated with previous or ongoing healthcare institution exposure or outpatient encounters; consequently, CA-bloodstream infections are more accurately termed either healthcare-associated (HCA)-community onset or CA-community onset bloodstream infections.[440] HCA-bloodstream infections that arise in the community generally occur in patients with indwelling catheters and other access devices and in patients on dialysis or undergoing outpatient treatment protocols. Community-onset CA-bloodstream infections arise in previously healthy persons and develop from localized infection elsewhere (e.g., skin and soft tissue infections, abscesses, ulcers, bone infection, burns). HCA-bloodstream infections arising in the hospital or in the community usually involve more resistant *S. aureus* strains than infections with onset occurring outside the healthcare inpatient/outpatient settings. Patients with staphylococcal bacteremia usually present with chills, rigors, and fever. Small hemorrhagic skin lesions may be present and those on the skin may evolve to form larger, necrotic ulcers. Endocarditis following *S. aureus* bacteremia occurs in about 10% of patients and may present in a subtle manner, or with multiple signs and symptoms.[86] With infection of the heart valves, more subtle hemorrhagic manifestations (e.g., Janeway lesions, Roth spot, splinter hemorrhages) may be apparent. Heart murmurs, pericardial friction rubs, and pericardial effusions may be present and, depending on the type of murmur, often suggest the likely location(s) of bacterial involvement and valvular insufficiency. Transthoracic and transesophageal echocardiography aid in delineating the extent of cardiac involvement and help to determine the need for and immediacy of surgical interventions. Thrombocytopenia is frequently present, and disseminated intravascular coagulation represents a rare but fatal complication occurring early in the clinical course. Metastatic foci of infection may develop in multiple organ systems, and hematogenous seeding of the kidney can result in mild-to-severe renal insufficiency, abscesses and pyelonephritis. If bloodstream infection is associated with infected access devices, the removal of these materials is essential for organism clearance from the bloodstream.[111] During and following removal, an 8- to 10-day course of parenteral antimicrobial therapy is recommended with follow-up including collection of additional blood cultures and exclusion of infective endocarditis. Other complications of *S. aureus* bloodstream infection may require longer antibiotic courses ranging from 10 to 14 days following cutaneous infections to 4- to 6-week regimens involving deep-seated infections (e.g., abscesses, bone infections).[111] Prior to the availability of antimicrobial susceptibility information, initial therapeutic coverage is directed against MRSA.

Endocarditis

S. aureus is a cause of native valve endocarditis and is also a leading cause of prosthetic valve endocarditis.[161,381,378,586] *S. aureus* infective endocarditis usually presents as an acute septic syndrome with fever, nonspecific symptoms (i.e., arthralgias, myalgias, pleuritic pain), and the appearance of a new cardiac murmur. Petechial skin lesions and Janeway lesions may be present; these indicate the development of septic emboli. Large vegetations may form on the valves, break off, and embolize to the coronary and peripheral arteries and to various organ systems. Septic emboli and mycotic aneurysms may be carried to the central nervous system where they can cause cerebral infarcts, abscesses, and intracerebral or subarachnoid hemorrhages. Diagnosis is aided by echocardiography, with transesophageal approaches being the most useful for detection of mycotic aneurysms. Right-sided endocarditis is seen primarily in injection drug users. For native valve endocarditis due to MSSA, a penicillinase-resistant penicillin (e.g., oxacillin, nafcilllin) or a first-generation cephalosporin (e.g., cefazolin) with or without an aminoglycoside is generally used.[440] Vancomycin is recommended for those with allergy to β-lactam antibiotics and in those with endocarditis due to MRSA. Therapy must be continued for 4–6 weeks. For prosthetic valve endocarditis due to MSSA, a penicillinase-resistant penicillin is administered along with rifampin and gentamicin. Vancomycin with rifampin and gentamicin is recommended for those with β-lactam allergy and in those with endocarditis due to MRSA. Treatment of prosthetic valve endocarditis must be continued for at least 6 weeks.

Meningitis	Meningitis due to *S. aureus* may occur as a complication of bacteremia or local trauma due to neurosurgical interventions, placement of shunts, surgery or injury (e.g., severe head trauma).[44,432] Meningitis complicating *S. aureus* bacteremia usually occurs in compromised individuals with a focus of infection elsewhere (i.e., abscess, osteomyelitis, pneumonia).[419] Although the signs and symptoms are similar to other meningeal infections, comorbidities (e.g., immunodeficiency, underlying disease) are present more frequently. Meningitis due to *S. aureus* is rare (2%–8% of all meningitides), but the mortality rate is high.
Pericarditis	Pericarditis (infection of the membranes surrounding the heart) due to *S. aureus* may arise hematogenously or secondary to local infection from penetrating chest trauma, surgery, or septic emboli.[463] Pericarditis may also occur as a complication of staphylococcal endocarditis. Patients present with chest pain, friction rubs, generalized heart failure and/or mediastinitis.
Pulmonary infections	Pulmonary infections caused by *S. aureus* may arise from aspiration or by hematogenous spread from another site. Aspiration pneumonia acquired in the community is usually seen in the elderly as a complication of influenza pneumonia, while that acquired in the hospital is usually secondary to intubation and ventilatory assistance.[332] *S. aureus* is emerging as the most frequent cause of nosocomial pneumonia, and more than half of these cases are due to MRSA.[385] Pneumonia due to *S. aureus* progresses rapidly, with tissue necrosis and cavitations, and complications of both types of pulmonary infections include bacteremia, lung abscesses and development of pleural empyemas.[192] Chest X-rays from these patients may show scattered, patchy infiltrates, defined abscesses or nodules, consolidation, or cavitation. In small children, thin-walled pneumatoceles with air–fluid levels may be seen. Pneumonia due to CA-MRSA presents with severe respiratory symptoms, fever, hypotension, and purulent, bloody respiratory secretions.[185,375] In these cases, the chest X-ray usually shows multiple cavitating infiltrates and abscesses. This clinical presentation is typically seen in young adults with CA-MRSA infection following an initial, influenza-like illness. Hematogenous pulmonary infection occurs as a complication of right-sided endocarditis and is secondary to embolization from valvular lesions, resulting in pulmonary infarcts. Complications of *S. aureus* pneumonia include pleural empyema and bronchopleural fistula formation.[67] These complications usually result from direct extension of infection from a contiguous site in the lung tissue and various scanning procedures (e.g., ultrasound, CT scans) can be diagnostically useful.
Bone and joint infections	*S. aureus* is the most common pathogen associated with bone and joint infections, and the relative frequency of MRSA involvement has increased in both hospital-acquired and CA infections.[3] *S. aureus* osteomyelitis most often occurs as a complication of local infection via direct extension or via hematogenous seeding. Hematogenous osteomyelitis is usually seen in children and characteristically involves the long bones, although rib and the lumbar spine involvement also occurs. Various types of scans, including MRI and CT, are helpful in ascertaining the extent of involvement and, in the case of spinal lesions, the presence of paravertebral abscesses. Abscesses and lytic bone lesions must be aspirated and cultured to provide a definitive diagnosis. Patients usually present with nonspecific symptoms of fever, weight loss, and bone pain. Osteomyelitis due to *S. aureus* can mimic malignancies such as osteosarcoma on radiologic studies, and needle biopsies are essential in identifying the microbial cause.[137] Septic arthritis due to *S. aureus* occurs primarily in prepubertal children and is usually a complication of bacteremia in both children and adults.[404] In these cases, the patient presents with a swollen, hot joint (most often involving the knee, hip, elbow, shoulder, or interphalangeal joints) that is painful on movement. Aspiration of the joint, Gram's stain, and culture of the purulent exudate are required for management. Osteomyelitis and septic arthritis resulting from CA-MRSA and CA-MSSA are seen primarily in children and are rare among adults.[137,570] In most cases, no underlying disease is present. In the case of CA-MRSA, skin and soft tissue infections are usually the source of the organism. Treatment of these infections usually involves vancomycin, and clindamycin has also been effective when isolates were demonstrated not to have inducible clindamycin resistance.[24,349,559] MSSA and MRSA infections of prosthetic joints has also been reported.[295] These infections usually develop shortly after joint replacement and require antimicrobial therapy and replacement of the prosthesis to effect cure.
Pyomyositis	Pyomyositis is an infection of the skeletal muscles that usually occurs secondary to trauma, often in the neighborhood of an existing focus of infection (e.g., a furuncle).[413] Patients usually present with fever and muscle pain; aspiration and culture yield *S. aureus*. Cure is effected by incision and drainage in association with the use of an appropriate antimicrobial agent.
Staphylococcal food poisoning	Staphylococcal food poisoning results from ingestion of food contaminated with *S. aureus* strains that produce heat-stable enterotoxins. The implicated foodstuff usually becomes contaminated by a food handler, with subsequent organism growth and toxin production. Implicated foods include potato salads, ice cream, custards, bakery goods, canned foods, and processed meats. Symptoms, including nausea, vomiting, abdominal cramps, and diarrhea, usually begin 2–6 hours after ingestion and abate after 8–10 hours. Fever and neurologic signs are characteristically absent. Treatment involves replacement of fluids lost due to vomiting and diarrhea until recovery is complete.

Staphylococcal scalded skin syndrome	Staphylococcal scalded skin syndrome (SSSS) is caused by *S. aureus* strains that produce exfoliative toxins and is usually seen in neonates and infants less than 5 years[304]. In cases of SSSS, infections with *S. aureus* may involve the conjunctivae, the nares, the umbilicus, and perineum. SSS may also result from extracutaneous staphylococcal infections, including urinary tract infection, septic arthritis, pneumonia, and endocarditis.[500] With localized infections, SSSS may appear as bullous lesions resembling bullous impetigo, When the toxins are absorbed systemically by the bloodstream, generalized SSSS results, with the development of widespread erythema and the appearance of bullous lesions over large areas of the body, with subsequent sloughing of the superficial skin layers. This results in the exposure of large areas of denuded and raw skin. In most cases, desquamation occurs and symptoms wane over 5–7 days. The responsible toxins are antigenic and antibodies against them are protective (Box 12-4). Outbreaks of SSSS have occurred in neonatal intensive care units and nurseries.[147] Culture of lesions may be noncontributory, so other possible sites of infection (i.e., nares, conjunctivae, umbilicus, nasopharynx) should be sampled.[305] SSSS caused by MSSA requires therapy with a penicillinase-resistant penicillin or clindamycin based on the susceptibility of the isolate. Assiduous supportive care (e.g., contact isolation, pain management, gentle skin cleansing) is necessary to prevent secondary superinfections.
Staphylococcal toxic shock syndrome	Toxic shock syndrome (TSS) is a multisystem illness characterized by a clinical syndrome that includes fever, hypotension, orthostatic dizziness, erythroderma (blanching rash), and varying degrees of vomiting, diarrhea, renal failure, headache, chills, sore throat, and conjunctivitis.[97,446,489] Initially, the disease was noted most frequently in women, with onset mainly occurring during menstruation. Investigations of the initial cases noted an association between the onset of disease and the use of high-absorbancy tampons during menses (menstrual TSS).[489] The fiber material used in high-absorbancy tampons created the ideal milieu for toxin production when introduced in the vagina. Elevated levels of oxygen trapped within the absorbent material along with the protein- and CO_2-rich, pH-neutral environment provided by the blood stimulated the production of TSS toxin (TSST-1) by *S. aureus* The toxin is produced locally and passes through the mucous membranes into the bloodstream; positive blood cultures are generally not seen in patients with TSS. Subsequently, nonmenstrual-associated TSS was reported in males and females as a complication of staphylococcal abscesses, osteomyelitis, postsurgical wound infections, burns, nasal packing, and postinfluenza pneumonia.[446] About 20% of *S. aureus* isolates from carriers and from clinical specimens produce TSST-1.[35] Although TSST-1 is implicated in most cases of menstrual TSS, nonmenstrual TSS may result from enterotoxins SEB and SEC as well.[357] The role of TSST-1, SEB, SEC, and other superantigens (SAgs) in the disease is supported by the acute onset of symptoms, with vascular congestion developing over a 1- to 2-day period. With the increase in capillary leakage and the system-wide decrease in vascular resistance, there is loss of intravascular fluids into the interstitial spaces. Fluid loss is also exacerbated by the presence of diarrhea in some patients. Loss of intravascular volume leads to hypotension and tissue hypoxia. Acute respiratory distress syndrome and disseminated intravascular coagulation are common and life-threatening complications of TSS. TSST-1 also appears to have some direct toxic effects on the myocardium, skeletal muscle, liver and kidney tissue. Interestingly, patients who develop TSS lack or have low titers of antibody against TSST-1 and other implicated SAgs before and during acute disease, and more than half of the patients remain susceptible to TSS as protective antibody levels are not produced.[521] Other conditions that may be included in the differential diagnosis of TSS include other toxin-mediated infections, (e.g., scalded skin syndrome, gastroenteritis, scarlet fever), local infections with shock and/or acute abdominal pain (e.g., infectious gastroenteritis, salpingitis, septic abortion, acute urinary tract infections) and multisystem illnesses of infectious (e.g., septic shock associated with pneumococci, meningococci, or *Haemophilis influenzae* type b; rubeola, Rocky Mountain spotted fever, tick-borne typhus, leptospirosis, *Legionella* infection; toxoplasmosis; and adenoviral or enteroviral syndromes) and noninfectious (Kawasaki's disease, systemic lupus erythematosus, acute rheumatic fever, rheumatoid arthritis, drug reactions) etiologies. Diagnosis of TSS is generally based on the clinical signs and symptoms according to the case definition (Box 12-3). Treatment of TSS involves antibiotic therapy, supportive care and, in some cases, surgery. Antimicrobial agents that inhibit toxin synthesis (e.g., clindamycin, fluoroquinolones, rifampin) may occasionally be recommended. Immune serum globulin may be helpful in severe cases. Prevention of TSS involves rapid attention to *S. aureus* wound and soft tissue infections and discontinuation of hyperabsorbent tampons. Nasal carriage can be eliminated by use of mupirocin. The mortality associated with staphylococcal TSS is about 3%.
Bactiuria and urinary tract infections	Isolation of *S. aureus* from urine can be associated with bacteremia and invasive disease with the urinary tract as the source of the organism.[9] Conversely, presence of the organism in the urinary tract may result from hematogenous dissemination to the kidney from another source. The emergence of methicillin resistance was associated with a concomitant increase in the frequency of MRSA in urine cultures. Most patients with *S. aureus* in the urine are asymptomatic, but up to one-third may present with symptoms suggesting urinary tract infection (i.e., fever, dysuria, hematuria, and pyuria.[377] Risk factors for *S. aureus* bactiuria include advanced age, urinary tract catheterization or instrumentation, residence in long-term-care facilities, and urinary tract surgery, obstruction, or malignancy.

predisposing factor. Nosocomial pneumonia due to *S. aureus* occurs in the clinical settings of obstructive pulmonary disease, intubation, and aspiration. Underlying malignant diseases are recognized as important risk factors for the development of *S. aureus* bacteremia. Bacteremia may also "seed" distant sites throughout the body, leading to endocarditis, osteomyelitis, pyoarthritis, and metastatic abscess formation, particularly in the skin, the subcutaneous tissues, lungs, liver, kidneys, and brain.[311,347] *S. aureus* meningitis occurs in patients with central nervous system abnormalities related to trauma, surgery, malignancy, and hydrocephalus. *S. aureus* is also one of many microorganisms associated with peritonitis in patients receiving continuous ambulatory peritoneal dialysis. Toxins produced by *S. aureus* are responsible for toxic epidermal necrolysis (staphylococcal scalded skin syndrome) and toxic shock syndrome (Boxes 12-3, 12-4, and 12-5) (Image Plate 12-1C).[168] *S. aureus*strains may also cause food poisoning because of the elaboration of exotoxins during growth in contaminated

BOX 12-4

Virulence Factors of *Staphylococcus aureus*

Virulence Factor	Comments
Capsular polysaccharides and biofilm formation	Over 90% of *S. aureus* produce exopolysaccharides that act to promote adherence of the organisms to host cells and prosthetic devices and prevent ingestion of the organism by polymorphonuclear cells.[261,316,380,541] Capsular polysaccharides of *S. aureus* are also able to alter the binding and functionality of complement. These exopolysaccharides have been observed by electron microscopic examination of *S. aureus*-infected pacemaker leads, peritoneal catheters, and intravenous lines and have been demonstrated immunologically in vitro. To date, 13 capsular polysaccharide types have been described in *S. aureus*; about 70%–80% of significant clinical isolates belong to capsular serotypes 5 or 8.[456,582] The other capsular serotypes are very rare among clinical isolates. These capsular types are found in both MRSA and MSSA isolates. In one study, 64% of MRSA were capsular types 5, whereas 60% of MSSA strains were capsular type 8.[575] About 10%–20% of clinical isolates are nontypeable by the methods used to characterize these capsular polysaccharide, and nontypeable strains often possess the genes for capsular types 5 or 8 but these genes are not expressed.[108] Some nontypeable isolates express a cell surface polysaccharide termed 336PS that is structurally similar to polyribitol-phosphate *N*-acetylglucosamine and teichoic acids of *S. aureus*.[334,394] Capsular polysaccharides are major components of biofilms, which are surface-attached organism clusters encased in an extracellular polymeric matrix consisting of exopolysaccharides, teichoic acids, proteinaceous material, and extracellular DNA. The DNA found in *S. aureus* biofilms originates from thermonuclease degradation of cellular DNA. Organisms embedded in the matrix display decreased metabolism and are recalcitrant to antimicrobial penetration and activity. The initiation of biofilm formation involves attachment via serum proteins (e.g., fibrinogen, fibronectin, vitronectin) coating catheters or other indwelling devices or via attachment directly to the plastic indwelling material itself. This attachment is promoted by a group of surface molecules belonging to the MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) family of adhesins. Following initial surface interactions, intercellular cross-linking via PIA occurs. Nuclease activity from lysed staphylococcal cells degrades the extracellular DNA in the biofilm and helps disperse the biofilm surface. There has been renewed interest in these capsular polysaccharides because they are essential factors for virulence and antibodies against them appear to be protective. As a result, a capsular antigen-based vaccine for serotypes 5 and 8 has been developed that contains these particular polysaccharides chemically conjugated to a haptenic protein carrier (StaphVAX, Mabi Biopharmaceuticals, Rockville, MD).[159,491,492] A conjugate vaccine against *S. aureus* 336PS is also in development.
Adhesins	The MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) adhesin family includes over 20 small proteins that are bound to the cell wall peptidoglycan of *S. aureus*.[415] Genes for these molecules are located on the chromosome and are present in all *S. aureus* strains. These proteins mediate adherence to host-matrix proteins (e.g., fibronectin, fibrinogen, elastin, other cellular lipids) and plastic implanted devices. Other adhesins function in binding hemoglobin, hemin, transferrin, and haptoglobin. Some adhesins are virulence factors that correlate with staphylococcal ecology and specific disease conditions. For example, clumping factor B (ClfB), fibronectin binding proteins A and B (FnPBA, FnBPB), and collagen-binding protein (Cna) are associated with *S. aureus* nasal colonization, ventricular assist device-related infections, and osteomyelitis, respectively.[25,433,598] Several other proteins (*S. aureus* surface proteins B, C, D, F, G, H, and K) have been described that have as-yet undetermined targets and ligand specificities.
Peptidoglycan, teichoic and lipoteichoic acids	*S. aureus* cell walls contain peptidoglycans (cross-linked polymers of *N*-acetylglucosamine and *N*-acetylmuramic acid), which are similar to those found in other gram-positive bacteria, and teichoic acids, which are unique ribitol (five-carbon monosaccharide)-phosphate polymers (see Chapter 5). Teichoic acids function in promoting adherence to mucosal surfaces and anchoring cell wall-associated enzymes. Lipoteichoic acids are found in the plasma

membrane and maintain linkage of the peptidoglycan to the cell membrane. In addition to providing rigidity and resilience to the staphylococcal cell wall, peptidoglycan components, and teichoic and lipoteichoic acids also have biologic activities that may contribute to virulence. These properties include the ability to activate complement, enhancement of chemotaxis of polymorphonuclear cells, elicitation of interleukin-1 production by human monocytes, and stimulation of the production of opsonic antibodies. Serologic tests for antibodies to these molecules have been investigated for their possible diagnostic and/or prognostic value; results have been disappointing because of considerable overlap of antibody titers among infected and noninfected individuals, the wide spectrum of infection and disease caused by *S. aureus*, and the host-dependent variability of the immune response due to age, other infections, and general immunocompetence. Several other proteins, including MSCRAMM adhesins, are covalently incorporated into the structure of the *S. aureus* peptidoglycan.[415]

Protein A

Protein A is a 42,000-Da MSCRAMMS adhesin that is found on both the cell surface and in medium during growth. This unique protein binds to the Fc region of all human IgG subclasses except IgG_3. Protein A interferes with opsonization and ingestion of the organisms by polymorphonuclear cells, activates complement, and can elicit immediate and delayed type hypersensitivity reactions. Protein A is also a "B-cell superantigen" because it can bind to certain B-cell receptors and consequently limits their ability to synthesize specific antibodies.[194] Protein A also binds vonWillebrand factor and complement protein C3, thereby promoting adherence of bacteria to platelets and is able to catalyzing the conversion of C3 to C3b.[106] The protein A molecule also binds to the receptor for TNF-α and, in so doing, reduces proinflammatory cytokine signaling.[191] Protein A is encoded by the chromosomal *spa* gene. The presence of protein A on *S. aureus* provides the basis for coagglutination test procedures that are used in many clinical laboratories for organism identification and for detection of bacterial antigens in body fluids.

Enzymes

S. aureus produces several enzymes that contribute to its virulence. **Catalase** production by these organisms may function to inactivate toxic hydrogen peroxide and free radicals formed by the myeloperoxidase system within phagocytic cells after ingestion of the microorganisms. **Clumping factor**, a cell-bound material that is able to bind fibrinogen, is responsible for the binding of *S. aureus* to both fibrin and fibrinogen. **Coagulase**, which can exist free in the medium or in a cell-bound form, binds and activates prothrombin and promotes the conversion of fibrinogen to fibrin. This enzymatic activity may act to coat the bacterial cells with fibrin, rendering them more resistant to opsonization and phagocytosis.[356] **Fibrinolysins** break down fibrin clots and facilitate the spread of infection to contiguous tissues. Similarly, **hyaluronidase** hydrolyzes the intercellular matrix of acid mucopolysaccharides in tissue and, thus, may act to spread the organisms to adjacent areas in tissue.[212] Strains of *S. aureus* causing chronic furunculosis have been found to be producers of potent **lipases** that may help to spread the organisms in cutaneous and subcutaneous tissues.[459,469] A **phosphatidylinositol-specific phospholipase C** has also been described that is associated with strains recovered from patients with adult respiratory distress syndrome and disseminated intravascular coagulation. Tissues affected by this enzyme become more susceptible to damage and destruction by bioactive complement components and products during complement activation. *S. aureus* also produces a **nuclease** or **phosphodiesterase** having both exo- and endonuclease activities. Immunologic and substrate-specificity studies indicate that several different types of **β-lactamase enzymes** are produced by *S. aureus*. Production of these enzymes may be inducible (i.e., they are produced only in the presence of β-lactam antimicrobial agents) or constitutive (i.e., they are produced continually) and render these organisms resistant to penicillin and ampicillin. Genes coding for these enzymes usually reside on plasmids (extrachromosomal DNA) that also carry genes for resistance to several antibiotics, such as erythromycin and tetracycline. These resistance genes may be transferred to other bacteria by transformation and transduction.

Hemolysins and leukocidins

S. aureus hemolysins have several biologic activities.[557] **α-Hemolysin** is lethal for a variety of cell types in vitro and will lyse erythrocytes from several animal species, but human erythrocytes are much less sensitive. Human monocytes, macrophages, and lymphocytes are susceptible to lysis by α-hemolysin, but granulocytes are highly resistant. This toxin is a protein with a molecular weight of 33,000 Da and is secreted into the medium during late logarithmic growth. Clusters of phosphatidylcholine, various membrane-associated lipids, and other cell-associated proteins act in concert for specific binding of α-hemolysin to susceptible target cells.[552,553] Individual monomers interact and aggregate on the target cell membrane, forming cylindrical homoheptamers with an aqueous central pore. These pores allow rapid efflux of potassium ions and other small molecules and influx of sodium and calcium ions, leading to osmotic swelling and rupture of the cell. This toxin is also active against platelets and may contribute to septic thrombotic events during *S. aureus* bacteremia.[483] α-Hemolysin is dermonecrotic on subcutaneous injection, and is lethal for animals when administered intravenously. α-Hemolysin toxin is responsible for the zone of hemolyzed red blood cells observed around colonies of some *S. aureus* strains growing on SBA. **β-Hemolysin** is a neutral sphingomyelinase (hydrolyzes the lipocarbohydrate sphingomyelin) that is active on a variety of cell types. β-Hemolysin is a protein exotoxin with a molecular weight of 35,000 Da and is secreted into the medium towards the end of the logarithmic growth phase.[237] Hemolytic activity requires magnesium ions and substrate specificity is restricted to sphingomyelin, which is hydrolyzed to form ceremide and phosphorylcholine. *S. aureus* β-hemolysin is cytotoxic for human monocytes and is inactive against granulocytes, lymphocytes,

and fibroblasts.[584] The varying susceptibilities of erythrocytes from different animal species to β-hemolysin-mediated lysis is probably due to differences in membrane sphingomyelin content. β-Hemolysin is a "hot–cold" hemolysin; that is, its hemolytic properties are enhanced by subsequent exposure of the red blood cells to low temperatures. This property may be caused by initial disruption of cohesive forces within the membrane and subsequent phase separation within the membrane as the temperature is lowered. Cell death probably results from disruption of host cell plasma membrane fluidity.[246] The β-hemolysin, along with the CAMP factor produced by group B streptococci, is responsible for the synergistic hemolysis observed in a positive CAMP test for presumptive identification of group B streptococci. **δ-Hemolysin** and **γ-hemolysin** are found in some *S. aureus* strains and also cause lysis of a variety of cell types. The δ-hemolysin is a small amphipathic peptide with a molecular weight of 3,000 Da that is secreted into the medium towards the end of the exponential growth phase. This hemolysin is produced by over 97% of *S. aureus* strains and is also found in 50%–70% of coagulase-negative staphylococci. The δ-hemolysin acts primarily as a surfactant or detergent-like molecule that interacts with cell membranes and forms channels that destabilize the membrane and increase in size over time, resulting in slow leakage of cellular contents.[576] The γ-hemolysin and the **PVL** are both bi-component pore-forming toxins that are composed polypeptides that are differentiated in the laboratory by the rate of migration on gel electrophoresis into "slow" (S) or fast (F) components.[199] The γ-hemolysin is composed of combinations of either HlgA or HlgC polypeptides (S components) with HlgB (F component). **PVL** is transmitted among staphylococcal strains by lysogenic bacteriophages (φSLT phage) and is composed of a combination of LukS and LukF. Luk ED and LukGH (also called LukAB) are additional bi-component pore-forming toxins. Monomers of these toxins bind sequentially to susceptible cell membranes and oligomerize into pores composed of four "S" components and four "F" components each. Affected cells undergo degranulation of the cytoplasm, cell swelling, and lysis.[397,418] The components of PVL are secreted, extracellular proteins, although LukGH is found as a predominant cell surface protein during the late exponential growth phase.[574] PVL is lytic for a variety of cell types including human neutrophils, murine alveolar macrophages, and rabbit neutrophils, but the role of PVL in the pathogenesis of human infections is unclear.[328] Animal models (i.e., mice, rabbits, monkeys) and studies with human cell types used in PVL research have produced conflicting results and have raised concerns about applicability of these models to human disease. A role for PVL in the pathogenesis of CA-MRSA infections, particularly skin and soft tissue infections, hemorrhagic pneumonia, and osteomyelitis, has been proposed and several studies have examined this association.[121] Large patient-centered studies have not documented a specific role for PVL in association with adverse clinical outcomes in skin and soft tissue infections or hospital-acquired pnemonias.[27,490] PVL genes are highly conserved and are present in nearly every CA-MRSA, including the USA300 strain that causes most CA-MRSA infections in the United States.[49,373,540] HCA-MRSA isolates and MSSA have a low prevalence of PVL gene carriage.[558,580] PVL-producing CA-MRSA strains usually have SCC*mec* type IV. Because the structural genes of PVL in CA-MRSA are found on lysogenic phages, lineages of PVL types reflect the phage type, the location of PVL gene insertion in the staphylococcal chromosome, and the degrees of PVL gene expression.[58] A group of small cytotoxic peptides termed PSMs have been described in *S. aureus, S. epidermidis,* and other coagulase-negative staphylococci.[588] PSMs are secreted α-helical peptides related to δ-hemolysin that also act by destabilizing membranes and causing cellular leakage. The role of PSMs in human staphylococcal infections is unclear, but one PSM gene, *psm-mec* is found within the SCC*mec* mobile genetic element in SCC*mec* types II, III, and VIII.[90] Several superantigen-like molecules have also been described in *S. aureus* that are able to inhibit the migration of neutrophils.[617]

Toxins

Exfoliatins or **epidermolytic toxins** are produced by 1%–2% of *S. aureus* strains and consist of two proteins, designated ET-A and ET-B, each of 24,000 Da molecular weight. The gene for ET-A (*eta*) is carried on a bacteriophage integrated into the chromosome, while the ET-B gene (*etb*) is on a plasmid[303]. These molecules are biochemically and immunologically distinct, have similar biologic activities, and also have superantigenic activities. Both ET-A and ET-B have serine protease activity and target desmoglein 1 (Dsg1), one of four adhesion glycoproteins that are located within desmosomes in the epidermis.[387,611] Dissolution of the mucopolysaccharide matrix of the stratum granulosum in the epidermis results in intraepithelial splitting of cellular linkages in the stratum granulosum.[305] The exfoliatins are responsible clinical presentation of "SSSS". Both exfoliatins also can function as superantigens. Other epidermolytic toxin isoforms (i.e., EF-C and EF-D) have also been identified. **Enterotoxins A through E, H, and I** are heat-stable molecules which are responsible for the clinical features of staphylococcal food poisoning, which is the most common cause of food poisoning in the United States. The exact mode of action of these enterotoxins is unknown, but they have been shown to increase intestinal peristalsis. Ingestion of preformed enterotoxins in food supporting staphylococcal growth (e.g., bakery goods, custards, potato salad, processed meats, ice cream) results in vomiting with or without diarrhea within 2–8 hours. These toxic conditions are self-limited (within 24–48 hours) and require only supportive therapy. Inflammatory changes (e.g., a hyperemic mucosa, neutrophilic infiltrates, mucopurulent duodenal exudates, brush border disruption) are seen throughout the gastrointestinal tract, with the more severe and extensive lesions being found in the stomach and the upper small intestine.

Superantigens

The **staphylococcal enterotoxins** (SEs) belong to a group of at least 15 toxins collectively known as pyrogenic exotoxins, which also includes the **toxic shock syndrome toxin** (TSST-1, see below). The SEs include SE A, B, C_n, D, E, G, H, I, J, K, L, M, N, and O. All of these molecules behave as **superantigens (SAgs)**, and, consequently, have parallel capital-letter designations as SAgs. All of these toxins share three biologic characteristics: pyrogenicity, superantigenicity, and the ability to enhance the lethal effects of minute amounts of endotoxin in rabbits up to 100,000-fold. Most superantigens are small (20–28 kDa) secreted proteins and most are encoded on mobile genetic elements.[398] **Superantigenicity** refers to the ability of these toxins to stimulate the proliferation of T lymphocytes without regard for their antigenic specificities. All of these toxins induce polyclonal T-cell proliferation through co-ligation between major histocompatibility complex class II molecules on antigen-presenting cells and the variable portion of the T-cell antigen receptor β-chain.[169] Following activation by superantigens, a large burst of cytokine release leads to an overwhelming inflammatory response and shock syndrome. In toxic shock syndrome, TSST-1, a small protein with a molecular weight of 22,000 Da, is able to cross adjacent mucosal membranes and enter the circulation after being produced locally by TSST-1-producing strains of *S. aureus*. Systemic release of cytokines and their broad physiologic effects results in rapid development of multisystem disease with TSS.

BOX 12-5

Case Definition of Toxic Shock Syndrome

Feature	Comment
Fever	Temperature ≥38.9°C (102.0°F)
Rash	Diffuse macular erythroderma
Desquamation	1–2 weeks (usually 10–14 days) after onset of illness; particularly of palms, soles, fingers, and toes
Hypotension	Systolic blood pressure ≤ 90 mm Hg for adults or less than fifth percentile by age for children less than 16 years of age. Orthostatic drop in diastolic pressure of ≥15 mm Hg from lying to sitting; Orthostatic syncope or orthostatic dizziness.
Involvement of Three of More Organ Systems:	
Gastrointestinal	Vomiting or diarrhea at onset of illness
Muscular	Severe myalgia or creatinine phosphokinase greater than twice the upper limit of normal
Mucous membranes	Vaginal, oropharyngeal, or conjunctival hyperemia
Renal	Blood urea nitrogen (BUN) or serum creatinine greater than twice the upper limit of normal for laboratory *or* urinary sediment with pyuria (≥5 leukocytes per high power field) in the absence of urinary tract infection
	Total bilirubin, alanine aminotransferase, or aspartate

Hepatic	aminotransferase levels at least twice the upper limit of normal for laboratory
Hematologic	Platelet count <100,000/mm^3
CNS	Disorientation or alterations in consciousness without focal neurologic signs when fever and hypotension are absent
Laboratory Criteria	Negative Results on the Following Tests:

Blood, throat, and spinal fluid cultures (blood cultures may be positive for *S. aureus*)

Serologic tests for Rocky Mountain spotted fever, leptospirosis, and measles

Case Classification

Confirmed	A case in which all six of the clinical findings described above are present
Probable	A case with five of the six clinical findings described above are present

Additional Laboratory Findings Pathognomic for TSS, but Presently Not Included in the Case Definition:

- Isolation of *S. aureus* from a mucosal or normally sterile body site
- Production by an incriminated staphylococcal isolate of TSST-1 or an alternative toxin known to cause TSS
- Serologic susceptibility to the relevant toxin at the time of acute illness
- Development of antibody to the relevant toxin during convalescence

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foods. *S. aureus* possesses several properties which are believed to contribute to their ability to cause disease (Box 12-4). These virulence factors are not found in all strains of *S. aureus*, however, and this organism continues to be a constant source of surprise as new and different pathogenic properties are discovered.[592] Additional information regarding infections associated with *S. aureus* is presented in Box 12-3. The case definition of toxic shock syndrome can be seen in Box 12-5.

Penicillin was the original drug of choice for treatment of serious *S. aureus* infections and the emergence of resistance was due to acquisition of plasmid-borne genetic elements encoding β-lactamases. Semisynthetic, penicillinase-resistant penicillins (i.e., oxacillin, methicillin, nafcillin) were developed for treatment and introduced for clinical use in 1959. During the 1980s, resistance to these agents also

emerged. Resistance to methicillin is due to the presence of an altered penicillin-binding protein called PBP2a (or PBP2′) that results from acquisition of a chromosomal gene called *mecA,* which is located within the large mobile staphylococcal chromosomal element known as the SCC*mec* (staphylococcal cassette chromosome). Many SEC*mec* types and subtypes have been identified since these genetic elements were first described (see typing section, below).[195] Interestingly, it is believed that *S. aureus* acquired the SCC*mec* transposon from *S. sciuri*, a coagulase-negative staphylococcal species found on animals and in the environment.[609] The PBP2a protein has a low affinity for all β-lactam agents, including cephalosporins. Once the normally present PBPs have been inactivated by a β-lactam agent, PBP2a continues to function and allows synthesis of a stable peptidoglycan structure, thereby allowing the organism to grow and divide. *S. aureus* strains that contain SCC*mec* are termed *methicillin-resistant S. aureus* (MRSA) in order to distinguish them from *S. aureus* that lack this element and are therefore susceptible to methicillin (i.e., MSSA, or methicillin-susceptible *S. aureus*).

In order to further characterize these organisms, MRSA from US hospitals were examined by pulsed-field gel electrophoresis (PFGE) and eight clusters—USA100 through USA800—were delineated.[358] All but two PFGE types—USA300 and USA400—were from infections associated with healthcare facilities and nosocomial infections. USA300 subsequently emerged as the predominant clone causing community-acquired MRSA infections, with severe skin and soft tissue infections as the predominant clinical presentation.[401,540,558] At present, two types of MRSA infections are recognized: healthcare-associated MRSA (HA- or HCA-MRSA) and community-acquired MRSA (CA-MRSA) infections.[34] HCA-MRSA infections occur mostly among elderly individuals and include predominantly bloodstream infections, pneumonia, and co-infections with other nosocomial agents. Individuals at risk for these infections usually have a history of recent hospitalization, living in a long-term-care facility, having an indwelling catheter in place, or recent surgery. HCA-MRSA isolates also tend to be multiresistant. Infections with CA-MRSA occur in those without healthcare-associated risk factors, and infections include soft tissue infections (including necrotizing fasciitis), severe necrotizing pneumonia, sepsis, and bone and joint infections.[34,68,87,121,149,267,269,571] CA-MRSA outbreaks have occurred among diverse populations in the community such as prisoners, children in day care centers, participants in contact sports, and military recruits, and are disproportionately represented among medically underserved populations in urban areas.[34,68,87,121,149,267,269] Reported increases in the incidence of pediatric skin/soft tissue infections caused by MRSA have largely been driven by the emergence of CA-MRSA.[178] The community-associated USA300 MRSA clone has now spread from the community and back into healthcare facilities, where it has now become a significant

cause of healthcare-associated and nosocomial bloodstream infections.[485] A study examining bloodstream and skin/soft tissue infections due to MRSA from 2000 to 2008 found that USA300 accounted for 55% of bacteremias and was the predominant strain causing community-associated, healthcare-associated, community-onset, and hospital-onset bloodstream infections.[537] The USA300 MRSA clone has now been reported on 5 continents and in over 36 countries.[324,386] HCA-MRSA and CA-MRSA also differ in their SCC*mec* types. Types I, II, and III are found in HCA-MRSA strains, while types IV, V, and VII predominate among CA-MRSA. The predominant subtype of the USA300 CA-MRSA clone in the United States, USA300-0114, possesses SEC*mec* type IV and SCC*mec* type 4a. Recently, several new SCC*mec* types and subtypes (SCC*mec* IIA-IIE, SCC*mec* types IVa–IVg, SCC*mec* types IX, X, V [5C2 and 5], subtype C) have been described.[174,323] The SCC*mec* types seen on CA-MRSA strains do not carry multiple antibiotic-resistant genes as seen in HCA-MRSA strains, and CA-MRSA SCC*mec*types are more likely to carry genes for the phage-associated Panton–Valentine leukocidin (PVL).[558] Subtypes of the USA300 clonal type have been reported in other countries (e.g., USA300-LV, USA300-P) and appear to cause diseases similar to the predominant USA300 subtype in the United States, with an emphasis on skin and soft tissue infections (e.g., furunculosis).[12,184,268,571]

In colonized individuals, *S. aureus* is carried predominantly in the anterior nares, but it can also be isolated from other sites, including the groin, axilla, and the external auditory canal. Healthy infants become colonized during the second or third month of life with strains that are usually acquired from the mother or the environment. Carriage rates at this time may be as high as 60%. With the development of the normal oropharyngeal microbiome and maturation of the immune system, carriage rates usually decrease several-fold after this time. Children and teenagers have higher rates of carriage than adults, and rates in the latter group are influenced by many factors (age, general health, comorbidities, chronic diseases). Currently, prevalence rates of *S. aureus* nasal colonization in healthy adults range between 25% and 35%, depending on the population surveyed. Individuals may persistently carry the same strain for extended periods, while others carry a single strain for a few months that is eventually replaced by another.[291] Nasal carriage of *S. aureus* can have serious clinical implications as well. Carriers have higher rates of staphylococcal infections than noncarriers, and nosocomial *S. aureus* infections are often caused by the strain being carried in the nares of the patients themselves. Reduction or eradication of nasal carriage can have significant effects on the rates of nosocomial infections. Carriage of MRSA, in particular, emerged early in the last decade and

presents particular issues for healthcare facilities since these colonized individuals are the most significant reservoirs of MRSA in both healthcare facilities and in the community upon discharge of carriers from hospitals or long-term-care facilities.[291]

Because MRSA causes severe infections with significant morbidity and mortality, preventing the spread of MRSA has become a priority over the last 10 to12 years. As with MSSA, MRSA carriers or those with MRSA infections are the most significant reservoirs of the organism in the hospitals and other healthcare facilities.[471] In 2001, the Veterans' Administration Hospital in Pittsburgh, PA, implemented a multifaceted MRSA surveillance program involving active surveillance for MRSA nasal colonization, isolation and contact precautions for colonized patients, and attention to hand hygiene. This approach succeeded in decreasing HCA-MRSA infections in the intensive care unit by 75%.[251] In 2003, a preventive strategy for both MRSA and vancomycin-resistant *Enterococcus* (VRE) was proposed by the Society for Healthcare Epidemiologists (SHEA) and the Centers for Disease Control and Prevention (CDC), but a proactive approach was not documented at that time. In 2004, Davis and colleagues[122] at Brooke Army Medical Center demonstrated that 19% of patients who were colonized with MRSA in the nares on hospital admission and 25% who become colonized with MRSA during hospitalization developed MRSA infection, compared with the 1.5% and 2% of patients colonized with MSSA, respectively, on admission. During 2007, the Veterans Administration became the first healthcare system to mandate surveillance testing for MRSA, and subsequently Illinois passed legislation requiring MRSA screening and reporting of at-risk patients. Legislation was subsequently passed in Pennsylvania, New Jersey, Minnesota, Maine, and California. The point of these proactive preventive strategies is to rapidly detect individuals who are colonized with MRSA, and several studies have documented that surveillance for MRSA in patients being admitted to the hospital is associated with reduction in MRSA-associated infections and disease.[120,210,426,452,522,591] In some institutions, every individual admitted to a healthcare facility is screened, while other programs screen only those patients who are considered high risk for MRSA colonization. Such patients include those who have been transferred from a long-term-care facility or from another healthcare facility, those who have been hospitalized within the past 6 months, and those who have a documented history of MRSA colonization or infection. Some healthcare facilities also screen individuals who have come from or traveled to countries with high MRSA prevalence rates.[452] Screening methods for detection of MRSA colonization include culture-based and molecular-based techniques (see sections covering culture- and molecular-based methods below). Molecular approaches for detection of MRSA offer several advantages over culture-based methods, including lower limits of detection, higher-throughput screening, and shorter times to detection. Reduced transmission of MRSA has been demonstrated when surveillance testing was done using real-time PCR, but no significant impact on

transmission was seen when culture-based testing was performed.[210] Detection of MRSA results in isolation of the patient and, in some cases, decolonization protocols to reduce the burden of MRSA prevalence and to decrease transmission. Decolonization protocols usually involve treatment with mupirocin nasal ointment and bathing with chlorhexidine.

With the emergence of resistance to the penicillinase-resistant penicillins, the glycopeptide agent vancomycin became the treatment of choice for infections due to MRSA.[563] Vancomycin-susceptible *S. aureus* (VSSA) has vancomycin minimum inhibitory concentrations (MICs) of ≤2 μg/mL.[107] However, in May of 1996, the first documented infection caused by an *S. aureus* strain with intermediate resistance to vancomycin was reported from Japan.[229] These isolates have been given the mnemonic "GISA" for "glycopeptide-intermediate *S. aureus*" as these strains have diminished susceptibility to both vancomycin and teicoplanin glycopeptide antibiotics; VISA (vancomycin-intermediate *S. aureus*) is the name used in the United States.[374] These strains have vancomycin MICs of 4 to 8 μg/mL and display a homogeneous phenotype. Strains of VISA have thickened cell walls, which is thought to prevent vancomycin from reaching its site of action in the bacterial cell wall division septum.[236] The Clinical and Laboratory Standards Institute (CLSI) defines *S. aureus* isolates requiring concentrations of vancomycin of ≤ 2 μg/mL for growth inhibition as "susceptible," those requiring 4 μg/mL to 8 μg/mL as intermediate, and those requiring ≥16 μg/mL vancomycin as resistant.[19,107,202] Among VISA strains, a heterogenous phenotype has also been recognized. Those isolates appear susceptible to glycopeptides (vancomycin MIC ≤ 4μg/mL) but contain subpopulations of cells with reduced susceptibility to vancomycin (vancomycin MIC 8 to 16 μg/mL) that occur at low frequencies. These strains are termed hVISA. Rare isolates of vancomycin-resistant *S. aureus* (VRSA) have been reported, and these isolates will have vancomycin MICs of ≥16 μg/mL. The vancomycin-resistant strains contain the *vanA* resistance determinant found in vancomycin-resistant enterococci. Reduced susceptibility to vancomycin is not reliably detected by disk diffusion; this method cannot differentiate vancomycin-intermediate and vancomycin-susceptible strains and the zone diameters do not correlate with staphylococcal vancomycin MICs. The disk diffusion test will only detect VRSA containing the *vanA* determinant and will show no zone around the 30-μg disk. VRSA strains can be reliably detected by current reference and commercial methods, including reference microbroth dilution, agar dilution, Trek Sensititre MIC panels, Microscan overnight and Synergies-Plus panels, Etest strips, and vancomycin screen agar.[202] Both the BD Phoenix and the VRSA screen on the Vitek 2 will also detect these strains. VISA strains are most easily detected by reference broth microdilution, Etest with a 0.5 McFarland-standardized inoculum, and agar dilution. Automated methods may detect VISA strains with MICs

of 8 μg/mL, but may not detect those with MICs of 4 μg/mL. The use of BHI agar with 6 μg/mL vancomycin is a useful adjunctive test for detection of both VISA and VRSA strains. In fact, CDC recommends that laboratories incorporate the vancomycin agar screening plate for all *S. aureus* isolates.[202] BHI agar with 6 μg/mL vancomycin is spot-inoculated with a 10 μL inoculum from a suspension equivalent to a 0.5 McFarland turbidity standard and inspected for growth after a full 24 hours' incubation. All isolates with vancomycin MICs of 8 μg/mL and some with

MICs of 4 μg/mL will grow on these screening plates. Isolates that grow on the screening plate should then be tested by a broth microdilution procedure to confirm resistance. Etest performed on Müeller–Hinton agar with an inoculum prepared from a 0.5 McFarland-equivalent suspension and incubated a full 24 hours is also a very sensitive and specific method for detection of these strains; with Etest, VISA strains have vancomycin MICs of ≥4 μg/mL and VRSA have MICs of ≥16 μg/mL. According to the CDC, three criteria must be met to verify that a given strain is a VISA: broth microdilution vancomycin MICs of 8 to 16 μg/mL, Etest vancomycin MICs of ≥6 μg/mL, and growth within 24 hours on a BHI agar plate containing 6 μg/mL vancomycin (Table 12-1).[202] Prompt and accurate detection and confirmation of these strains is necessary and critical for optimal treatment of individual patients and for prevention of transmission of these strains to other patients. Jacob et al.[249] have reviewed and performed a meta-analysis of the clinical outcomes of adults with MRSA infections wherein the isolates had a high MIC to vancomycin.

Staphylococcus aureus *subsp.* anaerobius

S. aureus subsp. *anaerobius* causes Morel's disease in sheep, which is characterized by subcutaneous abscess formation in and around the superficial lymph nodes.[525] This bacterium has not been isolated from human infections or clinical specimens. However, in 2006, a similar obligately anaerobic staphylococcal isolate was recovered from a 45-year-old male farm hand who presented with septicemia, septic arthritis, and multiple pulmonary abscesses.[416] While true isolates of *S. aureus* subsp.*anaerobius* are catalase-negative, the isolate from this patient was weakly catalase-positive and differed from *S. aureus* subsp. *anaerobius* and *S. saccharolyticus* (the other obligately anaerobic *Staphylococcus* species) by several phenotypic characteristics. Genotypic methods could not differentiate this organism from *S. aureus* subsp. *aureus,* subsp. *anaerobius,* or *S. saccharolyticus;* the authors surmised that this isolate was either a variant strain of *S. aureus* subsp. *anaerobius* or an undescribed subspecies of *S. aureus*.

Coagulase-Negative Staphylococci

In the past, coagulase-negative staphylococci were generally considered to be contaminants having little clinical significance. Some of these are important in the cheese industry.[434,435] Over the last four decades, however, these organisms have become recognized as important agents of human disease.[33,186,585] While several different species of coagulase-negative staphylococci have been described (Box 12-2), relatively few of them cause infections in humans. However, as more laboratories have attempted to identify the coagulase-negative staphylococci, infections caused by other species are being recognized more frequently. The types of infections associated with coagulase-negative staphylococci are described in Box 12-6.

Staphylococcus epidermidis. When clinical findings are correlated with the isolation of coagulase-negative staphylococci, *S. epidermidis* is by far the most frequently recovered organism, accounting for 50% to over 80% of isolates. Almost all infections caused by *S. epidermidis* are nosocomially acquired, except for native valve endocarditis and infections of semipermanent venous access devices. In addition to both native valve and prosthetic valve endocarditis, *S. epidermidis* has been isolated and documented as a pathogen in urinary tract infections, surgical wound infections, infections of various prosthetic devices, cerebrospinal fluid (CSF) shunt infections, peritoneal dialysis-related infections, and ophthalmic infections (Box 12-6).[103] Several virulence factors have now been described in *S. epidermidis*.[400,601] Scanning and transmission electron microscopic studies and immunologic examination of *S. epidermidis* strains from infections of indwelling medical devices demonstrated that these bacteria produce cell surface and extracellular macromolecules that initiate and subsequently enhance bacterial adhesion to foreign bodies, eventually forming a biofilm.[469] When plastic prosthetic devices are implanted they become rapidly coated with serum proteins, and *S. epidermidis* is able to specifically bind to fibronectin, fibrinogen, collagen, and vitronectin as well as to the plastic catheter material itself. A wide spectrum of adhesin molecules that are able to bind these serum proteins are found on the cell surface of *S. epidermidis* and include Aae and AltE (autolysin-adhesins), Ebp (Elasin-binding protein), Empb (extracellular matrix-binding protein), Fbe (fibrinogen-binding protein), and GehD (glycerol ester hydrolase).[60,213,223] Several staphylococcal surface proteins (e.g., Ssps) that bind directly to polystyrene have also been

TABLE 12-1 CDC Confirmatory Test for Detection of VISA and VRSA[202]

Method	Results for VRSA	Results for VISA	Comments
Reference broth microdilution	Vancomycin MIC ≥16 μg/mL in Mueller–Hinton broth	Vancomycin MIC of 4–8 μg/mL in Mueller–Hinton broth	Incubate for a full 24 hours.
Brain–heart infusion agar with 6 μg/mL vancomycin (commercial source)	Growth of >1 colony in 24 hours	Growth of >1 colony in 24 hours	Two or more colonies is a positive test.[a]
Etest	Vancomycin MIC ≥16 μg/mL on Mueller–Hinton agar	Vancomycin MIC ≥4 μg/mL on Mueller–Hinton agar	Use a 0.5 McFarland standard to prepare the inoculum suspension and incubate for a full 24 hours.

[a]For QC, use *Enterococcus faecalis* ATCC 29212 as a susceptible control and *Enterococcus faecium* ATCC 51299 as a resistant control.

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BOX 12-6

Infections Associated With *Staphylococcus epidermidis* and Other Coagulase-Negative Staphylococci

Infection	Comments
Urinary tract infections	Among the coagulase-negative staphylococci, *S. saprophyticus* causes both upper and lower urinary tract infections, primarily in young, sexually active women. This organism is the second most common cause of urinary tract infections after *Escherichia coli*, and is found in 3%–9% of acute, uncomplicated cystitis.[219,444] Complications of *S. saprophyticus* urinary tract infections include pyelonephritis, sepsis, kidney stones, and, rarely, endocarditis.[100,165,219,496] *S. saprophyticus* may also cause urinary tract infections with or without attendant complications in male children and men.[163,544] In clean-voided urine or catheterized specimens, colony counts of *S. saprophyticus* may be less than 100,000 cfu/mL and, consequently, the organism may be dismissed as a contaminant. Other coagulase-negative staphylococci are rare causes of urinary tract infections, and about 80%–90% of these infections are caused by *S. epidermidis*. Urinary tract infections caused by *S. epidermidis* and other coagulase-negative staphylococci are usually nosocomial and associated with indwelling urinary catheters. These infections occur in elderly male patients with prior urinary tract instrumentation, renal transplantation, urolithiasis, or other urological abnormalities. Pyuria and significant upper tract disease are usually seen in only about 10% of the latter group of patients. Coagulase-negative isolates from these patients are often multiply-resistant.
Implanted prostheses-related infections	Device-related infections in this category include various types of prostheses (e.g., knee and hip replacements). Coagulase-negative staphylococci, particularly *S. epidermidis*, are the most common cause of infections of implanted prosthetic devices.[126,581] Small numbers of organisms may be introduced during device placement or manipulation, leading to device loosening and failure, usually within 2 months to 2 years after implantation. Patients may also have histories of underlying diseases, including malignancy, corticosteroid use, sickle cell anemia, solid organ transplantation, and HIV infection. Other conditions such as obesity also may contribute. Patients usually present with dull recurring pain at the affected area. Prostheses-associated infections are diagnosed by direct examination and culture of needle aspirates from the affected site, by bone biopsy, or removal and culture of the implanted material. It has been shown that sonication of the explanted device prior to culture may increase the yield of organisms.[545] Following device removal and tissue debridement, patients are generally treated with antibiotics (i.e., usually vancomycin alone or combined with rifampin), and the device is reimplanted. Osteomyelitis due to coagulase-negative staphylococci may occur as a complication of prostheses implantation. Hematogenous osteomyelitis may result from bacteremia with coagulase-negative staphylococci arising from infections of shunts or other prosthetic devices.
Native valve endocarditis	Coagulase-negative staphylococci are rare causes of native valve endocarditis, accounting for about 7%–11% of all cases, excluding those cases associated with intravenous drug use.[10,102,104,128,378,381,430] Pathogenesis involves seeding of previously damaged heart valves or endocardium during transient bacteremia, although rare cases of native valve infection without prior underlying cardiac disease do occur.[10] *S. epidermidis* causes about 85% of cases of native valve endocarditis, with the remainder being caused by *S. hominis* (~6%), *S. lugdunensis* (~5%), *S. haemolyticus*, *S. capitis*, *S. caprae*, and *S. simulans*.[116,156,258,495,520] *S. lugdunensis* is the cause of 3%–8% of native valve endocarditis, often in the setting of preexisting cardiac and valvular disease (e.g., mitral valve prolapse). *S. lugdunensis* infective endocarditis can be unusually severe, with massive valvular destruction.[167,417,495] Complications (e.g., embolic phenomena, conduction defects, congestive heart failure) occur commonly in these patients and more than 50% of patients ultimately require valve replacement.[73,102,417] Mortality associated with this condition may be as high as 36%–40%.[104] Infections of cardiac pacemakers may occur at the time of placement or via hematogenous seeding of the device from a distant site.
Prosthetic valve endocarditis	In contrast to native valve endocarditis, coagulase-negative staphylococci are common etiologic agents of prosthetic valve endocarditis and cause from 20%–40% of cases, with *S. epidermidis* being the most frequently isolated species, almost to the exclusion of all others.[104,308,586] In this infection, organisms infect the ring of sutures holding the valve in place, causing the formation of micro-abscesses that are relatively protected from antibiotics. These organisms are usually inoculated into the site during surgery with symptoms becoming apparent 8–12 months later. The patient usually presents with fever and evidence of valve dysfunction. Diagnosis is made by blood cultures, which will be repeatedly positive with the same organism, and by transesophageal echocardiography. Complications are common and include dehiscence of the valve, intracardiac abscesses, valve obstruction due to formation of bulky vegetations, and occasionally cardiac dysrhythmia.[104,308,586] Since most of these infections are probably due to inoculation of small numbers of organisms into the site at the time of valve placement, the symptoms of endocarditis appear slowly over a period of several months. In addition to prosthetic valves, staphylococci may also infect pacemaker wires, implanted defibrillators, and vascular grafts. These infections also have an indolent presentation and transesophageal echocardiography is very helpful for delineating the extent of infection. Sequential blood cultures are usually positive. Treatment involves removal of the device and antimicrobial therapy, followed some time later by device replacement.

Intravenous catheter infections	Coagulase-negative staphylococci are responsible for 50%–70% of nosocomial bloodstream infections involving intravascular catheters, including both central venous and peripheral intravenous catheters.[209] Other implicated catheter types include central hyperalimentation catheters, subclavian hemodialysis catheters, Hickman and Broviac central lines, and Swan–Ganz catheters. For peripheral catheters, infection usually results from skin organisms that enter via the cutaneous surface along the catheter, thereby gaining access to the bloodstream. For longer-term and tunneled catheters, the hub of the catheter becomes colonized with skin or environmental bacteria. Organisms then gain access to the bloodstream via the lumen of the catheter itself.[146] Infected catheters may be the source of bacteremias, which may seed distant sites to create additional infectious complications. Infected catheters and catheter insertion sites often do not appear infected (e.g., erythema or purulence may not be present) and catheter-related bacteremia may be minimally symptomatic. While assiduous care of catheters may decrease the incidence of these bacteremic episodes, other innovations, such as antibiotic-impregnated catheters (e.g., rifampin- or minocycline-impregnated catheters) appear to significantly decrease the incidence of catheter-related infections.[118] Therapy for these infections includes removal of the catheter, if possible, along with systemic antimicrobial therapy. Documentation of intravenous catheter-related infections and bacteremias involves culture of peripheral blood by venipuncture, culture of blood drawn through the catheter, and, oftentimes, culture of the catheter tip itself. Several protocols have been proposed for documentation of catheter-related infections. Incubation time to positivity of less than 25 hours has been considered as an indicator of catheter-related bacteremia in immunocompromised pediatric patients, as the blood collected through the catheter would have a larger microbial burden than the peripheral blood collection.[337,348,614] A difference in detection time of 2 hours or more between blood cultures collected through the catheter and a peripheral blood culture has also been promulgated as a sensitive and specific way to diagnose catheter-related bacteremia.[176,441]
Cerebrospinal fluid shunt infections	*S. epidermidis* and other coagulase-negative staphylococci are common causes of infection associated with ventricular shunts, indwelling CSF catheters, intrathecal pumps, and ventriculostomy sites.[439,587] These infections usually occur within 2 weeks to 2 months after shunt placement, but may also occur during other shunt manipulations. Patients with CSF shunt infections may be asymptomatic, although some may present with subtle symptoms, including low-grade fever, nausea, vomiting, and headache. Examination of CSF aspirated from the shunt may show mild pleocytosis, with glucose being normal or slightly low. Diagnosis is made by culture of CSF from the shunt or from the cerebral ventricles. Prophylactic antimicrobial therapy following shunt placement may reduce shunt-related infection in up to 50% of patients.[443] Shunt removal and subsequent replacement is often necessary to effect cure, although some staphylococcal shunt infections have been effectively treated with antimicrobial agents alone.[482] Therapeutic agents usually include vancomycin, rifampin, and an aminoglycoside administered intraventricularly, since these nosocomially acquired infections are often due to staphylococci that are resistant to methicillin.
Peritoneal dialysis catheter-associated infections	Coagulase-negative staphylococci are the most frequently isolated organisms from patients with CAPD-associated peritonitis.[155,526] Peritonitis is characterized clinically by abdominal pain, nausea, vomiting, fever, and a cloudy effluent after dialysis, although many patients may have only a few or very mild symptoms. Peritoneal fluid may appear cloudy and may contain >100 leukocytes per mL. Gram stains of unconcentrated fluid may be negative, necessitating centrifugation or filtration for recovery of small numbers of organisms. Antimicrobial therapy of CAPD-associated staphylococcal peritonitis may be administered parenterally, orally, and/or intraperitoneally and usually includes oxacillin or methicillin, cephalosporins, vancomycin, or trimethoprim-sulfamethoxazole.
Vascular graft infections	Vascular graft infections are often caused by coagulase-negative cocci, particularly *S. epidermidis*.[84,320] The incidence of vascular graft infections ranges from 0.6% to 3% and depends on the graft location, with aortic vascular grafts having the highest morbidity and mortality rates, and inguinal grafts involving the groin area having higher infection rates. Vascular graft infections that occur early after graft placement are more acute in clinical presentation and usually involve gram-negative bacteria (e.g., *Enterobacteriaceae*, *Pseudomonas aeruginosa*) and *S. aureus,* while infections occurring months later usually involve coagulase-negative staphylococci. Staphylococcal isolates recovered from these infections are often multiply-resistant, suggesting that the infections are due to the presence of contaminating organisms in the grafts themselves. Patients may be considered to have definite vascular graft infection if the patient has clinical signs of infection (e.g., fever, chills, shock, pain/erythema in the area of the prosthesis), biologic evidence of infection (e.g., elevated white blood cell count, elevated C-reactive protein, abscess, perigraft air or fluid on CT scans), and positive blood cultures and/or positive intraoperative specimens from the graft itself.[319] In addition to blood cultures, the most suitable specimens for microbiologic diagnosis of vascular graft infections include explanted graft tissue, perigraft fluid obtained by CT-guided aspiration, or specimens from the graft obtained during surgery.[320] Oftentimes, removal of the vascular graft along with antimicrobial therapy, is necessary to prevent additional morbidity and motality.[470]

Bloodstream infections	In some medical centers, coagulase-negative staphylococci (particularly *S. epidermidis*) are the most common agents of bloodstream infections, being responsible for 30%–40% of these cases.[602] These organism usually reach the bloodstream via infected vascular access devices.[362] Patients who develop catheter-related staphylococcal bacteremia usually have multiple access devices in place for treatment of hematologic malignancies or administration of chemotherapeutic agents for bone marrow transplantation. Patients usually present with signs and symptoms of bacteremia, with fever, hypotension, and leukocytosis, but obvious clinical signs and symptoms may be absent.[595] The issue is complicated by the fact that coagulase-negative staphylococci are the most common blood culture contaminants and intravenous catheters often become contaminated with coagulase-negative staphylococci.[38] Blood cultures collected through catheters should be accompanied by peripheral cultures collected by venipuncture to assess whether the catheter is the source of possible bloodstream infection.[362] Semiquantitative cultures of intravenous catheter tips are commonly performed in laboratories. The catheter roll technique described by Maki and colleagues[337] indicates that colony counts of 15 cfu or greater suggests a possible diagnosis of catheter-related bloodstream infection. Positive semiquantitative cultures with negative peripheral blood cultures indicate catheter colonization.[468] Empiric therapy should be directed against methicillin-resistant coagulase-negative staphylococci, but also requires catheter removal, if possible.
Neonatal and pediatric infections	Most pediatric infections are nosocomial staphylococcal bacteremias occurring in neonatal intensive care units.[264,573] These infections are associated with prematurity, low birth weight, the presence of indwelling peripheral and/or central lines, ventilatory assistance, and administration of total parenteral nutrition.[169,581] While most staphylococcal agents in such infections are *S. epidermidis*, other species (e.g., *S. haemolyticus*, *S. hominis*, *S. warneri*, *S. cohnii*, *S. capitis*) have also been involved. In some instances, coagulase-negative staphylococci causing infections in neonatal intensive care units originate from nurses and other caregivers or from the hospital environment. Neonates with bacteremia due to coagulase-negative staphylococci may present with neutropenia, thrombocytopenia, metabolic acidosis, and apnea. In addition to bacteremia, coagulase-negative staphylococci can also cause pneumonia, meningitis, urinary tract infections, and cutaneous manifestations.
Ocular infections	Coagulase-negative staphylococci are the most frequently implicated pathogen causing postsurgical endophthalmitis. Surgical procedures associated with these infections include lens implantation and cataract surgeries.[1,46,119,309] The diagnosis is made by aspiration and culture of vitreous fluid. Treatment of coagulase-negative staphylococcal endophthalmitis requires both parenteral and intraventricular administration of antimicrobial agents, including linezolid, rifampin, and/or vancomycin. Coagulase-negative staphylococci have also caused hospital-acquired conjunctivitis in neonatal ICUs, suture-related corneal infections following penetrating keratoplasty, blepharokeratoconjunctivitis, and purulent conjunctivitis.[59,235,607] Coagulase-negative staphylococci may also be a cause of bacterial keratitis, particularly in the settings of previous corneal surface disease and ocular trauma.[197] Coagulase-negative staphylococci are the predominant organisms causing chronic dacryocystitis (inflammation of the lacrimal sac). In a study conducted in India, 44.2% of isolates from patients with chronic infection were coagulase-negative staphylococci, while *S. aureus* and *S. pneumoniae* accounted for 10.8% and 10% of isolates, respectively.[29] Coagulase-negative staphylococci are also significant causes of conjunctivitis in newborns.[583] A susceptibility survey of 117 coagulase-negative staphylococcal corneal isolates found that all were susceptible to vancomycin, linezolid, and rifampin, and that susceptibility to gentamicin, tetracycline, and fluoroquinolones varied considerably.[150] The ARMOR ocular surveillance studies have noted that resistance among coagulase-negative staphylococci to ophthalmic fluoroquinolones (e.g., ofloxacin, gatifloxacin, moxifloxacin) will need to be monitored prospectively to prevent treatment failures and provide guidance for empiric therapy of ocular infections.[201]
Cutaneous infections	Coagulase-negative staphylococci may be recovered from a variety of cutaneous lesions (cysts, carbuncles, furuncles) both alone and along with other pyogenic bacteria, including *S. aureus* and β-hemolytic streptococci. Coagulase-negative staphylococci are also common in polymicrobial surgical site infections, particularly following cardiothoracic surgery.[502] The most commonly implicated species have included *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, and *S. hominis*. Unusual cutaneous infections involving *S. epidermidis* include malignant external otitis.[504]

discovered. Following initial adhesion, the microorganisms synthesize a polysaccharide intercellular adhesin (PIA) that crosslinks cells together within the developing biofilm. PIA is a linear polysaccharide composed of β-1,6-linked 2-deoxy-2-amino-D-glucopyranosyl residues, of which 80% to 85% are *N*-acetylated, with the rest being either non–*N*-acetylated and positively charged or modified with phosphate or

succinate residues and negatively charged.[335,465,466]PIA structural genes reside within a four-gene chromosomal operon called *ica*, with the four genes being designated *icaA*, *icaB*, *icaC*, and *icaD*. *S. epidermidis* also produces phenol-soluble modulins (PSMs) like *S. aureus* that act to disperse the outer biofilm layers, which subsequently interact with neutrophils, causing degranulation and cell lysis.[400] *S. epidermidis* also

produces a variety of bacteriocins that bind to and lyse other bacteria, allowing the organism to compete more effectively with other bacteria and efficiently colonize skin surfaces. Once established on cutaneous surfaces, some *S. epidermidis* produce proteases, lipases, and fatty acid modifying enzymes that contribute to cutaneous staphylococcal and polymicrobial infections.[469] Resistance to methicillin is also highly prevalent among *S. epidermidis* isolates, with rates ranging from 75% to over 90%, and SCC*mec* types I, IIa, IIb, III, IV, IVa-IVd, V, and several variants of these types have been detected in *S. epidermidis* isolates from humans.[174,239,372,467,621] SCC*mec* types conferring resistance to methicillin are also found in other human- and animal-associated coagulase-negative staphylococcal species, including *S. haemolyticus* (SCC*mec* types I-V, III variant), *S. warneri* (SCC*mec* type IVE), *S. saprophyticus* (SCC*mec* type III, *S. hominis* (SCC*mec* III), *S. capitis* (SCC*mec* types I, IA, II-V), *S. sciuri* (SCC*mec* type III),*S. xylosus* (SCC*mec* type III), and *S. lentus* (SCC*mec* type III).[174,342,467,618,621]

Staphylococcus saprophyticus* subsp. *saprophyticus. The coagulase-negative species *S. saprophyticus* deserves special mention as this species is a well-documented cause of acute urinary tract infections. In 1996, a new subspecies *of S. saprophyticus* was characterized among coagulase-negative, novobiocin-resistant strains recovered from 7% of nostril cultures from healthy cows.[207] Phenotypic characteristics, cell wall and fatty acid analysis, and genetic relatedness studies showed that these isolates were similar to but distinct from *S. saprophyticus* isolates recovered from humans. These bovine strains have since been named *S. saprophyticus* subsp. *bovis*, while human isolates are now called *S. saprophyticus* subsp. s *aprophyticus* (hereafter referred to as *S. saprophyticus*).

S. saprophyticus causes both upper and lower urinary tract infections, primarily in young, sexually active women of childbearing age. This organism is the second most common cause of urinary tract infections after *Escherichia coli* and is found in 3% to 9% of acute, uncomplicated cystitis.[221,444,601] In urine specimens from these patients, the organism is usually present in quantities ≤100,000 cfu/mL, but will be detected in sequential specimens. Patients usually present with dysuria, pyuria, and hematuria.

Upper urinary tract infections (i.e., pyelonephritis) may be seen in 41% to 86% of patients and, occasionally, *S. saprophyticus* bacteremia may occur as a complication of upper urinary tract infection.[100,188] Complications of *S. saprophyticus* urinary tract infections include pyelonephritis, sepsis, and kidney stones.[100,165,219,496] *S. saprophyticus* may be a cause of acute, symptomatic urinary tract infections in both male and female children and teenagers in the absence of urinary tract structural abnormalities.[4,263,544] This organism has also caused acute urinary tract infections, urethritis, and catheter-associated urinary tract infections in men, prostatitis in elderly men, and rarely, bacteremia, sepsis, and endocarditis.[50,188,263,382,496] In 1999, Hell and colleagues[224] reported the isolation of *S. saprophyticus* as a cause of nosocomially acquired pneumonia. The rectal canal likely serves as the reservoir for the organism and food may be the ultimate source for the organism, as it has been isolated from farm animals, raw beef and pork, pasture grass, and fodder.[220,221] Molecular studies of isolates from urinary tract infections indicate that multiple clonal types of *S. saprophyticus* are capable of causing disease and these pathogenic clones are widely disseminated and persist in the population for several years.[601]

Other Coagulase-Negative Staphylococci. Other staphylococcal species are found in humans and animals as part of the normal microbiome and as causes of several types of infections (Box 12-2). Some species are environmental and have uses in various industries, including food processing. While coagulase-negative species other than *S. epidermidis* and *S. saprophyticus* are frequently found as contaminants in clinical specimens, medical progress has created an important role for many other coagulase-negative staphylococci in human infections and disease. Several species have been reported as causes of human infections, principally in wounds, urinary tract infections, bloodstream infections, osteomyelitis, catheter-related sepsis, ventriculoperitoneal shunt infections, and both native valve and prosthetic valve endocarditis (Boxes 12-2 and 12-6). They are important opportunistic pathogens in immunocompromised patients, including premature neonates, neutropenic cancer patients, elderly persons with serious underlying diseases, and hospitalized patients following invasive procedures and with indwelling plastic devices. Infections with many of these other species are acquired in the hospital setting. Commonly implicated species include *S. haemolyticus*, *S. lugdunensis*, *S. schleiferi*, *S. warneri*, *S. hominis*, *S. simulans*, *S. capitis*, *S. cohnii*, *S. xylosus*, and *S. saccharolyticus*.[455] Some of these agents, such as *S. schleiferi* and *S. warneri*, produce a variety of extracellular products (i.e., glycocalyx, DNase, lipase, esterase, protease, and α- and β-hemolysins) that contribute to virulence. *S. haemolyticus* has demanded increased interest due to emergence of glycopeptide resistance in this species. *S. lugdunensis* has emerged as a significant pathogen causing primarily endocarditis and skin/soft tissue infections. The

ecological niches and clinical significance of staphylococci other than *S. aureus*, *S. epidermidis*, and *S. saprophyticus* are described in Box 12-2, while infectious processes associated with *S. epidermidis* and other coagulase-negative staphylococci are described in Box 12-6.

Micrococcus *Species and Related Genera*

Several micrococcal species have been recovered from human clinical specimens, particularly *Kocuria* species, *Kytococcus* species, and *Micrococcus* species. *Kocuria kristinae* has been isolated as a cause of catheter-related bacteremia, infective endocarditis, peritoneal dialysis-associated infections, and cholecystitis-associated infections.[11,32,307,333] *K. kristinae, K. marina*, and *K. rosea* have all been described as causes of peritonitis in CAPD-patients.[75,141,317] *K. rhizophila* also caused persistent bacteremia related to a damaged central line, and, in another case, this organism colonized the Port-A-Cath of a pediatric patient, resulting in septic episodes over a 2-year period when both line and peripheral blood cultures were positive.[36,371] *K. varians*was

isolated as the sole agent in a brain abscess in a diabetic patient.[549] *Kytococcus* species, particularly *K. schroeteri*, have emerged as unusual agents in a variety of human infections. Several cases of aortic- and mitral-prosthetic and bioprosthetic valve endocarditis due to *K. schroeteri* have appeared since 2002.[37,314,370,447,616] Therapy often requires surgical intervention, although successful treatment outcomes with vancomycin/gentamicin plus rifampin and daptomycin monotherapy have been reported.[326] Bacteremic pneumonia due to *K. schroeteri* was documented in three patients with acute myeloid leukemia. These isolates were generally susceptible to linezolid, trimethoprim-sulfamethoxazole, rifampin, gentamicin, imipenem, meropenem, and vancomycin.[57,231,321] *K. schroeteri* has also been isolated from cases of chronic implant-related arthritis, osteomyelitis, and postoperative spondylodiscitis.[85,250] In the latter case, the patient had undergone surgical discectomy for treatment of chronic sciatica.[250] *K. schroeteri* has been isolated from shunt-associated ventriculoperitoneal fluid specimens, and both *K. schroeteri* and *K. sedentarius* have been isolated from peritoneal fluids of patients who are treated with continuous ambulatory peritoneal dialysis (CAPD).[93,255,476] *Micrococcus* species have been isolated from cases of bacteremia, peritoneal dialysis-associated peritonitis, septic arthritis, meningitis, and both native valve and prosthetic valve endocarditis.[260,367,551]

Rothia mucilaginosa

Since 1978, *R. mucilaginosa* has been reported as an opportunistic agent in a variety of clinical scenarios. Several cases of native valve and prosthetic valve endocarditis caused by *R. mucilaginosa* have appeared in the literature; in some cases, cardiac infection was associated with use of intravenous drugs.[112] *R. mucilaginosa* sepsis has been reported in association with head-and-neck trauma, central and peripheral access devices, cancer chemotherapy, cardiac catheterization following myocardial infarction, CAPD, and extreme prematurity.[232,369,436,464] *R. mucilaginosa* also has emerged as a significant pathogen in both adults and children with underlying malignancies (e.g., leukemia, lymphoma, breast cancer, Hodgkin's disease, squamous cell carcinoma, osteosarcoma, rhabdomyosarcoma, and AIDS) and in recipients of bone marrow transplants.[2,17,190,294,315,546,550] Infections have included transient and sustained bacteremias, peripheral and central line-associated sepsis, pneumonia, meningitis, vertebral osteomyelitis, and cholangitis.[2,17,158,190,315] Recovery of *R. mucilaginosa* from these patients was associated with severe neutropenia as a result of underlying disease, immunosuppression, or cytotoxic chemotherapy. Colonized indwelling access devices (e.g., intravenous or intraarterial lines, Brouviac and Hickman catheters) were the source of the organism in some patients, while mucosal or esophageal ulcerations secondary to cytotoxic chemotherapy, periodontal disease, or dental infections or procedures were believed to be possible sources in others. *R. mucilaginosa* may rarely cause bone and joint infections, including septic arthritis, vertebral osteomyelitis, and postoperative spondylodiskitis.[69,257] In rare cases, *R. mucilaginosa* may cause severe disease (e.g., meningitis) in healthy individuals.[451]

Isolation and Preliminary Differentiation of Staphylococci and Related Gram-Positive Cocci

Direct Gram-Stained Smears

On direct Gram-stained smears from clinical specimens, staphylococci appear as gram-positive or gram-variable cocci ranging in size from 0.5 µm to about 1.5 µm in diameter (Image Plate 12-1D and 12-1F). Larger cells are seen among members of the genus *Macrococcus*, but these species are not causes of human infections.[281] The organisms may appear singly, in pairs, in short chains, or in clusters, both within and outside of polymorphonuclear cells. Variations in cell size and Gram reaction are probably due to the action of the inflammatory cells and their hydrolytic enzymes on the bacterial cells. On direct smears, pairs and short chains of organisms cannot be differentiated from streptococci, micrococci, or peptostreptococci, although

streptococci frequently appear as chains of diplococci rather than as chains of discrete, individual cells. Reports of direct smears should include quantitation of cell types and microorganisms (e.g., "many PMNs, moderate gram-positive cocci"). If the Gram's stain appearance is more typical, a report of "gram-positive cocci resembling staphylococci" can be issued, with culture confirmation to follow. Gram's stain morphology cannot be used to differentiate staphylococci from micrococci and related genera or from planococci.

Isolation From Clinical Specimens

For recovery of staphylococci and related organisms, clinical specimens should be inoculated onto sheep blood agar (SBA) and other bacteriologic media. For isolation of organisms from heavily contaminated specimens, specimens should also be inoculated onto Columbia colistin-nalidixic acid (CNA) media or phenylethyl alcohol (PEA) agar, which inhibit growth of gram-negative bacteria and allow the growth of gram-positive organisms. Mannitol salts agar is a good selective medium for assessing the presence of *S. aureus* in specimens such as nasal cultures. On SBA, most staphylococci produce good growth within 24 hours, although micrococci may require 48 hours for the growth of sizable colonies. Some species of staphylococci may also require more than 24 to 48 hours' incubation in order to discern whether a specimen contains a pure or a mixed culture. Longer incubation—up to 72 hours—may be necessary to ensure that identification and susceptibility tests are being performed on a pure culture, especially if multiple colonies are being sampled to obtain a representative inoculum.

Colony Morphology

Micrococcus and *Staphylococcus* species form distinctive colonies on SBA. Colonies of most staphylococcal species grow more rapidly than micrococci, and are 1 to 3 mm in diameter after 24 hours' incubation, although some (e.g., *S. warneri, S. simulans, S. auricularis, S. vitulinus, S. lentus*) may form smaller colonies during this time. Strains of some staphylococcal species will show considerable variation in the size of colonies on the same culture plate, giving

the appearance of a mixed culture. Staphylococcal colonies are usually smooth, butyrous, and have a low-convex profile with an entire edge. Colonies of some *S. aureus* strains are usually large (4 to 6 mm in diameter) smooth, entire, and butyrous in consistency, although some strains may be wet looking or "sticky." Some strains may be pigmented yellow or yellow-orange (hence the name "aureus," meaning "golden"), while other strains may produce off-white or gray colonies. The latter strains may resemble group D streptococci and enterococci (catalase-negative).

Pigment production in both *S. aureus* and among the coagulase-negative staphylococci usually becomes more pronounced after incubation at room temperature for 2 to 3 days. Some *S. aureus* and some coagulase-negative species may have a distinct or hazy zone of β-hemolysis around the colonies (Image Plate 12-1E); this hemolytic property may become apparent only after prolonged incubation.

Isolates of *S. aureus* from patients who have been treated with antimicrobials for long periods or who have certain persistent underlying conditions such as cystic fibrosis may grow as small, atypical colonies that are known as small-colony variants. These colonies are usually very small, nonpigmented, and grow better in the presence of CO_2. These isolates may also be difficult to test for antimicrobial susceptibility due to their poor growth. Broth used in microdilution susceptibility testing of these isolates may require supplementation, and agar dilution, disk diffusion, and Etest-susceptibility methods should be performed on Müeller–Hinton agar containing 5% sheep blood.

Micrococci and related species generally grow more slowly, often requiring 48 hours' incubation before typical colony morphology can be discerned. After this time, micrococcal colonies are 1 to 2 mm in diameter, are dull or matte in textural appearance, and have a high-convex profile with entire edges. Colonies of *Micrococcus*, *Nesterenkonia, Dermacoccus*, *Kocuria* species, and *A. agilis* may be smooth or matte in appearance. Some species are nonpigmented (*M. lylae, N. halobia*), or may produce yellow (*M. luteus*, *K. sedentarius*, *K. varians*, *K. palustris*, *K. rhizophila*), orange (*D. nishinomiyaensis*, *K. kristinae*), or pink-to-red (*K. rosea*, *A. agilis*) colonies on agar media (Image Plate 12-1H). Pigmentation usually becomes more obvious or intense if plates are incubated at room temperature for several days. Colonies of *R. mucilaginosa* are gray to white and may be mucoid in appearance. They tend to adhere to the agar and, when removed from the media, are rubbery and difficult to emulsify.

The Catalase Test

Staphylococci and micrococci are differentiated from the streptococci, enterococci, and "*streptococcus*-like" bacteria by the catalase test. This test detects the presence of cytochrome oxidase enzymes (see Chart 1-1). The catalase test is performed with 3% hydrogen peroxide (H_2O_2) on a glass slide. Immediate and vigorous bubbling indicates conversion of the H_2O_2 to water and oxygen gas (Image Plate 12-1G). Ideally, the catalase test should be performed from a medium that does not contain blood, since red blood cells themselves may produce a weakly positive catalase reaction. However, since most clinical laboratories recover staphylococci on either nonselective or selective blood-containing media (e.g., SBA and CNA agar, respectively), care should be taken to sample only the tops of colonies for the catalase test to avoid carry-over of blood and possible false-positive reactions. This can be done most expeditiously with a wooden applicator stick. Rare strains of staphylococci may be catalase-negative, and

some enterococci produce a "pseudocatalase" and are weakly reactive with H_2O_2. *R. mucilaginosa* is usually catalase-negative or weakly positive.

Methods for Differentiating Micrococci and Staphylococci

Several methods are available for differentiating *Micrococcus* and related species and *Staphylococcus* species, the two catalase-positive genera most frequently seen in the clinical laboratory, from other catalase-positive, gram-positive cocci. Some require special media and prolonged incubation, while others are commercially available and provide results within 18 to 24 hours or less. Table 12-2 lists the phenotypic tests for differentiation of *Micrococcus*, *Macrococcus*, and *Staphylococcus* species. Although *Macrococcus* species are primarily equine isolates, reactions for these species are included for primary recognition of macrococcal isolates recovered in laboratories that provide services to veterinary practices.

Fermentation of Glucose. This test is performed in a manner similar to the oxidation-fermentation (OF) tests for nonfermentative gram-negative bacilli. The OF medium for staphylococci contains additional nutrients such as yeast extract to fulfill the more exacting growth requirements of staphylococci. This method, regarded as the reference procedure for differentiation of the *Micrococcaceae*, requires prolonged incubation and is not readily adaptable or amenable to routine laboratory use.

Susceptibility to Lysostaphin. Lysostaphin is an endopeptidase that cleaves the glycine-rich pentapeptide cross-bridges in the staphylococcal cell wall peptidoglycan. This activity renders the cells susceptible to osmotic lysis. Certain staphylococcal species (i.e., *S. aureus*, *S. simulans*, *S. cohnii*, *S. xylosus*) are more susceptible to lysostaphin than others (e.g., *S. hominis*, *S. saprophyticus*, *S. haemolyticus*), hence standardization and interpretation of this test is sometimes difficult. A tube test for lysostaphin susceptibility is commercially available from Remel (Remel Inc., Lenexa, KA). A heavy suspension of the organism is prepared in 0.2 mL of sterile saline, after which 0.2 mL of the Remel lysostaphin solution is added. The suspension is incubated at 35°C for 2 hours. Clearing of the suspension indicates susceptibility to lysostaphin. Lysostaphin susceptibility can also be determined using a filter paper-disk diffusion method. A plate of Müeller–Hinton agar is inoculated with the organism to be tested (0.5 McFarland turbidity standard) and a disk impregnated with 10 µg of lysostaphin filter-sterilized solution, 287 units/mL) is placed on the plate. The plate is incubated for 24 hours at 35°C. *Staphylococcus*species will generally show zones of inhibition of 10 to 16 mm in diameter. *Micrococcus* and related species will show no zones. In order to obtain optimal results with the lysostaphin susceptibility test, the organism should be grown on a beef peptone-based medium rather than on a casein peptone- or soy peptone-based medium. The crucial factor is the glycine content of the medium,

since glycine is an

TABLE 12-2 Phenotypic Characteristics for the Differentiation of *Staphylococcus, Micrococcus* and Related Species, and *Macrococcus*

Characteristic	Reaction/Description for: *Staphylococcus*	*Micrococcus* and Related Species[a]	*Macrococcus*
Organism size	0.6–1.6 µm	1–1.8 µm	1.3–2.5 µm
Colony profile	Raised, low convex	Convex	Low convex, domed
Growth rate	Slow to rapid	Very slow	Slow
Fermentation of glucose	+	–	–
Furazolidone susceptibility (100 µg furazolidone disk)	S	R	S
Bacitracin susceptibility (0.04 unit TaxoA disk)	R	S	R
Modified oxidase test	–[b]	+	+
Susceptibility to lysostaphin	S	R	S
Facultative growth under a coverslip on blood agar	1+ to 4+	–/1+	NA
Acid production aerobically from glycerol in the presence of 0.4 µg/mL erythromycin	+	–	NA

[a] Includes the genera *Micrococcus, Kytococcus, Dermacoccus, Nesterenkonia,* and *Kocuria.*
[b] All *Staphylococcus* species are modified oxidase-negative except for *S. vitulinus, S. sciuri,* and *S. lentus.*
+, positive reaction; –, negative reaction; S, susceptible; R, resistant; NA, data not available.

important part of the staphylococcal cell wall and is essential for the action of lysostaphin.

Production of Acid From Glycerol in the Presence of Erythromycin. In this test, medium containing glycerol (1%) and erythromycin (0.4 µg/mL) is prepared with an enriched agar base containing bromcresol purple indicator and poured into Petri plates. Several colonies of the isolate are streaked as a single line on the medium and the plate is incubated for up to 3 days at 35°C. Staphylococci will produce acid on this medium, while micrococci and related species will not.

Susceptibility to Furazolidone. This test is performed as a disk susceptibility procedure using commercially available disks (FX disks, 100 µg, BD Biosciences, Sparks, MD). The procedure for this test is described in detail in Chart 12-1. Staphylococci are inhibited by furazolidone and show zones of 15 mm or more, while micrococci and related species are resistant and show zones of 6 mm (no zone) to 9 mm (Image Plate 12-2A and 12-2B).[28] However, coagulase-negative staphylococci that are resistant to furazolidone may be seen occasionally.

Modified Oxidase Test.[28,157] This test is also commercially available from Remel

(Microdase Test Disks, Remel, Inc.). Filter paper disks impregnated with tetramethyl-*p*-phenylenediamine dihydrochloride (oxidase reagent) in dimethyl sulfoxide (DMSO) are used. The DMSO renders the cells permeable to the reagent. A colony from the growth medium is removed with an applicator stick and rubbed onto the disk. The development of a blue-purple color within 30 seconds is a positive test. No color development within this time is a negative test (Image Plate 12-2C). *Micrococcus* species, *N. halobia*, *D. nishinomiyaensis*, *A. agilis*, *K. kristinae*, and *Macrococcus* species are modified oxidase-positive, while strains of *K. sedentarius*, *K. rosea*, *K. varians, K. palustris*, and *K. rhizophila* are modified oxidase-negative.[281,292,512] All *Staphylococcus* species are modified oxidase-negative except for strains of *S. sciuri*, *S. lentus*, and *S. vitulinus*.[282]

Susceptibility to Bacitracin. This procedure employs the same bacitracin disk used for the presumptive identification of group A β-hemolytic streptococci. A lawn of growth is prepared on a Müeller–Hinton agar or a blood agar plate as described above for the FX disk test, and a bacitracin differential disk (Taxo A, 0.04 units bacitracin, BD Biosciences) is placed on the inoculum. After overnight incubation, zone sizes are measured. Staphylococci are resistant and grow to the edge of the disk, while micrococci and related species are susceptible, producing zones of 10 mm or greater.[28]

Coverslip Growth Method. This method takes advantage of the strictly aerobic character of micrococci and related species and the facultative metabolism of the staphylococci.[360] To perform the test, the organism is streaked for isolation onto a blood agar plate and a sterile coverslip is placed onto a heavily inoculated area of the plate (i.e., the second quadrant of a four-quadrant inoculation). After overnight incubation at 35°C to 37°C, the plate is checked for growth underneath the coverslip. In the original description of this test, 15 *S. aureus* strains, 20 coagulase-negative staphylococcal isolates, 18 *M. luteus* isolates, and single isolates of other

Micrococcus-like organisms (*K. rosea*, *D. nishinomiyaensis, K sedentarius*, *K, kristinae*, *K. varians*) were tested. All micrococci and related species failed to grow under the coverslip, while all staphylococci except a few animal isolates (e.g., *S. equorum*, *S. intermedius*, *S. kloosii*, *S. lentus*, *S. sciuri*) demonstrated at least 1+ growth. The test correlated well with furazolidone and bacitracin susceptibility tests.

The choice of the method used in a given laboratory depends upon the type of work performed (e.g., reference laboratory, environmental microbiology, clinical laboratory). In most clinical microbiology laboratories, the modified oxidase test, the furazolidone disk test, and the Taxo A bacitracin disk test are probably the most logical choices,

since they are rapid, reliable, inexpensive, and commercially available.

Methods for Direct Detection of MRSA in Clinical Specimens

Chromogenic Media for Surveillance of MRSA Colonization

Several chromogenic media are available for detection of MRSA in nares specimens and other sites that are sampled to determine colonization with this organism (e.g., axilla, groin). These media contain cefoxitin for detection of methicillin resistance and chromogenic substrates that, when hydrolyzed by specific bacterial enzymes, produce colored colonies. Currently available chromogenic media include Spectra MRSA agar (Remel, Lenexa, KA), BBL CHROMagar MRSA II (Becton-Dickinson, Sparks, MD), MRSA*Select* (Bio-Rad, Hercules, CA), chromID MRSA agar (bioMérieux, Durham, NC), Brilliance MRSA agar (Oxoid/Remel) CHROMagar (CHROMagar Microbiology, France), and Oxacillin-Resistance Screening Agar (ORSA, Oxoid, dehydrated form only). Brilliance MRSA obtained FDA clearance as Spectra MRSA (Remel) in the United States, so these two media are the same. Chromogenic media are inoculated and incubated for specified periods of time as recommended by the manufacturer. For example, Spectra MRSA, chromID MRSA, and Brilliance MRSA are read after 24 hours' incubation at 35°C to 37°C, while MRSA*Select* agar can be read after 18 hours and incubated up to 28 hours before being considered negative for MRSA. In most evaluations of these media, incubation periods of up to 48 hours have been generally used, with a first reading occurring at 18 to 24 hours, depending on the product in use. The sensitivity of these chromogenic media for detection of MRSA varies from as low as 78% to 80% up to 97% to 99% when compared with conventional methods for detection of MRSA (Table 12-3).[79,129,214,217,298,340,376,424,448,564,566,597,604] The specificities of these MRSA media are in the 92% to 99% range. In some cases, prolonging the incubation of these cultures results in a slight increase in sensitivity with a concomitant decrease in specificity. For example, in one study of MRSA*Select* agar, the specificity of the medium for detection of MRSA decreased from 97.2% after 24 hours' incubation to 92.1% after 48 hours while the sensitivity increased from 80.7% to 92.6% during that time period.[340] In the same study, the sensitivity of chromID MRSA increased from 82.8% at 24 hours to 93.5% after 48 hours, with a decrease in specificity of from 96.3% after 24 hours to 89.7% after 48 hours. Chromogenic media for detection of MRSA has also been shown to increase the yield of MRSA over conventional methods (i.e., blood and CNA agar) for polymicrobial wound and selected pulmonary specimens and has been used as a method for easy detection of MRSA in blood cultures.[211,425] In some institutions, nares and other surveillance

specimens are inoculated into enrichment broth (usually TSB with 6.5% NaCl) and incubated for 18 to 24 hours prior to plating on chromogenic media. While this may increase the yield of positive specimens, the advantage of a rapid turnaround time is lost, particularly if chromogenic plates are incubated for 48 hours before final reading.

Molecular Methods for Surveillance of MRSA Colonization

Molecular approaches are also available and FDA-cleared for detection of MRSA in surveillance and infection control programs. These assays are real-time PCR-based assays with short turnaround times compared with culture-based methods. Currently available tests include the BD GeneOhm MRSA assay (Becton-Dickinson Diagnostics, San Diego, CA), the BD Max MRSA assay (Becton-Dickinson), GeneXpert MRSA (Cepheid, Sunnyvale, CA), and the Roche LightCycler MRSA Advanced assay. The BD GeneOhm assay was first marketed as the IDI-MRSA assay.[462] These assays are FDA-cleared for nares specimens only, although studies have examined use of these tests with groin, axilla, and throat specimens, or combined nares/groin specimens.[56,215,234,272] Both the BD GeneOhm and the Cepheid GeneXpert assays co-amplify the extremity of SCC*mec* and part of the adjacent *S. aureus*-specific *orfX* gene at the SCC*mec* insertion site; these assays do not specifically target the *mecA* gene. The BD GeneOhm assay is performed with the SmartCycler instrument (Cepheid) while GeneXpert MRSA is performed with the GeneXpert real-time PCR platform. In this assay, the specimen is placed in a single-use disposable cartridge, which contains freeze-dried beads with all of the reagents needed for specimen DNA extraction, amplification, and detection. This assay requires minimal hands-on time and can be performed in less than 90 minutes. Rates of specimen inhibition for the BD GeneOhm assay are 1.3% to 1.7%, while rates for the GeneXpert MRSA test are 1.6% to 1.8%. The LightCycler MRSA Advanced test is a carousel-based thermal cycler system that can run up to 32 specimens (30 tests and 2 control) at a time and is performed with the LightCycler instrument.[22] The LightCycler MRSA Advanced assay uses swab extraction and mechanical lysis for specimen prep, real-time PCR amplification of MRSA DNA and fluorogenic target-specific hybridization probes for detection of amplified DNA. Each reaction mixture contains an internal control to detect specimen inhibition. These molecular assays have been evaluated individually and in several side-by-side comparisons (Table 12-4).[22,56,61,95,117,139,215,234,248,272,312,341,392,412,427,457,461,518,590,591,605] These studies differ in the populations tested (both low and high prevalence), comparator methods used (direct culture on chromogenic medium, enrichment culture followed by subculture to chromogenic medium), and methods for analyses of discrepant results. In general, these molecular assays have high sensitivity and specificity, with negative predictive values ranging from 93% to 100%.

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TABLE 12-3 Chromogenic Media for Isolation of MRSA[a]

Medium	Manufacturer	Incubation[b] (Hours)	MRSA	Non-MRSA	Sensitivity (%)		Specificity (%)	
					24 Hours	48 Hours	24 Hours	48 Hours
MRSASelect	Bio-Rad	18–28	Small pink/mauve colonies	White, colorless	81–98.2	92.6–98.7	91.4–100	88–90.6
Spectra MRSA	Remel	24	Small-to-medium denim-blue colonies against a white opaque background	Inhibited or white colonies	83.6–96	99	92.1–99.7	98.5–99.2
BBL CHROMagar MRSA II	BD	20–26	Mauve colonies	No growth, nonmauve colonies	84.6–87.7	92.4–96.8	99.2–99.8	97.8–100
ChromID MRSA	bioMérieux	24	Green colonies	Nongreen colonies	81.4–97.4	88.6–98	96.3–100	89–99
ChromID MRSA New	bioMérieux	18 and 24	Green, blue–green, dark green (24)	Other	94.3	NA	95.4	NA
HardyCHROM MRSA	Hardy Diagnostics	24	Pink-to-magenta colonies	Purple colored film	93.3	97	99.7	99.7
Brilliance MRSA	Oxoid	24	Small-to-medium denim-blue colonies against a white opaque background	Inhibited or white colonies	90	96.4	86.9	69
CHROMagar	CHROMagar Microbiology	24	Mauve colonies	Blue, colorless, or inhibited	81.9	93.1	99.1	97.4
Oxacillin-Resistance Screening agar	Oxoid	24–48	Intense blue	No growth, straw-colored colonies	96.1	99.3	96	76
Denim-Blue MRSA	Oxoid	24	Blue	White	96	NA	98	NA

[a] From references 79, 129, 214, 217, 298, 340, 376, 424, 425, 448, 566, 597, and 606.
[b] Incubation time recommended by the manufacturer.

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TABLE 12-4 Performance of Molecular Assays for Detection of MRSA in Surveillance Specimens

References	Culture Method	Site of Culture				BD GeneOhm MRSA (%)				GeneXpert MRSA (%)				LightCycler MRSA Advanced (%)				Comments
		Nares	Axilla	Groin	Throat	SENS	SPEC	PPV	NPV	SENS	SPEC	PPV	NPV	SENS	SPEC	PPV	NPV	
95	• 498 paired nasal swabs cultured on blood agar • Low prevalence of MRSA (2.8%)	X				92.9	95.7	38.2	99.8									
117	• Low prevalence of MRSA (4.1%)	X				93.9	99.2	83.8	99.7									• BDMax fully automated platform • Fewer unresolved results than GeneOhm
412	• Evaluated the achromopeptidase lysis procedures compared with the MRSA lysis kit method • Tested 1,216 nasal swabs (in-, out-, and nursing home patients) • Direct culture on CHROMagar and read at 24 and 48 hours • Enrichment in TSB with 6.5% NaCl, incubated 24 hours, subculture to CHROMagar, incubated for 24 and 48 hours	X				92	94.6	75.4	98.5									• The ACP procedure reduces hands-on time as three steps (two centrifugation steps and one wash step) are omitted • Median time to positivity were 69.3 hours for culture and 2.83 hours for BD.
215	• Collected 728 nasal and 1,539 groin swabs • Direct culture onto blood agar, mannitol salts, MH agar with 4% NaCl • Prevalence of 4.8%	X				97.2	99.4	89.7	99.9									• Sequencing of discordant samples confirmed MRSA in 40% of specimens that were culture-negative and PCR-positive by both methods
				X		100	98.7	61.5	100									
234	• Enrichment in TSB with 7.5% NaCl for 24 hours • Subculture to chromID chromogenic medium and incubated 24 hours	X				100	99.5	93.8	100	100	99.4	91.7	100					• Data shown after resolution of discrepant PCR results by culture positivity at any site • Collecting swabs from nose and groin resulted in a higher rate of detection of MRSA carriers • TAT for BD, Xpert and Culture were 5 hours 40 minutes, 2 hours and 20 minutes, and 54 hours and 30 minutes, respectively
				X		100	99.5	93.3	100	100	99.4	88.9	100					

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References	Culture Method	Site of Culture				BD GeneOhm MRSA (%)				GeneXpert MRSA (%)				LightCycler MRSA Advanced (%)				Comments
		Nares	Axilla	Groin	Throat	SENS	SPEC	PPV	NPV	SENS	SPEC	PPV	NPV	SENS	SPEC	PPV	NPV	
272	• Specimens from 43 patients • Known high rate of MRSA colonization (12%–15%) • Nares, groin, and combined swabs collected • Cultured directly on chromogenic MRSA agar, incubated 24–48 hours • Swab then placed in TSB with 6.5% NaCl, incubated 48 hours, then subcultured to chromogenic MRSA agar	X		X		84.8/ 73.2	92.7/ 93.5	76.5/ 80.4	95.6/ 90.6	87.0/ 75.0	93.8/ 94.7	80.0/ 84.0	96.2/ 91.1					• Combined specimen data shown • Data: Performance compared with 48 hour enrichment culture • Similar levels of accuracy using the combined specimen approach. Use of combined specimens enhances MRSA colonization detection.
392	• Belgian study • 500 nares specimens • Prevalence of 1.6% • Enriched culture in TSB-3.5% NaCl, incubated overnight • Subcultured to MRSA*Select* and incubated 48 hours	X				62.5	99.0	50.0	99.4	62.5	97.7	31.3	99.4					• Sensitivity noted to be lower in the low prevalence population being tested.
590	• Study performed in the Netherlands • 1,764 at-risk patients • Prevalence of 3.3% • Broth enriched for 24 hours and subcultured to chromogenic MRSA-ID agar and incubated 24 hours	X				82.5	96.5	44.2	99.5	75.0	94.5	33.3	99.1					• Sensitivity, specificity, PPV, and NPV of culture reported as 85.7%, 96.6%, 46.2%, and 99.5%, respectively.
312	• Belgian study • 246 specimens from high-risk patients • Prevalence rates have increased from 4.7% to 19% from 2002 to 2005 • Direct culture on MRSA*Select* incubated 24 hours • Swab placed in TSB with 6.5% NaCl incubated 24 hours • Broth subcultured to MRSA*Select* and incubated 24 hours	X								78.3/ 89.2	97.7/ 97.7	78.3/ 78.3	97.7/ 96.3					• Data: Performance with direct culture/performance with enriched culture. • In vitro data showed Xpert MRSA detected 39/40 representative MRSA strains belonging to SCC*mec* types I, II, IV, and V. • Of the seven isolates missed by Xpert, four were SCC*mec* type IV variant not targeted by the current genXpert MRSA assay.

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605	• Multicenter evaluation of Xpert MRSA • 1,077 nares specimens from seven US sites • Prevalence: 5.2%–44% • Direct culture on CHROMagar incubated 18–24 hours • Enrichment in TSB-6.5% NaCl and subculture to CHROMagar, with incubation for 24 and 48 hours	X								94.3/ 86.3	93.2/ 94.9	73.0/ 80.5	98.8/ 96.6					• Data: Performance with direct culture/performance with enrichment culture.
139	• Netherlands study • Low prevalence, of 3.9% ("search and destroy" national policy) • Enrichment in phenyl-mannitol broth with ceftizoxime and aztreonam • Subculture to chromogenic medium with confirmation 24 and 48 hours	X		X	X					91.6	97.0	54.3	99.7					• Combined performance data for three body sites • Four of 12 positives detected only after enrichment culture.
461	• Study performed in Ireland • Swab specimens from four sites from 204 patients • Direct cultures on MRSA*Select* agar • Enrichment with subculture to MRSA*Select*	X X		X X	X X					88/ 79 84 92 63	92/ 94 92 94 98	53/ 71 67 69 83	99/ 96 97 99 93					• Data: Performance with direct culture/performance with enrichment culture • Performance data for individual body sites compared with enriched culture results only • Evaluated Xpert with 114 MRSA representing SCC*mec* types IVa, IVb, IVc, IVd, Vt and Vi, and all 114 MRSA isolates and seven control strains were detected. • Level of detection of detection (LOD) was 610 cfu/mL (equiv to 58 cfu/swab)
56	• Eswab fluid used for PCR and enriched culture • Staph broth incubated overnight and subcultured to MRSA*Select* • 50 known MRSA carriers	X	X	X	X					89 68 78	99 98 98							• Pooled results from separate analysis of all three sites increased sensitivity to 92% • Testing of pooled specimens over 12 months had PPV of 77% and a NPV of 99%

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341	• Belgian study • 52 nose and groin specimens from previously-identified MRSA carriers • Direct culture and after overnight enrichment on CHROMagar	X	X	90.9/ 71.4 100/ 90.5		100/ 57.1 88.2/ 76.1							• Data: Performance with direct culture/performance with enrichment culture • Overnight enrichment drastically increased the number of MRSA true-positive status of screening samples. • For 14 samples that did not show MRSA on direct culture, Xpert detected 2 and BD detected 7 • Compared diagnostic sensitivities of BD and Xpert assays. Sensitivities were high at 10^3 cfu/mL MRSA concentrations (92.3% for BD, 96.3% for Xpert)
457	• 1,891 nasal swabs collected • Direct culture on chromID MRSA (bioMerieux) and incubated 48 hours					60.7	97.3						• Of 24 MRSA isolated from Xpert-Negative sample 3 harbored composite SCC*mec* • Among 61 samples that were culture-negative and Xpert-Positive, 15 MSSA tested positive with pure colonies on Xpert • MSSA included strains with SCC*mec* deletions encompassing *mecA* and MLST clonal complexes
22	• 1,202 collections of double swabs used for PCRs culture • Direct culture on MRSASelect • Cultures incubated 18–28 hours • No enrichment • Prevalence not stated	X				99	95.5	95.2	95.5				• Sequencing of discordant samples confirmed MRSA in 40% of specimens that were culture-negative and PCR-positive by both methods
427	• Multicenter study comparing LightCycler Advanced assay to BD GeneOhm • 1,402 nares specimens from five centers • Direct culture on CHROM agar MRSA read after 24 and 48 hours • Enrichment in TSB-6.5% NaCl, with subculture on CHROMagar and incubation for 24 and 48 hours			95.7/ 88.8	91.7/ 91.8			95.2/ 89.9	96.4/ 96.8	37.8	99.9		• Data: Performance with direct culture/performance with enrichment culture • Following discrepancy analysis, the relative sensitivity of LightCycler and GeneOhm were 92.2% and 93.2% respectively • Specificity was significantly better with the LightCycler Advanced assay • Sensitivity of direct culture was 80.4%.

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As experience with these assays has accumulated, so has the recognition of staphylococcal isolates that have lost all or part of SCC*mec*. These isolates are termed *mecA* "dropouts," because they lack a functional *mecA* gene, but contain the SCC*mec*junction sequence. These junction sequences can also vary, and coagulase-negative staphylococci may contain similar sequences that can lead to false-identifications of coagulase-negative staphylococci as MRSA.[31,55,312,421,514] MSSA isolates can arise from MRSA following partial or complete excision of SCC*mec*, and, conversely, MRSA can result from MSSA by site-specific integration of SCC*mec* into the *orfX* chromosomal site. Because assays like the BD GeneOhm and the GeneXpert MRSA target the SCC*mec*-*orfX* junction and do not specifically target the *mecA* gene, MSSA strains that lack a functional *mecA* gene may be detected as *mecA* positive ("false-positive MRSA").[20] Shore and colleagues[493] investigated 25 isolates of multiresistant MSSA from hospitals in Ireland for the presence of SCC*mec* and performed multilocus sequence and *spa* typing. Several of these isolates contained remnants of SCC*mec,* and three of the MSSA isolates were detected as MRSA by both the GeneXpert and BD GeneOhm assays. The clinical sensitivities of these direct molecular MRSA assays is influenced by the prevalence of these aberrant isolates. In a report from Belgium, the Cepheid GeneXpert MRSA performed with a sensitivity of only 60.7% due to the presence of new SCC*mec* variants and some MSSA isolates

that carried cross-reactive SCC*mec*-like genetic sequences.[457] Based on such reports, it is possible that these direct molecular tests may someday outlive their usefulness unless MRSA and MSSA strains are monitored for the loss or acquisition of SCC*mec*, SSC*mec*-like, and neighboring sequences (e.g., *orfX*) that may affect the sensitivity and specificity of these gene target-specific assays.

Molecular Methods for Detection of MRSA and MSSA in Blood Cultures and Skin/Soft Tissue Infections

Commercially available molecular assays are also available and FDA-cleared for detection of MSSA and MRSA in positive blood cultures and in specimens from skin and soft tissue infections. Tests include the GeneXpert MRSA/SA BC and GeneXpert MRSA/SA SSTI (Cepheid) and the BD GeneOhm StaphSR assay (Becton-Dickinson). The GeneXpert assay contains primers and probes that simultaneously detect three targets—sequences within the *spa* (protein A) gene, the SCC*mec cassette*, and the gene for PBP2a-mediated methicillin resistance (*mecA).* Wolk et al.[606] evaluated both GeneXpert assays and found that the BC test performed with sensitivities of 100% for MSSA and 98.3% MRSA, with specificities of 98.6% and 99.4%, respectively. For wound specimens, MSSA and MRSA were detected with sensitivities of 100% and 97.1%, respectively, with specificities in excess of 96%. Spencer and colleagues[510] evaluated the GeneXpert MRSA/SA when gram-positive cocci in clusters were observed on Gram stains from positive blood cultures. The assay was 100% sensitive and specific for detection of MRSA and was 100% sensitive and 99.5% specific for MSSA. Another study of the GeneXpert MRSA/SA system found phenotypic and genotypic concordance of results in 94.6% of cases.[53] In an evaluation by Kelley and coworkers,[271] 21 of 102 blood culture specimens were positive with *S. aureus*; 17 were MSSA and 4 were MRSA. The GeneXpert currently identified 20 (95.4%) of 21 MSSA and 3 (75%) of 4 MRSA. A single MRSA strain was identified as an MSSA. These workers also challenged the system with spiked blood cultures containing 28 heterogeneously vancomycin-intermediate *S. aureus* (i.e., VISA and hVISA). The GeneExpert test identified 84.6% of VISA and 80% of hVISA isolates as MRSA. The Xpert MRSA/SA SSTI test has also been evaluated and compared with culture for the diagnosis of staphylococcal osteoarticular infections.[144] Sensitivities of 100% and specificities of 95.3% to 100% were obtained for detection of MSSA, MRSA, and methicillin-resistant coagulase-negative staphylococci. The rapidity of the assay (72 minutes) versus culture (requiring 1 to 4 days for completion) enables targeted therapy to be initiated much earlier in cases of staphylococcal bone infections.

The BD GeneOhm StaphSR has also been evaluated by several groups. Using spiked blood culture media, Grobner and colleagues[198] found the test to be 100%

sensitive and specific for detecting *S. aureus*. For MRSA detection, the assay was 95.6% sensitive and 95.3% specific. Of the five discrepant MRSAs, three were methicillin-susceptible revertant MRSAs and two were not detected.[198] A 2007 clinical evaluation using consecutive blood cultures positive for gram-positive cocci in clusters found the BD test to have a sensitivity and specificity of 98.9% and 96.7%, respectively, for MSSA detection, and 100% and 98.4%, respectively, for detection of MRSA.[513] In a 2009 study of 100 blood cultures containing gram-positive cocci in clusters, 23 yielded MSSA, 36 grew MRSA, and 41 grew non–*S. aureus* isolates. The BD GeneOhm StaphSR test misidentified 3 (8.3%) of 36 MRSA as MSSA and 1 (4.3%) of 23 blood cultures that grew MSSA tested negative.[501] The three MRSAs that were misidentified as MSSA were SCC*mec* variants that lacked part of the SCC*mec* integration site, the molecular target used in the BD assay. Frey and colleagues[172] used the BD GeneOhm StaphSR assay to detect and differentiate staphylococci in 24 valvular tissue specimens (12 fresh and 12 formalin-fixed, paraffin-embedded specimens) from patients with *S. aureus* endocarditis.[172] The assay was 100% sensitive and specific in detecting *S. aureus* in valvular tissues, including specimens from deparaffinized tissue blocks. Twenty-two valvular specimens (11 fresh and 11 embedded) from those without staphylococcal endocarditis were uniformly negative. In this study, there was an 87.5% correlation between presence of the *mecA* gene and the oxacillin result using BD GeneOhm MRSA/SA BC.

Identification of *Staphylococcus aureus*

The single most reliable characteristic for identifying *S. aureus* is the coagulase test. The conventional coagulase test may be performed by the slide or tube procedures. The medium for both procedures is rabbit plasma with EDTA. Citrated plasma should not be used, as organisms that are able to utilize citrate (e.g., *Enterococcus* species) will yield

positive results if they are mistaken for staphylococci; always perform a catalase test first. Human plasma (e.g., outdated material from blood banks) may contain anti-staphylococcal antibodies and should not be used to perform coagulase test procedures.

Slide Coagulase Test

Most strains of *S. aureus* have a bound coagulase or "clumping factor" on the surface

of the cell wall. This factor reacts directly with fibrinogen in plasma, causing rapid cell agglutination (Image Plate 12-2D). Performance of this test is described in detail in Chart 1-3. The test can be performed with growth from blood agar, CNA agar, or other nonselective nutrient medium, but should not be performed from media having a high salt content (e.g., mannitol salts agar) since the high salt causes some strains of *S. aureus* to autoagglutinate. Strains that are negative with the slide coagulase test must be confirmed with a tube coagulase test; strains deficient in clumping factor will usually produce free coagulase. Some human coagulase-negative species (e.g., *S. lugdunensis* and *S. schleiferi* subsp *schleiferi)* produce clumping factor and may be slide coagulase-positive. With chromogenic media for detection of MRSA, consult the package insert regarding whether coagulase testing can be performed directly from the media or whether subculture is required.

Tube Coagulase Test

The coagulase detected by this method is secreted extracellularly and reacts with a substance in the plasma called "coagulase-reacting factor (CRF)" to form a complex, which, in turn, reacts with fibrinogen to form fibrin (clot formation) (Image Plate 12-2E). Tests that are negative after 4 hours incubation at 35°C should be held at room temperature and read again after 18 to 24 hours, because some strains will produce fibrinolysin on prolonged incubation at 35°C, causing dissolution of the clot during the incubation period. Rare *S. aureus* strains may be coagulase-negative, and some animal isolates (*S. intermedius*, *S. hyicus*, S. delphini, and *S. schleiferi* subsp. *coagulans)* may be tube coagulase-positive.[134,204,205,556,569] Strains of *S. schleiferi* subsp. *schleiferi* that are clumping factor- and tube coagulase-positive and also produce heat-stable endonuclease have been recovered from human infections and may be misidentified *S. aureus*. These strains can be differentiated by failure to produce acid from maltose, lactose, mannitol, and sucrose. *S. schleiferi* subsp. *coagulan*s, a cause of canine external otitis, has been isolated from humans and may be misidentified as clumping factor-negative *S. aureus*. Phenotypic tests distinguish these strains.

Alternative Coagulase Test Procedures

Agglutination Tests. Several products that use this approach are commercially available and include Staphaurex and Staphaurex Plus (Remel, Lenexa. KA), Slidex Staph-Kit (bioMérieux), BBL Staphyloslide latex test (BD), Pastorex Staph Plus (Bio-Rad) (Image Plate 12-2F). Dryspot Staphytect Plus, Staphytect Plus, and Staphylase Test (Oxoid, Basingstoke, England). These procedures use latex beads coated with plasma except the Staphylase Test, which uses erythrocytes as the carrier particles and the Slidex Staph-Kit, which uses both latex beads and erythrocytes as the carrier particles. Fibrinogen bound to the carriers detects clumping factor and IgG molecules

also present on the carriers detect protein A, the staphylococcal cell wall protein that is able to bind immunoglobulin G molecules by the Fc region.[16] Mixing of these latex reagents with colonial material from agar media and rapid agglutination is a positive test and identifies the isolate as *S. aureus*. These reagents may also provide identifications from agar media inoculated with pellets obtained from blood cultures positive for gram-positive cocci in clusters and incubated for 4 to 6 hours.[273] Early evaluations comparing latex agglutination kits with 4-hour and 24-hour tube coagulase tests reported that false-negative latex agglutination results may be encountered when MRSA are tested. Among MRSA, the predominate capsular type is capsular serotype 5, with most others being capsular serotype 8.[163,164] Hence, some manufacturers have added antibody to the serotype 5 capsular polysaccharide as one of the detection molecules bound to the latex or stabilized red blood cells. Thus, the Dry Spot Staphytect Plus, the Staphytect Plus, the Pastorex Staph Plus also have antibody against staphylococcal capsular polysaccharide types 5 and 8 bound to the latex beads, while the Staphaurex Plus has antibodies against capsular type 5 and staphylococcal glycopolysaccharide antigen 18 bound to the latex beads. The Slidex Staph-kit consists of a mixture of latex particles sensitized with monoclonal IgG antibodies and antibody against cell surface proteins and stabilized red cells sensitized with fibrinogen to detect clumping factor. The Staphaurex test and the Pastorex Staph Plus are single-reagent tests, while the Staphaurex Plus, BBL Staphyloslide, Slidex Staph-kit, Staphytect Plus, DrySpot Staphytect Plus, and Staphylase include a negative control reagent with nonsensitized latex particles and/or stabilized red blood cells. Mixing of the test reagent with growth from an agar plate results in rapid clumping of the carrier-organism suspension, while no clumping occurs with the nonsensitized latex/red blood cell control reagents. Comparative evaluations of these tests have shown sensitivities of between 93% to 100% for identification of MSSA, and 82% to 100% for MRSA isolates.[14,596] Staphaurux Plus and the Staphylase test showing markedly lower sensitivities for identifying MRSA isolates. The specificity of these tests are generally in the range of 96% to 99%, with some having lower specificities (i.e., 91% to 92%) in some evaluations. Depending on the kit used, false-positive test results may occur with some strains *S. intermedius*, *S. lugdunensis, S. schleiferi, S. saprophyticus, S. hyicus, and S. delphini*.

Additional Confirmatory/Identification Tests for Staphylococcus aureus

Deoxyribonuclease Test. Some *S. aureus* strains may produce weak or equivocal tube coagulase reactions and rare isolates may indeed be coagulase-negative. Fortunately, other tests correlate highly with coagulase production. *S. aureus* produces both deoxyribonuclease (DNase) and a thermostable nuclease having endo- and

exonucleolytic

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activities (Image Plate 12-2G). DNase can be detected by heavily spot-inoculating the organism on DNase test medium containing the metachromatic dye toluidine blue O (commercially available from several vendors). After 24 hours' incubation at 35°C, the medium under and around the inoculum turns from azure blue to pink, indicating DNA hydrolysis. The toluidine blue O content in the medium should not exceed 0.005%; higher concentrations may mask detection of DNase activity. Spot inoculation is necessary because *S. aureus* strains do not grow well on the media and growth is not required for enzyme detection. While this test is a helpful adjunct for identification of *S. aureus*, other staphylococci may also produce DNase.

Thermostable Endonuclease Test. The same DNase test medium is used, only 3-mm holes are cut into the agar with a sterile cork borer, and the wells are filled with a 24-hour broth culture of the test organism that has been boiled in a water bath for 15 minutes. The plate is incubated overnight at 35°C. *S. aureus* strains will show a pink zone surrounding the well containing the boiled suspension. Certain animal isolates (e.g., *S. caprae*, *S. schleiferi*, *S. intermedius*,*S. hyicus*) also produce heat-stable thermonuclease, and some coagulase-negative staphylococci (i.e., *S. epidermidis*, *S. simulans*, *S. capitis*, *S. carnosus*) may produce weak positive reactions. Specificity of this assay for *S. aureus*thermostable endonuclease may be confirmed by sero-inhibition of the reaction by polyclonal or monoclonal antibodies directed against the *S. aureus* enzyme, or by PCR amplification and sequencing of the *nuc* gene that encodes the *S. aureus* heat-stable endonuclease (see below).[63] A modification of this method was superior to the tube coagulase test for direct identification of *S. aureus* in blood cultures.[306] Primers and probes for amplification and detection of the *nuc* gene of *S. aureus* have also been synthesized and examined for their utility in culture confirmation and direct detection.[62] For identification, the lower limit of sensitivity of the PCR assay was 5 to 20 cfu of the organism. Using staphylococci-free clinical specimens that were seeded with serial dilutions of *S. aureus*, the lower limit for direct detection of *S. aureus* by the PCR assay ranged from 10 to 20 cfu up to 1,000 cfu, depending on the type of clinical specimen. The PCR-based assay was 100% specific for *S. aureus*.[62]

Mannitol Fermentation. *S. aureus*, unlike *S. epidermidis* and several other coagulase-negative species, produces acid from mannitol. This property is exploited in epidemiologic studies to detect *S. aureus* in soil, feces, and in screening nasal carriers of *S. aureus*. **Mannitol salts agar** contains mannitol (1%), 7.5% NaCl, phenol red,

and peptones. The high salt concentration discourages growth of other organisms (except for enterococci) and selectively recovers staphylococci. *S. aureus* colonies can be detected by a yellow zone around isolated colonies. Other infrequently isolated staphylococcal species may also ferment mannitol, so isolates recovered on this medium must be confirmed by other tests.

AccuProbe *Staphylococcus aureus*. The AccuProbe *Staphylococcus aureus* test (Gen-Probe, San Diego, CA) uses a chemiluminescent probe directed against rRNA genes to provide an identification from colonies on agar media.[88] The test uses a chemiluminescent acridinium ester-labeled, single stranded DNA probe that is complimentary to specific ribosomal RNA sequences of *S. aureus*. Cells from colonial growth are lysed and the probe is added. The probe binds to the rRNA sequence in a specific manner and unhybridized probe is removed using a proprietary hybridization-protection step. After removal of the unhybridized probe, a Leader 50i luminometer (Gen-Probe) is used to detect hydrolysis of the acridinium ester present on the nucleic acid sequence that is hybridized to the rRNA of the organism. A flash of light from probe hydrolysis is reported in relative light units (RLUs), with a result of ≥50,000 RLUs being considered positive. The test performs well for identification of *S. aureus* from agar media, but less well with pellets obtained from blood cultures positive with gram-positive cocci in clusters. For testing from blood culture pellets, sensitivities and specificities of 72.4% to 95% and 99.1% to 99.8%, respectively have been reported.[325,499] This test has also performed well when used as a direct detection method for *S. aureus* in other specimen types (e.g., bronchoalveolar lavage fluids).[6]

Rapid Tests for Detection of Methicillin Resistance

Although not an identification method, rapid detection of methicillin resistance in *S. aureus* is as critical as species identification. The *mecA* gene is the structural gene for a low-affinity penicillin-binding protein (PBP2′ or PBP2a) that is found in methicillin/oxacillin-resistant *S. aureus* (MRSA) strains. Initially, kits for *mecA* detection used cycling probe amplification (Velogene Rapid MRSA identification assay, ID Biomedical Corp., Vancouver, BC, Canada) or fluorescence technology (Crystal MRSA-ID System, BD Biosciences) for detection of methicillin resistance. The Velogene assay required PCR expertise followed by detection methods using fluorescein-conjugated probes.[21,331] The Crystal MRSA-ID system depended on detection of fluorescence during growth of a putative MRSA isolate in broth. These assays performed well and provided accurate results within 2 (Velogene) to 4 hours (Crystal MRSA). These kits were marketed in the late 1990s but are no longer available in the United States. Current commercially available methods use latex agglutination (MRSA-Screen, Denka-Seiken Co., Niigata, Japan, marketed in the United States by Hardy Diagnostics, Santa Maria, CA; Oxoid Penicillin-Binding

Protein latex agglutination test, Oxoid, Ltd., England, marketed in the United States by Remel; Slidex MRSA Detection, bioMérieux) or immunochromatographic strip approaches for detection of PBP2a (Clearview Exact PBP2a, Inverness Medical Innovations, Scarborough, MA; BinaxNOW PBP2a test, Alere Healthcare). These tests provide results in 15 to 30 minutes, with the Oxoid product being most widely used at present.

Both MRSA-Screen and Oxoid PBP2′ use latex beads that are coated with monoclonal antibodies against PBP2′ and the tests are performed similarly. Sufficient colonial growth is suspended in four drops of extraction reagent #1 (0.1 mol/L NaOH) in a microfuge tube, and the suspension is boiled for 3 minutes. After cooling, a single drop of extraction reagent #2 (0.5 mol/L KH_2PO_4) is added. The tube is centrifuged at

1,500×*g* for 5 minutes and the supernatant is removed. Aliquots (50 μL) of the supernatant are placed in "control" and "test" circles of a reaction card. A single drop of the test latex reagent (latex beads coated with monoclonal antibodies against PBP2a) is placed on the "test" circle and a single drop of control latex reagent (no anti-PBP2a antibodies) is added to the "control" circle. Each reaction area is mixed with a loop and the card is rocked for 3 minutes and observed for visible agglutination. Agglutination with the test but not the control latex reagent is a positive test for PBP2a (MRSA), while no agglutination in test or control areas on the card is a negative test (MSSA). These tests are not substitutes for specific identification tests for *S. aureus*, since MSSA isolates will be latex agglutination negative for *mecA*, and methicillin-resistant coagulase-negative staphylococci that also carry the *mecA* gene will also be positive. Evaluations of the MRSA-Screen have reported sensitivities and specificities of 97% to 100% and 99% to 100%, respectively, for detection of *mecA* in *S. aureus*, and sensitivity/specificity of 100% for *mecA* detection in coagulase-negative staphylococci.[21,330,331,565,613] A study of the Oxoid product from the Netherlands reported a sensitivity of 97% and a specificity of 100% when testing 90 genetically diverse MRSA and 106 MSSA isolates. All 10 methicillin-resistant *S. epidermidis* isolates were also positive with the PBP2a latex test.[565] A Canadian evaluation of the MRSA-Screen latex agglutination assay evaluated 71 MSSA, 213 MRSA, and 25 BORSA isolates and found a sensitivity of 99% and a specificity of 100%.[21] Similar high sensitivities and specificities have been obtained with the Oxoid latex agglutination test for PBP2′.[64,74,138,345] The Oxoid product was evaluated using growth from blood agar subcultures of positive blood culture bottles showing gram-positive cocci in clusters that had been incubated for only 3 hours. The results of the PBP2′ latex test correlated 100% with oxacillin-resistance determined by microbroth dilution.[345] Discrepancies have been noted between the two latex agglutination kits when testing

directly from blood cultures.[89,138] Chapin and Musgnug evaluated the Oxoid latex test using bacterial pellets obtained from positive blood cultures showing gram-positive cocci in clusters. Seventy cultures were positive for *S. aureus*, including 44 MRSA and 26 MSSA. The PBP2a assay performed on these pellets was 100% specific, but had a sensitivity of only 18%, so the predictive value of a negative test was very low (42%).[89] Diab and colleagues[138] examined 56 blood cultures positive for gram-positive cocci in clusters, performed direct tube coagulase tests and Oxoid latex tests on pellets from these positive blood cultures, and compared the results with those obtained from colonial growth the next day. Of these 56 isolates, 25 were *S. aureus* and 31 were coagulase-negative staphylococci. Compared with *mecA* detection by PCR, the direct Oxoid latex agglutination test was 100% sensitive for detection of *mecA* in both *S. aureus* and coagulase-negative staphylococci, while the specificity for *S. aureus* and coagulase-negative staphylococci were 100% and 75%, respectively. The Oxoid assay has been used for detection of methicillin resistance in veterinary isolates (*S. intermedius* and *S. schleiferi* subspecies) and was found to correlate highly with the *mecA* PCR "gold standard."[40] The bioMérieux Slidex MRSA Detection latex test is similar in format and procedural details to the Denka-Seiken and Oxoid tests, but it is not available in the United States at present. More information on methicillin resistance in staphylococci and the methods for its detection are available in Chapter 15 on antimicrobial susceptibility testing.

The Clearview Exact PBP2a and the BinaxNOW PBP2a immunochromatographic strip assays are rapid, qualitative tests for detection of PBP2a (PBP2′). The two assays are performed in a similar manner. Two drops of reagent 1 are placed in a small tube and three-well isolated colonies are emulsified in the liquid. Two or three drops of a second reagent are added to the tube and it is briefly vortexed. The immunochromatographic strip is placed in the tube and incubated at room temperature for 5 minutes. After this time, a positive control line appears along with a line for the presence of PBP2a if the isolate is *mecA* positive, while a single control line appears if the isolate is PBP2a-negative. No lines on the strip or absence of a control line are invalid tests. Evaluations of the Clearview assay have reported sensitivities of 96.6% to 100% and specificity of 100% compared with the Denka-Seiken and bioMérieux latex agglutination and cefoxitin disk susceptibility tests.[83,388,567] In an evaluation conducted in Spain, the BinaxNOW PBP2a test, 20 (95% sensitivity) of 21 MRSA isolates were positive, while none of 27 MSSA isolates were positive (100% specificity).[458] At the present time, neither of these kits are available in the United States.

Genotype MRSA. Genotype MRSA (Hain Life Science, Germany) is an immunochromatographic strip that detects the presence of the *mecA* gene and also identifies the organism as either *S. aureus* or *S. epidermidis*. Following cell lysis, DNA

extraction and amplification using biotinylated primers, amplicons are detected by hybridization to membrane-bound probes on the immunochromatographic strip using a streptavidin alkaline phosphatase conjugate and an alkaline phosphatase-mediated detection step. Each immunochromatographic strip has lines for a conjugate control (CC), a universal control (UC), and lines specific for *mecA*, *S. aureus*, and *S. epidermidis*, respectively. MSSA strains yield three lines on the strip (CC, UC, and an *S. aureus* line), while MRSA yield these lines, plus a fourth line for the *mecA* gene. Similar results for methicillin-susceptible and methicillin-resistant *S. epidermidis* are obtained, only the identification line on the strip corresponds with the *S. epidermidis*-specific probe immobilized on the strip instead. Non–*S. aureus*, non–*S. epidermidis* isolates (e.g., *S. warneri*, *S. hominis*, *S. haemolyticus*) will produce the control lines (UC and CC), plus a line for *mecA* if the isolate possesses the gene. An evaluation of the Genotype MRSA test found a 100% correlation with an in-house PCR-based method for detection of *mecA*.[437] No equipment is needed except for a thermocycler for the amplification steps, and results are available within 2 hours.

Identification of Coagulase-Negative Staphylococci

Because the importance of *S. epidermidis* as an agent involved in several infectious processes and the recognized clinical significance of *S. saprophyticus* in urinary tract infections, it is important for laboratories to employ methods for

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identification of these two species (Image Plate 12-3A). Kit systems are available that reliably identify *S. epidermidis, S. saprophyticus*, and several staphylococcal species. Relatively few species are seen with regularity in actual practice. A 1996 examination of 415 significant coagulase-negative staphylococci recovered from blood cultures in Denmark found that 68.7% were *S. epidermidis*, followed in frequency by *S. hominis* (14.7%), *S. haemolyticus* (10.4%), *S. warneri* (2.9%), and *S. cohnii* (1.7%), with *S. saprophyticus*, *S. capitis,* and *S. lugdunensis* each accounting for 1% or less of the total.[253] The distribution of clinically significant species reported in these studies generally reflects the literature that has accumulated regarding the clinical significance of the individual species.

Conventional Identification Methods

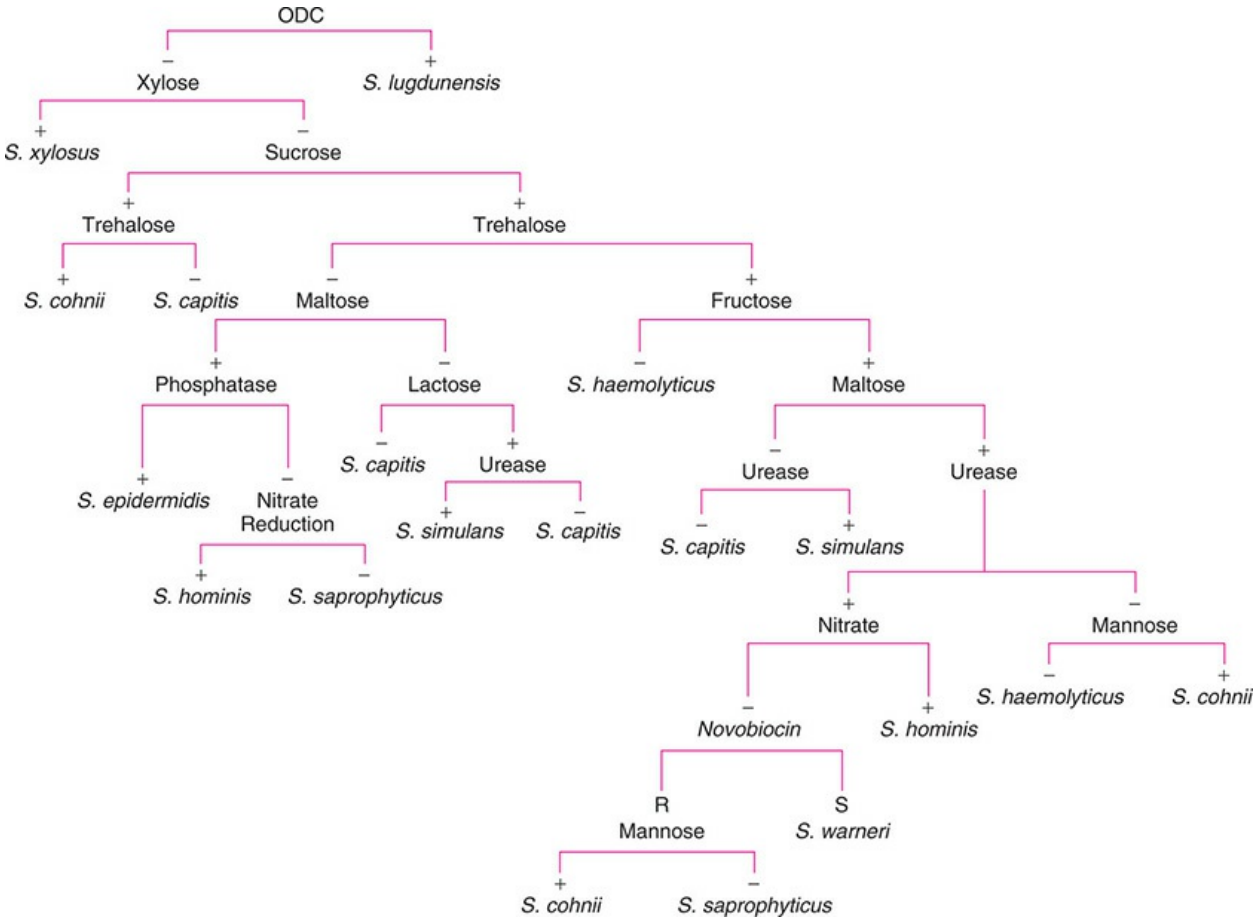
In 1975, Kloos and Schleifer published a scheme for the phenotypic identification of

coagulase-negative staphylococcal species.[284] With the coagulase test for identifying *S. aureus*, this scheme used a large number of physiologic and biochemical tests for differentiating coagulase-negative isolates. Updates and expansions of the scheme led to a 1999 publication by the Subcommittee on the Taxonomy of Staphylococci and Streptococci of the International Committee on Systematic Bacteriology setting forth minimum standards for genus *Staphylococcus* and for descriptions of new *Staphylococcus* species.[171] Modified conventional identification schemes incorporating other tests (e.g., PYR) desferrioxime susceptibility), have been published, and newer schemes have demonstrated improvements in accuracy and turnaround times. Modified conventional procedures use biochemical tests (i.e., PYR, alkaline phosphatase, urease, ornithine decarboxylase, arginine dihydrolase, nitrate reduction acetoin production), acid production from carbohydrates (i.e., D-cellobiose, lactose, maltose, D-mannitol, D-mannose, D-ribose, sucrose, D-trehalose, D-xylose), and susceptibility tests (i.e., susceptibility to desferroxamine, 100 µg, novobiocin 5 µg, and polymyxin B, 300 IU) for species identification. These tests are inoculated with heavy organism suspensions (e.g., a #2 McFarland turbidity standard). Disk tests are read after 24 hours and other tests are interpreted after 72 hours' incubation. Iorio and colleagues[245] developed a 9-test identification scheme and compared the identifications obtained with the modified conventional procedure. Tests included in this simplified scheme were clumping factor, novobiocin and desferrioxamine susceptibility, PYR, urease, alkaline phosphatase, and acid production from mannose, trehalose, and xylose. This simplified 9-test scheme was 98.5% accurate when compared to the reference method when challenged with 198 clinical staphylococcal isolates and 11 control strains. De Paulis and colleagues[130] evaluated a 5-test (novobiocin susceptibility, urease, PYR, ornithine decarboxylase, and aerobic acid production from mannose) conventional identification scheme and compared it to the reference method of Kloos and Schleifer. One or two additional tests were required for identification of certain species and species groups. This scheme showed excellent correlation with the reference phenotypic method, particularly for identifying *S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*. Conventional identification is too time-consuming and labor-intensive for clinical laboratories. Fortunately, a relatively small number of species are recovered from significant human infections, and most commercially available kit systems described briefly below can serve this purpose. Table 12-5 presents the phenotypic characteristics for identification of those staphylococci that are most commonly isolated from human clinical specimens. Table 12-6 includes the phenotypic criteria for identification of human, animal, and environmental species in the genus *Staphylococcus*. Figure 12-1 presents a dichotomous key that is helpful for conventional identification of several clinically important coagulase-negative staphylococci.

Pyrrolidonyl Arylamidase Activity. The PYR test is also useful as a phenotypic test for identifying coagulase-negative staphylococci. As with the enterococci, L-pyrrolidonyl-β-naphthylamide is cleaved by the arylamidase to L-pyrrolidone and free β-naphthylamide, which combines with the PYR reagent (*p*-dimethylaminocinnamaldehyde) to produce a red color. The test is performed with PYR broth inoculated to approximate a #2 McFarland turbidity standard. After 2 hours' incubation at 35°C, the PYR reagent is added. The appearance of a red color within 2 minutes constitutes a positive test, with a yellow, orange, or pink color being negative. Alternatively, any of the commercial rapid PYR disk tests may be used. Hydrolysis of PYR is also found on some of the kit systems that are available for identifying staphylococci. Both *S. aureus* and *S. epidermidis* are PYR-negative, while *S. haemolyticus*, *S. intermedius*, *S. lugdunensis*, and *S. schleiferi* subsp. *schleiferi* are PYR-positive.

Susceptibility to Polymyxin B. The disk diffusion test for susceptibility to polymyxin B is also helpful for identifying coagulase-negative staphylococci. The test can be done on SBA along with that for novobiocin susceptibility (see below). A suspension of the organism equivalent to a 0.5 McFarland turbidity standard is prepared in saline and swabbed onto a plate as for a Bauer–Kirby disk diffusion test. A polymyxin B disk (300 units, BD Biosciences) is applied to the inoculum and the plate is incubated overnight. Resistance is indicated by the presence of a zone of less than 10 mm. Susceptible strains have zones that are equal to or greater than 10 mm. *S. aureus*, *S. epidermidis*, *S. hyicus*, and some *S. lugdunensis* strains are polymyxin resistant.

Ornithine Decarboxylase Test. The ornithine decarboxylase (ODC) test is most useful for confirming the identity of *S. lugdunensis*, as this species is the only one that is consistently ornithine decarboxylase-positive. A tube of decarboxylase base and base with added ornithine (Remel) are inoculated heavily and overlaid with sterile mineral oil. On incubation at 35°C, both tubes will turn from pale-gray to yellow as glucose is fermented. Following this, the ornithine in the test tube will be decarboxylated, resulting in the ornithine broth turning a violet color. The base tube will turn and remain yellow, and the ornithine tube will remain yellow if ODC is not produced. While most positive tests may be detected within 6 to 8 hours, the test is read after 24 hours' incubation. The ODC test is not included in some of the kit systems for identifying staphylococci and must be set up as an additional test. This test is a useful indicator for *S. lugdunensis* as this species is the only coagulase-negative *Staphylococcus* that is ODC-positive.



■ **FIGURE 12-1** Dichotomous key for identification of the more common human coagulase-negative staphylococci.

Urease Production. Some staphylococci are urease-positive, including *S. epidermidis*, *S. intermedius*, and most isolates of *S. saprophyticus*. Regular urease broth or urea agar slants can be used for this test. The slant or the broth is inoculated and incubated at 35°C for 18 to 24 hours. A change in the phenol red indicator from yellow to pink/red is a positive test.

Acetoin Production. Production of acetoin (the Voges–Proskauer test) is helpful in distinguishing *S. aureus*, which are acetoin-positive, from other coagulase-positive species (*S. intermedius* and *S. hyicus*), which are both acetoin-negative. The conventional enteric acetoin production test (MR–VP broth) or the rapid disk method may be used with comparable results.

Susceptibility to Novobiocin for Presumptive Identification of Staphylococcus saprophyticus

Some human staphylococcal species (*S. saprophyticus, S. cohnii subspecies, S. hominis* subsp. *novobiosepticus, S. xylosus*, some *S. pseudolugdunensis*) and several animal species (*S. sciuri* subspecies, *S. lentus, S. gallinarum, S. kloosii, S. equorum, S. arlettae, S. vitulinus, S. nepalensis*, and *S. stepanovicii*) and one environmental species (*S. succinus*) are resistant to novobiocin, with MICs of ≥1.6 µg/mL (Image Plate 12-3B). Since novobiocin-resistant species other than *S. saprophyticus* are infrequently encountered in human clinical specimens, the novobiocin susceptibility test provides a useful method for presumptive identification of *S. saprophyticus*. The novobiocin test is performed as a disk susceptibility test using a novobiocin disk (5 µg) and is described in detail in Chart 12-2. Strains resistant to novobiocin will show zones measuring 6 mm (no zone) to 12 mm; susceptible strains will have zones of 16 mm to 27 mm. This test was originally described using a medium called P agar, which is not available commercially. However, studies with the routine media (e.g., SBA) have shown that comparable results are obtained.

Commercial Identification Systems

All of the kits available for identifying coagulase-negative staphylococci use modified carbohydrate fermentation tests, standard identification tests (e.g., nitrate reduction, urease, acetoin), and chromogenic enzyme substrates for organism identification. Test systems are adapted to manufacturer-specific formats.

Automated Identification Systems

Vitek 2 Gram-Positive (GP) Identification Card. The Vitek 2 Gram-Positive (GP) identification card (bioMérieux,

TABLE 12-5 Phenotypic Characteristics for Identification of Staphylococci Commonly Isolated From Human Clinical Specimens

Test	*S. aureus*	*S. epidermidis*	*S. saprophyticus*	*S. haemolyticus*	*S. warneri*	*S. hominis* subsp. *hominis*	*S. hominis* subsp. *novobiosepticus*	*S. lugdunensis*	*S. schleiferi* subsp. *schleiferi*
Clumping factor	+	–	–	–	–	–	–	+sl	+
Coagulase	+	–	–	–	–	–	–	–	–
Novobiocin	S	S	R	S	S	S	R	S	S
Polymyxin B	R	R	S	S	S	S	NA	S/R	S
PYR	–	–	–	+	–	–	–	+	+
ODC	–	V–	–	–	–	–	–	+	–
URE	V	+	+	–	+	+	+	V	–
ACET	+	+	+	+	+	V	V	+	+
PAL	+	+	–	–	–	–	–	–	+
Heat-stable nuclease	+	–	–	–	–	–	–	–	+
β-GAL	–	–	+	–	–	–	–	–	+sl
Acid produced aerobically from:									
GLU	+	+	+	+	+	+	+	+	+
MAL	+	+	+	+	+sl	+	+	+	–
SUC	+	+	+	+	+	+sl	+sl	+	–
MNTL	+	–	V	V	V	–	–	–	–
MANN	+	+sl	–	–	–	–	–	+	+
TREH	+	–	+	+	+	V	–	+	V

+, positive reaction; –, negative reaction; +sl, slow or delayed positive reaction; V, variable reaction; V–, variable reaction, most strains negative; S, susceptible; R, resistant; NA, data not available; PYR, Pyrrolidonyl arylamidase; ODC, Ornithine decarboxylase; URE, Urease; PAL, Alkaline phosphatase; ACET, Acetoin (VP); β-GAL, β-Galactosidase; GLU, Glucose; MAL, Maltose; SUC, Sucrose; MNTL, Mannitol; MANN, Mannose; TREH, Trehalose.

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TABLE 12-6 Phenotypic Characteristics for Identification of *Staphylococcus* Species

Species	Clumping Factor	Coagulase	NB	POLY B	Modified Oxidase	PYR	Heat-Stable Endonuclease	NO_3 RED	ACET	URE	ADH	ODC	PAL	β-GAL	β-GLU	β-GUR
Staphylococci Found in Humans and Nonhuman Primates																
S. aureus subsp. *aureus*	+	+	S	R	–	–	+	+	+	V	+	–	+	–	+	–
S. aureus subsp. *anaerobius*	–	+	S	NA	–	NA	+	–	–	NA	NA	NA	+	–	–	–
S. epidermidis	–	–	S	R	–	–	–	+	+	+	V	V	V+	–	V	–
S. saprophyticus subsp. *saprophyticus*	–	–	R	S	–	–	–	–	+	+	–	–	–	+	V	–
S. saprophyticus subsp. *bovis*	–	–	R	NA	–	+	–	+	V	+	–	–	–	V	V	–
S. auricularis	–	–	S	S	–	V	–	Vsl	–	–	V	–	–	Vsl	–	–
S. capitis subsp. *capitis*	–	–	S	S	–	–	–	V	V	–	V	–	–	–	–	–
S. capitis subsp. *urealyticus*	–	–	S	NA	–	V	–	+	V	+	+	–	–	–	–	–
S. caprae	–	–	S	S	–	V	–	+	+	+	+	–	+sl	–	–	–
S. cohnii subsp. *cohnii*	–	–	R	S	–	–	–	–	V	–	–	–	–	–	–	–
S. cohnii subsp. *urealyticum*	–	–	R	S	–	V	–	–	V	+	–	–	+	+	–	+
S. haemolyticus	–	–	S	S	–	+	–	+	+	–	+	–	–	–	V	V
S. hominis subsp. *hominis*	–	–	S	S	–	–	–	V	V	+	V	–	–	–	–	–
S. hominis subsp. *novobiosepticus*	–	–	R	NA	–	–	–	V	V	+	–	–	–	–	–	–
S. lugdunensis	+sl	–	S	S/R	–	+	–	+	+	V	–	+	–	–	+	–
S. massiliensis	–	–	S	NA	–	–	–	+	–	–	–	–	–	–	–	–
S. pasteuri	–	–	S	NA	–	–	–	V	V	+	V	–	–	–	+	+
S. petrasii subsp. *petrasii*	–	–	S	NA	–	+	NA	+	+	+	+	–	–	–	–	–
S. petrasii subsp. *croceilyticus*	–	–	S	NA	–	+	NA	+	+	+	+	–	–	–	–	+
S. pettenkoferi	–	–	S	NA	–	+	–	+	–	+	–	–	+	–	NA	–
S. pseudolugdunensis	–	–	R/I	S/I		+		V	V	V+	–	+	V	NA	NA	NA
S. saccharolyticus	–	–	S	NA	–	NA	–	+	NA	NA	+	NA	V	NA	NA	NA
S. schleiferi subsp. *schleiferi*	+	V	S	S	–	+	+	+	+	–	+	–	+	+	–	–
S. schleiferi subsp. *coagulans*	V	+	S	NA	–	NA	+	+	+	+	+	NA	+	NA	NA	NA
S. simulans	–	–	S	S	–	+	–	+	V	+	+	–	Vsl	+	–	V
S. warneri	–	–	S	S	–	–	–	V	+	+	V	–	–	–	+	V
S. xylosus	–	–	R	S	–	V	–	V	V	+	–	–	V	+	+	+

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Staphylococci Found in Other Animals																
S. arlettae	–	–	R	NA	–	–	–	–	NA	–	–	–	+[sl]	V	NA	+
S. chromogenes	–	–	S	R	–	V	–	+	–	+	+	–	+	–	V	–
S. delphini	–	+	S	NA	–	NA	–	+	–	+	+	NA	+	NA	NA	NA
S. equorum subsp. *equorum*	–	–	R	NA	–	–	–	+	–	+	–	–	+[sl]	V	NA	+
S. felis	–	–	S	NA	–	NA	–	+	–	+	+	NA	+	+	–	–
S. gallinarum	–	–	R	S	–	–	–	+	–	+	–	–	+[sl]	V	+	V
S. hyicus	–	V	S	R	–	–	+	+	–	V	+	–	+	–	V	+
S. intermedius	V	+	S	S	–	+	+	+	–	+	V	–	+	+	V	–
S. kloosii	–	–	R	S	–	V	–	–	V	V	–	–	V	V	V	V
S. lentus	–	–	R	S	+	–	–	+	–	–	–	–	V	–	+	–
S. lutrae	–	+	S	NA	–	NA	V	+	–	+	–	NA	+	+	NA	NA
S. microti	–	–	S	NA	–	+	–	+	+[w]	–	–	–	+	+	–	–
S. muscae	–	–	S	NA	–	NA	–	NA	–	NA	–	–	+	–	NA	NA
S. nepalensis	–	–	R	S	–	+	–	+	–	+	–	–	+	+	NA	+
S. pseudintermedius	–	+	S	NA	–	+	–	+	+	+	+	NA	NA	+	+	–
S. rostri	–	–	S	NA	–	+	–	+	–	–	V	–	+	–	NA	–
S. sciuri subsp. *carnaticus*	V	–	R	S	+	–	–	+	–	–	–	–	V	–	+	–
S. sciuri subsp. *sciuri*	–	–	R	S	+	–	–	+	–	–	–	–	+	–	+	–
S. sciuri subsp. *rodentium*	+	–	R	S	+	–	–	+	–	–	–	–	V	–	+	–
S. simiae	–	–	S	R	–	–	–	+	–	+	–	–	+	+[w]	V	NA
S. stepanovicii	–	–	R	S	+	–	–	+	–	+	–	–	–	–	NA	–
S. vitulinus	–	–	R	NA	+	–	–	+	–	–	–	–	–	–	V	–
Other Staphylococci (Mostly Environmental)																
S. carnosus subsp. *carnosus*	–	–	S	S	–	+	–	+	+	–	+	–	+	+	–	–
S. carnosus subsp. *utilis*	–	–	NA	NA	NA	NA	NA	V	NA	–	+	NA	–	–	NA	–
S. condimenti	–	–	NA	NA	NA	NA	NA	+	NA	+	+	NA	+	+	NA	–
S. equorum subsp. *linens*	–	–	S	NA	–	–	–	+	–	+	–	–	V	–	NA	+
S. fleurettii	–	–	R	NA	+	–	–	+	–	–	–	–	V	–	NA	–
S. piscifermentans	–	–	S	NA	–	NA	–	+	–	+	+	NA	+	–	+	–
S. succinus	–	–	NA	NA	–	–	NA	–	–	+	–	NA	+	NA	NA	NA

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Species	Acid Produced From:											
	GLU	SUC	LAC	MAL	MNTL	ARAB	MANN	XYL	TREH	CELL	RAFF	NAG
Staphylococci Found in Humans and Nonhuman Primates												
S. aureus subsp. *aureus*	+	+	+	+	+	–	+	–	+	–	–	+
S. aureus subsp. *anaerobius*	+	+	–	+	NA	–	NA	–	–	–	–	–
S. epidermidis	+	+	V	+	–	–	+[sl]	–	–	–	–	–
S. saprophyticus subsp. *saprophyticus*	+	+	V	+	V	–	–	–	+	–	–	V
S. saprophyticus subsp. *bovis*	+	+	–	+	+	–	–	–	+	–	–	+
S. auricularis	+	V	–	+[sl]	–	–	–	–	+[sl]	–	–	–
S. capitis subsp. *capitis*	+	+[sl]	–	–	+	–	+	–	–	–	–	–
S. capitis subsp. *urealyticus*	+	+	V[sl]	+	+	–	+	–	–	–	–	–
S. caprae	+	–	+	V[sl]	V	–	+	–	+[sl]	–	–	–
S. cohnii subsp. *cohnii*	+	–	–	V[sl]	V	–	V[sl]	–	+	–	–	–
S. cohnii subsp. *urealyticum*	+	–	+	V[sl]	+	–	+	–	+	–	–	V
S. haemolyticus	+	+	V	+	V	–	–	–	+	–	–	–
S. hominis subsp. *hominis*	+	+[sl]	V	+	–	–	–	–	V	–	–	V
S. hominis subsp. *novobiosepticus*	+	+[sl]	V	+	–	–	–	–	–	–	–	–
S. lugdunensis	+	+	+	+	–	–	+	–	+	–	–	+
S. massiliensis	–	–	–	–	–	–	–	–	–	–	–	–
S. pasteuri	+	+	V	V[sl]	V	–	–	–	+	–	–	NA
S. petrasii subsp. *petrasii*	+	+	V	+	V	–	+	–	+	–	–	–
S. petrasii subsp. *croceilyticus*	+	+	V	+	V	+[w]	–	–	+	–	–	–
S. pettenkoferi	+	+	–	–	–	–	–	–	–	–	–	–
S. pseudolugdunensis	+	+	V	–	V	NA	–	V	+	NA	–	–
S. saccharolyticus	+	–	–	–	–	–	+[sl]	–	–	–	–	NA
S. schleiferi subsp. *schleiferi*	+	–	–	–	–	–	+	–	V	–	–	+[sl]
S. schleiferi subsp. *coagulans*	+	V	V	–	V	–	+	–	–	–	–	NA
S. simulans	+	+	+	V[sl]	+	–	V	–	V	–	–	+
S. warneri	+	+	V	+[sl]	V	–	–	–	+	–	–	–
S. xylosus	+	+	V	+	+	V	+	+	+	–	–	+

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Staphylococci Found in Other Animals												
S. arlettae	+	+	+	+	+	+	+	+	+	–	+	–
S. chromogenes	+	+	+	V	V	–	+	–	+	–	–	V
S. delphini	+	+	+	+	$+^{sl}$	–	+	–	–	NA	NA	NA
S. equorum subsp. *equorum*	+	+	V	V	+	+	+	+	+	V^{sl}	–	V
S. felis	+	V	+	–	+	–	+	–	+	–	–	+
S. gallinarum	+	+	V	+	+	+	+	+	+	+	+	+
S. hyicus	+	+	+	–	–	–	+	–	+	–	–	+
S. intermedius	+	+	V	V^{sl}	V^{sl}	–	+	–	+	–	–	+
S. kloosii	+	V^{sl}	V^{sl}	V^{sl}	+	V	–	V^{sl}	+	–	–	–
S. lentus	+	+	V	V	+	V	$+^{sl}$	V^{sl}	+	+	+	V
S. lutrae	+	NA	+	+	V	NA	+	+	+	NA	NA	NA
S. microti	+	+	+	–	–	–	$+^{w}$	+	+	–	–	+
S. muscae	+	+	–	–	–	–	–	+	+	+	–	NA
S. nepalensis	+	+	+	+	+	–	+	V	+	–	–	+
S. pseudintermedius	+	+	+	+	$+^{w,sl}$	–	+	–	+	–	–	+
S. rostri	+	+	V	–	–	–	–	–	V	–	–	–
S. sciuri subsp. *sciuri*	+	+	V^{sl}	V^{sl}	+	V	V^{sl}	V^{sl}	+	+	–	–
S. sciuri subsp. *carnaticus*	+	+	V^{sl}	V^{sl}	+	V	V^{sl}	+	+	V^{sl}	–	–
S. sciuri subsp. *rodentium*	+	+	–	V^{sl}	+	V^{sl}	V^{sl}	V^{sl}	$+^{sl}$	V	–	–
S. simiae	+	+	+	+	+	V	–	V	+	–	–	$+^{sl}$
S. stepanovicii	+	+	–	$+^{w}$	+	–	–		+	–	–	
S. vitulinus	+	+	–	–	+	–	–	V	V^{sl}	V	–	–
Other Staphylococci (Mostly Environmental)												
S. carnosus subsp. *carnosus*	+	–	V	–	+	–	+	–	V	–	–	NA
S. carnosus subsp. *utilis*	+	–	–	–	–	–	–	NA	V	–	–	NA
S. condimenti	+	V	+	V	+	–	+	NA	+	–	–	NA
S. equorum subsp. *linens*	+	–	–	–	–	–	+	–	–	–	–	–
S. fleurettii	+	+	–	+	NA	V	+	V	+	–	–	NA
S. piscifermentans	+	V	V	V	V	–	–	–	+	–	–	NA
S. succinus	+	NA	+	NA	NA	NA	+	NA	+	NA	V^{sl}	NA

+, positive reaction; −, negative; V, variable reaction; $+^{sl}$, slow positive reaction; $+^{w}$, weak positive reaction; V+, Variable reaction, but most strains positive; V^{sl}, variable slow reaction; NA, not available; NB, Novobiocin; POLY B, Polymyxin B; PYR, Pyrrolidonyl arylamidase; NO_3 RED, Nitrate reduced to nitrite; ACET, Acetoin (VP); URE, Urease; ADH, Arginine dihydrolase; ODC, Ornithine decarboxylase; PAL, Alkaline phosphatase; β-GAL, β-Galactosidase; β-GLU, β-Glucosidase; β-GUR, β-Glucuronidase. GLU, Glucose; SUC, Sucrose; LAC, Lactose; MAL, Maltose; MNTL, mannitol; ARAB, Arabinose; MANN, Mannose; TREH, Trehalose; XYL, Xylose; Raff, Raffinose; NAG, N-acetyl-glucosamine.

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Marcy l'Étoile, France) is a gram-positive organism identification card designed for use with the automated Vitek 2 bacterial identification/susceptibility testing system. The 64-well card contains 43 colorimetric substrates for phenotypic identification of *Staphylococcus* species, *Streptococcus* species, and several gram-positive bacillary species (e.g., *Corynebacterium* species,*Erysipelothrix rhusiopathiae*, and *Listeria monocytogenes*). A suspension of the organism is prepared in saline (0.45% to 0.50% NaCl) in a polystyrene tube to a density equivalent to a McFarland 0.50 to 0.63, as determined with the Vitek 2 DensiChek spectrophotometer. The tube and card are inserted into the Vitek 2 cassette and the card is autoinoculated by a vacuum-release method within the Vitek 2 instrument. Wells in the card are optically scanned and read every 15 minutes, with a total incubation time of about 8 hours.

Several evaluations of the Vitek 2 GP identification card indicate that the system works well for identifying staphylococci. Funke and Funke-Kissling performed an

evaluation of the Vitek 2 GP identification card and compared the results to reference methods.[173] The card identified 100% of 45 *S. aureus*, 32 of 33 S. *epidermidis*, 25 of 29 *S. haemolyticus*, 13 of 13 *S. hominis* and all of five *S. lugdunensis* isolates. Single strains of several other species and all three *R. mucilaginosa* isolates were also identified correctly. Layer and colleagues[313] compared the GP identification card to the Phoenix system using 27 reference and type culture strains and molecular methods for reference identification. The card identified 70.1% of reference strains to the species level.Delmas and colleagues[125] compared the Vitek 2 GP identification card to an oligonucleotide array-based molecular identification method using 190 *Staphylococcus* strains, including 38 type strains representing 35 species and 152 previously characterized isolates from clinical, food, and environmental sources. The Vitek 2 GP card identified 93.3% of 60 clinical isolates and 73% of 92 environmental staphylococcal isolates. A comparative study of the GP identification card with 16S rRNA and MicroSeq 500 sequencing performed in Korea found that the card identified 87.5% of 120 clinical isolates correctly, including 88.7% of 53 *S. epidermidis*, 88% of 25 *S. hominis*, 100% of 16 *S. capitis*, and 83.3% of 6 *S. lugdunensis* isolates.[278] An evaluation performed in Greece with 147 clinical staphylococcal isolates and using molecular reference identification methods (i.e., *tuf* gene PCR–restriction fragment length polymorphism [RFLP]) found that the Vitek 2 card identified 98% of the 147 strains correctly, including 100% of 52 *S. aureus* and 96.8% of coagulase-negative staphylococcal species.[92] All 50 *S. epidermidis*, 13 *S. haemolyticus*, 8 *S. cohnii*, and 6 *S. lugdunensis* isolates were identified correctly. The Vitek 2 GP identification card was also examined in a 20-laboratory collaborative study for its ability to identify reference staphylococcal strains found in food products.[115] The card correctly identified 117 of 120 *S. aureus*, and 100% of 60 *S. epidermidis*, 60 *S. hyicus*, and 60 *S. intermedius* isolates, respectively. Lastly, Lee and colleagues[318] reported that the Vitek 2 GP identification card identified 86.6% of 34 presumptive *S. saprophyticus* isolates that were confirmed as such by the Bruker Biotyper MALDI-TOF MS instrument.

Phoenix (Becton-Dickinson Diagnostic Systems, Sparks, MD). The BD Phoenix system is an automated identification and susceptibility test system that uses an ID panel called PMIC/ID-13 for identification of gram-positive cocci. The panel contains 45 wells containing 20 enzymatic substrates, 16 carbohydrates utilization tests, and seven assimilation carbon sources, and susceptibility to colistin and polymyxin B. A standardized suspension of the bacterial isolate is prepared in broth and poured into the panel within 30 minutes of preparation. The panel is then loaded into the instrument where it is incubated and read by the optics of the instrument. Time to providing an identification ranges from 3 to 4 hours.

The BD Phoenix gram-positive identification panel has been evaluated and

compared with several other identification methods. In a study performed at Johns Hopkins University, the Phoenix system was evaluated using cell wall fatty acid analysis (i.e., Sherlock Microbial Identification System [MIDI, v 3.1]) and conventional methods for reference identification.[78] Phoenix identified 100% of 218 *S. aureus* isolates and 99% of 102 coagulase-negative staphylococcal isolates. The only misidentification was one of 12 *S. hominis* strains. In a German evaluation, the Phoenix identified 76.2% of 42 *S. epidermidis*, 90% of 30 *S. hemolyticus,* 100% of 6 *S. capitis*, and 75% of 4 *S. hominis* isolates.[313] Single isolates of *S. lugdunensis* and S. *warneri* were also identified. In this study, the BD Phoenix was tested side-by-side with the Vitek 2 GP identification card. The GP identification card correctly identify 80 of 86 clinical staphylococcal isolates and misidentified 6, while the BD Phoenix correctly identified 70 of the 86 isolates and incorrectly identified 12 isolates.[313] A 2008 study performed in Greece compared the Phoenix system with the API ID32 Staph with discrepancies being resolved by 16S rRNA and *tuf* gene sequence analysis.[65] The Phoenix system correctly identified 90.5% of 200 coagulase-negative staphylococcal isolates, with 6% being incorrectly identified, and 3.5% having no identification reported by the testing system. A 2012 study also done in Greece examined the BD Phoenix in comparison with the Vitek 2.[92] The BD Phoenix correctly identified all 52 *S. aureus* strains and 84.2% of 95 coagulase-negative staphylococci, while the Vitek 2 performed the same as Phoenix for *S. aureus* but identified 96.8% of the 95 coagulase-negative staphylococci correctly. The BD Phoenix identified 86.7% of *S. saprophyticus* isolates when compared with MALDI-TOF mass spectrometry.[318]

MicroScan Panels. Siemens HealthCare Diagnostics (Deerfield, IL), manufacturers of the Microscan WalkAway-96 automated system for identification and antimicrobial susceptibility testing using a microtiter format, has several panels for identification of gram-positive cocci, including conventional, overnight identification/antimicrobial susceptibility microtiter panels (Microscan Pos Combo ID/AST panels), rapid panels (2- to 2.5-hour identifications), and "Synergies-Plus" panels, which provide rapid identification along with certain antimicrobial susceptibility results within 4.5 hours. In the United States, only conventional (Microscan Pos Combo panels) and "Synergies-Plus" panels (Microscan "Synergies-plus" Pos Combo 2 panels) are available. The database for identification includes other gram-positive organisms, including micrococci, streptococci, enterococci, and listeriae. The Microscan Pos Combo panels are microtiter plate format combination ID/AST panels that have 26 conventional and chromogenic phenotypic tests plus various antimicrobial agents for simultaneous microbroth

dilution testing (six different panels are available). A Microscan Pos Breakpoint Combo ID/AST panel is also available and offers the same phenotypic identification tests but fewer dilutions of antimicrobial agents. Following manual inoculation and placement of the plates in the WalkAway-96 instrument, the plates are incubated for 16 to 24 hours at 35°C, and then the panels are read by the WalkAway optics. The biochemical profile of an isolate is compared with the database and an identification is generated.

The Microscan Pos Combo panels have been evaluated and are comparable to other commercially available systems. The Microscan Combo Pos panel was used as part of the reference identification in a study of a modified conventional identification method reported by Iorio and colleagues[245] in Brazil. The Microscan panel identified 59 of 69 *S. epidermidis*, 32 of 44 *S. haemolyticus*, 10 of 16 *S. hominis* subsp. *hominis*, 8 of 9 *S. hominis* subsp. *novobiosepticus*, and 5 or 6 *S. lugdunensis* strains. Overall, 157 (79.3%) of the 198 isolates were correctly identified. An evaluation of the Microscan Combo Pos panel was performed by Patteet and colleagues[414] in Belgium with 428 coagulase-negative staphylococcal isolates from a variety of clinical specimens. These isolates were identified by conventional and other commercial methods in past studies. The panel correctly identified 405 (94.5%) of the 428 isolates, with low-level (<85%) identifications and misidentification comprising 11 (2.6%) and 12 (2.8%) of isolates, respectively. For *S. epidermidis*, 97.3% were correctly identified, as were 96% of 50 *S. haemolyticus*, and 80% of 5 *S. lugdunensis*.

Sherlock Microbial Identification System. The Sherlock Microbial Identification System (MIDI, Microbial ID Inc., Newark, DE) uses high-resolution gas-liquid chromatography (GLC) of cellular fatty acid derivatives for the identification of bacteria. The database of the system is composed of libraries containing analyses of cellular fatty acid methyl ester profiles of various bacteria and compares the composition of individual isolates with those in the database using covariance matrix/pattern recognition software. A similarity index is used to express the relatedness of a profile of an unknown organism to representative profiles of known organisms. Recent evaluations of the MIDI system as a stand-alone method for identification of staphylococci are not available, although, when combined with a few phenotypic tests, the system appears to perform well as a for routine identification of coagulase-negative staphylococci.[78]

Biolog Microplate Identification System. The Biolog Microplate Identification System (Biolog, Inc., Hayward, CA) identifies microorganisms on the basis of oxidation of a variety of substrates. Manual, semiautomated, and automated configurations of this system are available. The automated versions of the system include the OmniLog Gen III data collection/data management software, a reader/incubator, computer, printer, pipettor, and turbidometer. The GEN III Omnilog system enables the identification of aerobic bacteria only, while the GEN III Omnilog-

Plus system also includes databases for identification of anaerobic bacteria, yeasts, and filamentous fungi. Additional software is available for phenotypic array analysis. The database for staphylococci includes human, animal, and environmental species. The system uses 96-well microplates with 95 substrates plus a control well lacking substrate. Combo plates and separate plates for identification of either gram-positive or gram-negative bacteria are available. Plates are inoculated with a suspension of the organism and incubated for 4 or 24 hours. If the organism oxidizes a substrate in an individual well, the respiration of the organism during oxidative substrate assimilation causes the reduction of a tetrazolium indicator dye, turning the well from colorless to purple. This system is not approved for human in vitro diagnostic work at the present time and no recent evaluations of this system are available. Results of a 1993 evaluation reported an accuracy of 69% to 73%, suggesting that the Biolog system is not acceptable as an identification method for these organisms.[366]

Sensititre GPID Plate (Trek Diagnostic Systems, Inc., Cleveland, OH). The Sensititre GPID plate is a identification system that was configured to identify the eight most common staphylococcal species encountered in clinical specimens (i.e., *S. aureus*, *S. epidermidis*, *S. capitis* subsp. *capitis*, *S. haemolyticus*, *S. hominis* subsp. *hominis*, *S. lugdunensis*, *S. saprophyticus* subsp. *saprophyticus*, and *S. warneri*). Garza-Gonzalez and colleagues[175] compared this system with API Staph, with discrepancies being resolved by manual phenotypic tests and partial sequencing of the 16S rRNA gene. While the API Staph (Image Plate 12-2H) currently identified 91% of 155 isolates tested, the Sensititre GPID plate identified 68%. The plate system correctly identified 73% of 86 *S. epidermidis*, 86% of 35 *S. haemolyticus*, 54% of 13 *S. hominis*, one of two isolates of *S. saprophyticus*, and one of four *S. capitis* isolates. None of 8 *S. warneri* strains were identified. Isolates belonging to other species included in the Sensititre GPID database were not tested.

Manual Identification Systems

API Staph-Ident. The API Staph-Idnet (bioMérieux, Inc.) is a 10-test gallery that is inoculated with a heavy organism suspension, and identification is determined by generation of a four-digit octal code derived from the positive tests on the strip and interpreted with an online database (i.e., APIweb) (Image Plate 12-3C and 12-3D). API Staph-Ident has been extensively evaluated, and agreement with conventional procedures has ranged from 43% to 95%, depending on the species tested. Rhoden and Miller published a 4-year prospective study comparing the API Staph-Ident with reference methods on 1,106 isolates and found an overall agreement of 81.1%.[449] Percent agreements for the five most common isolates were 97.1% for *S. epidermidis*, 82.5% for *S. hominis*, 77.2% for *S. aureus*, 75.8% for *S. haemolyticus*, and 64.1% for *S. warneri*. These workers concluded that the database of the current Staph-Ident system is inadequate for identifying both common and uncommon

staphylococcal species.[449]

API Staph. API Staph (bioMérieux, Marcy l'Étoile, France) is an 18- to 24-hour identification system for micrococci and staphylococci. This system contains 19 tests arranged in a strip format and is inoculated with a suspension of the organism (0.5 McFarland standard) prepared in a peptone yeast extract broth medium provided with the kit. After reading the biochemical reactions, a seven-digit octal code is generated and identification of the organism is obtained with the APIweb software-assisted database. The database consists of 25 taxa and includes staphylococci of human and veterinary origin, *Micrococcus* species, and *R. mucilaginosa*. Although the database of this system is large, it has not

been extensively evaluated. A study by Perl and colleagues[423] found that the API Staph system correctly identified 73% of 277 coagulase-negative staphylococci. (While 94% of 94 *S. epidermidis* isolates were correctly identified, the system performed poorly with less common isolates like *S. haemolyticus* (85% correct), *S. hominis* (75% correct), *S. simulans* (67% correct), and *S. warneri* (22% correct). The API Staph was used as an identification method to characterize 33 coagulase-negative, non-*epidermidis* staphylococci recovered from ocular infections and compared with microbial genotyping for identification.[338] The API Staph and genotyping corresponded for only 48.4% of the isolates. While most strains of *S. haemolyticus* were correctly identified, isolates of *S. warneri*, *S. hominis*, *S. capitis*, and *S lugdunensis* were identified with likelihoods ranging from 40% to 75%. More recent evaluations of this kit have dealt with veterinary isolates (i.e., goat milk isolates, bovine mastitis isolates); in these studies, overall agreement of the API Staph ID with molecular methods was only 72% to 76%.[293,411,535]

API ID32 Staph. ID32 Staph (bioMérieux, Marcy l'Étoile, France) is a 24-hour gallery system for identifying staphylococci and related species. The system can be read manually to generate a profile number that is interpreted by the APIweb online database or the test panel can be used with an automated bioMérieux ATB system, which includes a densitometer, an inoculator, a reader, a microcomputer, and a printer. ID32 Staph has the most extensive database of the manual systems, with many human staphylococcal species and several animal and environmental species included. The system also identifies six micrococcal species and *R. mucilaginosa*. In a study of 440 consecutive coagulase-negative staphylococcal isolates, Ieven and coworkers[240]reported that the ID32 Staph panel identified 95.2% of *S. epidermidis*; *S. haemolyticus*, *S. lugdunensis, S. schleiferi,* and *S. capitis* were identified with accuracies of 98% to 100% (Image Plate 12-3E, 12-3F, 12-3G and 12-3H). Layer and

colleagues[313] used the ID32Staph as a reference method in an evaluation of the BD Phoenix and Vitek 2 system, with arbitration of discrepant identifications by two molecular methods, and found that 89.4% of the reference and clinical isolates tested were correctly identified by ID32Staph. The ID32 Staph had more correct identification results than either of the two automated systems. In an evaluation of the Phoenix and the Vitek 2 automated identification systems, ID32 Staph was used as a reference system. Isolates were also analyzed by 16S rRNA and *tuf* gene sequencing. The ID32 Staph system identified 96.5% of the 200 isolates tested isolates tested and showed 93% to 100% concordance with molecular identifications.[65]

RapID Staph Plus (Remel). The RapID Staph Plus uses modified conventional phenotypic tests and chromogenic enzyme substrates for identification of clinically relevant *Staphylococcus* species. A suspension of the organism is prepared in inoculation fluid to match a #3 McFarland turbidity standard and poured into the inoculation port of the cuvette. Manual manipulation of the cuvette simultaneously hydrates and inoculates the substrates in the wells of the cuvette. After incubation for 3 to 6 hours at 35°C to 37°C, the reactions for the various substrates are recorded; 12 of the phenotypic tests are read directly, while tests 13 through 17 requires addition of RapID Staph Plus dimethylaminocinnamaldehyde reagent and test 18 requires nitrate reagents A and B. The reactions are used to derive a six-digit octal code which is entered into the Electronic RapID Compendium (ERIC) computer-assisted database. This database provides the organism identification. The database includes 33 species and subspecies of staphylococci, and also includes *Kocuria* species, *Kytococcus* sedentarius, *Micrococcus* species, and *R. mucilaginosa*. Evaluations of this system are not available.

BBL Crystal Gram-Positive (GP) Identification Kit. The Crystal GP Identification kit (BD Biosciences) is a miniaturized identification panel consisting of 29 fluorogenic, chromogenic, and modified conventional tests plus a negative control for the identification of gram-positive isolates belonging to several genera, including *Staphylococcus*, *Micrococcus*, *Stomatococcus*, *Streptococcus, Enterococcus*, *Aerococcus*, and *Lactococcus*. The system is inoculated with a suspension of organisms (a #2 McFarland turbidity standard), incubated for 4 hours at 35°C to 37°C in an ambient atmosphere, and read with a special Crystal system panel viewer. The Crystal GP kit was evaluated along with Vitek 2 and the MicroScan systems and compared with identifications obtained by 16S rRNA sequencing and an in-house molecular method using the MicroSeq 500.[278] The Crystal GP kit correctly identified 92.5% of 16 *S. epidermidis*, and correctly identified all *S. warneri* (6 isolates), *S. simulans* (2 isolates), *S. cohnii* (3 isolates), *S. haemolyticus* (4 isolates), and a single *S. saprophyticus* isolate. Only 2 of 6 *S. lugdunensis* and 1 of 25 *S. hominis* isolates were correctly identified; some *S. capitis* and all *S. caprae* isolates were misidentified.

Microbact Staphylococcal 12S Identification System (Oxoid). The Microbact

Staphylococcal 12S (Oxoid) is a 12-test identification system based on six carbohydrate utilization tests, arginine dihydrolase, urease, and four chromogenic enzyme substrates. Only 20 of the more common staphylococcal species are included in the database. The wells of the strip are inoculated with a suspension of the organism and the strip is incubated for 18 to 24 hours at 35°C to 37°C. In addition, the results of a coagulase/latex agglutination test, a DNase test, and notation of pigment production are also required. After addition of color development reagent to the enzymatic tests, the reactions are recorded on the report form along with results of the three additional tests and the resulting five-digit octal code is entered into the Microbact Computer Aided Identification software, which then provides a species identification. No evaluations of this system are available and this system is not available in the US market.

RapiDEC Staph. RapiDEC Staph (bioMérieux) is a manual kit for identifying *S. aureus*, *S. epidermidis*, and *S. saprophyticus* within 2 hours. An organism suspension is dispensed into four cupules, which include a negative control, a fluorescent coagulase substrate, an alkaline phosphatase substrate (PAL), and a β-galactosidase substrate (BGAL), respectively. "Aurease" is a proteolytic enzyme that reacts with prothrombin to form staphylothrombin that, in turn, cleaves a fluorescent peptide in the cupule, releasing a fluorescent radical. After 2 hours incubation, greater UV fluorescence in the "coagulase" cupule relative to the control identifies *S. aureus*. The other two cupules are read either directly or after reagent addition. A positive alkaline

phosphatase test or a positive β-galactosidase test identifies *S. epidermidis* and *S. saprophyticus*, respectively. The system does well for identifying *S. aureus*, but *S. epidermidis* isolates that produce low levels of or are negative for alkaline phosphatase are not identified by RapiDEC Staph.[252] Similarly, some *S. saprophyticus* isolates are not identified because they lack β-galactosidase activity or are misidentified since the enzyme is found in novobiocin-susceptible and -resistant staphylococci (e.g., *S. cohnii*, *S. hominis*, *S. simulans*). The control and "aurease" cupules have been used for identification of *S. aureus* directly in positive blood cultures using a centrifuged aliquot from the positive blood culture. With a protocol slightly modified from the suggested procedure, van Griethuysen and colleagues[562] identified 100% of 42 *S. aureus* isolates directly in blood culture bottles with a specificity of 96.6%. A similar study by Chapin and Musgnug[88] found that the "aurease" test had a sensitivity and specificity of 96% and 99%, respectively, when used directly from blood cultures growing gram-positive cocci in clusters.

Staphylococcus aureus/Coagulase-Negative Staphylococci PNA

FISH Culture Confirmation Kit (AdvanDx, Woburn, MA)

PNA FISH technology combines fluorescence in situ hybridization (FISH) with unique peptide nucleic acid (PNA) molecules wherein the negatively charged carbohydrate-phosphate moieties comprising the backbone of DNA and RNA are replaced with uncharged PNA probes containing the same nucleotide bases as DNA, allowing them to hybridize with complementary nucleic acid sequences. The uncharged, hydrophobic backbone of PNA probes allows the probes to penetrate the hydrophobic cell walls of bacteria and enables tighter and more specific hybridization to nucleic acid sequence targets.[603] The PNA probes hybridize with *S. aureus*-specific rRNA sequences or with rRNA sequences of selected coagulase-negative staphylococcal species. These PNA probes are labeled with tags that fluoresce under UV light. PNA FISH technology has found its greatest application in direct identification of pathogenic bacteria directly in positive blood cultures. The *S. aureus*/CNS PNA FISH test reagent contains fluorescein-labeled *S. aureus*-specific PNA probes and Texas Red-labeled PNA probes targeting sequences in other coagulase-negative staphylococci. A drop of blood/broth from a positive blood culture showing gram-positive cocci in clusters is mixed with fixative in the well of a single-well microscope slide provided in the kit and fixed with heat or methanol. One drop of the *S. aureus*/CNS PNA is added to the well on the smear and a coverslip is placed. The slide is incubated for 30 minutes at 55°C. The slide is the placed in wash solution, the coverslip is removed, and the slide is incubated in the wash solution for 30 minutes at 55°C in the slide warmer of the PNA FISH Workstation. Following the wash step, the slide is air-dried, a drop of mounting medium is placed on the smear and a coverslip is placed. The slide is examined under fluorescence microscope. *S. aureus* appears as bright, apple-green fluorescent clusters of cocci, while coagulase-negative staphylococci appear as multiple, bright-red fluorescent cocci. Other microbial, nonstaphylococcal cells are nonfluorescent. The test takes approximately 2½ hours to perform, with less than 30 minutes hands-on time.

More recently, the *Staphylococcus* QuickFISH BC was approved for use. This test identifies *S. aureus* and coagulase-negative staphylococci from blood cultures in less than 30 minutes by the elimination of a wash step.[124] This modification uses a drop from a positive blood culture that is prepared on FISH slides, with positive and negative controls already present on the slide. The slide is heat-fixed at 55°C for up to 5 minutes. Single drops of two PNA solutions—*Staphylococcus* PNA Blue and *Staphylococcus* PNA Yellow—are mixed together on a coverslip that is placed inside a template. Mixture of the two reagents in the oval window of the template results in a uniform green color. The coverslip is then turned over and applied to the dried/fixed blood culture drop on the specimen slide within the borders of the viewing area. The slide is incubated at 55°C for 15 to 20 minutes at the workstation. The slide is read on a fluorescence microscope by first inspecting the controls, then moving over to the

specimen well in the same plane of focus. As with the longer procedure, *S. aureus* appears as bright, apple-green fluorescent clusters of cocci, while coagulase-negative staphylococci appear as multiple, bright-red fluorescent cocci. This test has a turnaround time of less than 30 minutes with a hands-on time of less than five minutes.

Published evaluations of *S. aureus* PNA FISH over several years have shown very high sensitivity, specificity, and positive and negative predictive value.[226,396] A blinded multicenter comparative study of *S. aureus* PNA FISH was performed in eight different laboratories using a variety of blood culture media from three different manufacturers (ESP medium, Trek Diagnostics, Inc. Westlake, OH; BACTEC medium, Becton-Dickinson; BacT/Alert (bioMérieux). The sensitivity of the PNA FISH test for detection of *S. aureus* ranged from 98.5% to 100%, with specificities of 98.5% to 99.2% regardless of the blood culture medium being used.[395] A study performed in Spain evaluated 285 blood cultures containing gram-positive cocci in clusters on Gram's stain. The *S. aureus* PNA FISH assay demonstrated 100% sensitivity and 99.4% specificity with positive and negative predictive values for detection of *S. aureus* bacteremia being 99.2% and 100%, respectively.[193] A five-center study evaluated 722 positive blood culture bottles containing gram-positive cocci in clusters. For detection of *S. aureus,* sensitivity of the QuickFISH test was 99.5% with a specificity of 98.8%.[124] A single *Micrococcus* isolate fluoresced green (i.e., *S. aureus*) initially, but did not fluoresce when repeated. This study also addressed the specificity of the assay using reference strain-spiked blood cultures. Of 34 coagulase-negative staphylococcal strains representing 31 species, all produced positive (red fluorescence) except for single isolates of *S. simulans* and *S. felis*. Several other organisms (i.e., gram-positive and gram-negative coccal and bacillary bacteria) produced uniformly negative results. An Italian evaluation of the *Staphylococcus* QuickFISH BC test performed on 173 blood cultures found that 100% of 35 *S. aureus* were correctly identified by rapid PNA FISH, with a specificity of 100%. The test also identified 98.6% of coagulase-negative staphylococci, again with 100% specificity.[77]

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Molecular Identification and Typing Methods for Staphylococci

Several molecular methods using DNA as the basis for identification have been described for species level identification and strain typing of *Staphylococcus* species for epidemiologic studies and in outbreak investigations. Methods have included 16S rRNA gene sequencing, amplified fragment length polymorphism (AFLP) analysis of plasmid, ribosomal, and chromosomal nucleic acids, polymerase chain reaction/nucleic

acid probe hybridization procedures, PCR analysis of insertion elements and 16S to 23S rRNA intergenic spacer regions, *IS256* spacer length polymorphisms, and ribotyping.[76,131,132,222,230,486] Closely related species in the genus *Staphylococcu*s may not be adequately resolved by 16S rRNA sequence-based methods and these issues limit the usefulness of this approach for staphylococcal species identification.[76] Other gene sequences have been evaluated for their ability to robustly discriminate individual species and include the heat-shock protein 60 (*hsp60*) gene, the heat-shock protein 40 (*dnaJ*) gene, the gene for the β-subunit of RNA polymerase (*rpoB*), the thermostable endonuclease gene (*nuc*), the superoxide dismutase (*sodA*) gene, the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gap*), and the gene encoding elongation factor Tu (*tuf*).[142,181,182,222, 275,302,486,498] Sequencing of the *tuf* gene, in particular, is more discriminatory for species identification than 16S rRNA sequence analysis for staphylococcal identification.[238,429] The availability of various primary and secondary databases (e.g., GenBank, BIBI, EzTaxon, MicroSeq) containing published staphylococcal gene sequences complicates identifications and different databases may produce slightly different results. Hwang and colleagues[238] compared 16S rRNA and *tuf* sequences for 97 clinical coagulase-negative staphylococcal isolates and found slightly different results with each of the published and online databases. Ghebremedhin and colleagues[181] found that PCR amplification of the *gap* gene, direct sequencing of the amplicon, and comparison with the GenBank database-enabled reliable species identification. The standardization of databases, the adoption of reference or reference-equivalence agreement, and specific criteria for species designations will improve reliability.

A commercial molecular system for simultaneous identification and detection of *mecA* called the Templex StaphPlex I (Genaco Biomedical Products, Inc., Huntsville AL) is also available.[533] This multiplex uses PCR amplification primers specific for *tuf*gene sequences that are conserved for all coagulase-negative staphylococci, primers for genes specific to *S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis,* and *S. simulans,* and primers for SCC*mec* types I, II, III, and IV. In addition, primers are included for the detection of the *aac, ermA and ermC, tetM* and *tetK,* for detection of resistance to gentamicin, erythromycin, clindamycin, and tetracycline, respectively. Following extraction and amplification by PCR, identifications are obtained by detection of specific amplicons using a suspension array of labeled beads that are tagged with gene sequences that bind specifically with the amplicons. Detection of labeled amplicons is obtained using the Luminex 100 instrument (Luminex, Austin, TX). In an evaluation of this system with blood culture broths showing gram-positive cocci in clusters, the StaphPlex I system correctly identified 186 of 203 *S. epidermidis*, 10 of 12 *S. haemolyticus*, 25 of 34 *S. hominis*, and 1 of 1 *S. lugdunensis*. *S. capitis* (10 isolates), *S. warneri* (2 isolates), *S. pasteuri* (2 isolates),

and *S. pettenkoferi* (9 isolates) were identified as "other coagulase-negative staphylococci" by the system.[533] The StaphPlex I also detected SCC*mec* type II (most HCS-MRSA) and SCC*mec* type IV (most CA-MRSA) with 100% sensitivity and greater than 95% specificity. The StaphPlex also contains primers and probes for the detection of the PVL genes.

For strain typing of staphylococci, genotypic and molecular methods have largely supplanted older phenotypic methods, such as phage typing, plasmid profiles, and protein analysis. Genotypic typing methods are based on either DNA banding patterns as detected by electrophoresis or by DNA sequencing approaches. PFGE was used initially to delineate the eight MRSA clonal types USA100 through USA800.[358] The typing methods that are currently used for *S. aureus* include *spa* sequence typing, double-locus *spa-clfb* typing, multilocus sequence typing (MLST), and SCC*mec* typing.[515] The *spa* gene encodes protein A, which is found on the surface of *S. aureus*, and the typing method involves sequencing of the variable 24-nucleotide X region of *spa*.[208] Double-locus *spa-clfb* typing involves sequencing of both *spa* and the clumping factor B gene (*clfB*), which contains variable numbers of serine-aspartate repeating units depending on acquisition or loss of complete or partial gene segments.[300]MLST addresses the sequence variability of several core variable (CV) housekeeping genes (usually 7 to 10 genes) to determine a type. SCC*mec* typing deals with sequencing of the *mecA* gene. For these three typing methods, online databases are available that contain analyses of thousands of isolates from several countries (e.g., http://www.SeqNet.org, www.mlst.net, www.saureusmlst.net). Availability of online resources has enabled more standardized approaches to procedural testing issues and nomenclature. Standardization and reference databases are essential for worldwide communication regarding *S. aureus,* and are invaluable to public health officials, research laboratories, and published scientific communications. Additional markers (PVL typing, sequencing of the PVL genes *luk*S-PV and *luk*F- PV, and *arc*A typing (which encodes the arginine catabolic mobile element) may also be helpful for type delineation particularly in outbreak investigations and for nationwide and worldwide epidemiology.[386] Shallcross et al.[488] have reviewed the role of PVL in staphylococcal disease. Multilocus variable-number tandem repeat (MLVA, VNTR) analysis and PFGE are used less frequently for *S. aureus*, although various researchers have examined this approach. For MLVA, the procedure and the nomenclature are not standardized.

PFGE is the most discriminatory method for molecular typing of *S. epidermidis* and other coagulase-negative staphylococci.[368] Strains are identified based on RFLPs of the whole chromosome using restriction enzymes that produce rare DNA cuts and result in large DNA fragments that can be resolved by gel electrophoresis. PFGE analysis requires training and is technically demanding. Different laboratories may

produce slightly different results even with the same set of strains. Standards for interpretation are available, but slight procedural variations may result in slightly different results that may present difficulties

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in interpretation.[539] In addition to PFGE, several other methods have been described for genotyping of coagulase-negative staphylococci, including RFLP, random amplified polymorphic DNA (RAPD), AFLP, repetitive sequence-based PCR (rep-PCR), and multilocus variable-number tandem repeats (VNTR) analyses.[76,80,131,166,254,290,383] The DNA sequencing-based typing method of choice is MLST. This method involves sequencing a set of housekeeping genes that evolve slowly over time. For *S. epidermidis*, seven of these genes are sequenced and compared to published sequences in an online MLST database.[153,543] Because these genes are highly conserved, with very low mutation rates over time, this method can be used to establish lineages and relationships between and among different *S. epidermidis* isolates over months to years. Conversely, PFGE lends itself more readily to analysis of short-term hospital outbreaks (e.g., investigations of an outbreak of *S. haemolyticus* in a neonatal ICU). At the present time, MLST is only available for *S. epidermidis,* while PFGE can be used for other coagulase-negative staphylococci.[225,507,600]

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is currently being introduced as a method for bacterial identification in clinical laboratories. At this time two systems are available: the MALDI Biotyper (Bruker Daltronics, Bremen, Germany) and the AXIMA/Shimadzu mass spectrometer (marketed in the United States by bioMérieux). The latter system uses the SARAMIS (Spectral ARchiving And Microbial Identification System, AnagnosTec, Am Muehlenberg 11, 14476 Potsdam/Golm, Germany) as its reference database. A subset of SARAMIS referred to as Superspectra represents the automated system that is available for bacterial identification. The Bruker database currently contains 28 staphylococcal species, while the SARAMIS reference database contains 38 species and subspecies of staphylococci. DuPont and colleagues[145] in France evaluated the Bruker mass spectrometer for identification of 234 clinical coagulase-negative staphylococcal isolates representing 20 species, which were identified by *sodA* gene sequencing. Results were also compared with those obtained with the Vitek 2 and Phoenix systems. When species not common to the databases were eliminated, MALDI-TOF MS correctly identified 97.4% of the isolates, while the Vitek 2 and

Phoenix identified 78.6% and 79%, respectively. Dubois and coworkers[143] also examined the Bruker MS for the identification of 152 staphylococcal strains belonging to 22 different species. These isolates were characterized using a *sodA* gene-based oligonucleotide array for reference identification. Of these 152 isolates, 99.3% were correctly identified to the species level, with one strain identified to the genus level. An evaluation of the Bruker system conducted in the Netherlands found that MS identified 94.3% of 261 gram-positive cocci representing two genera and nine species, whereas phenotypic methods (Vitek 2 and API strips) identified only 63.2%.[568] An Italian evaluation used the Bruker MS for the identification of 450 bloodstream isolates, with *rpoB* gene sequencing as the reference method.[508] MS correctly identified 447 (99.3%) of the isolates with only three misidentifications. Bergeron and others evaluated the AXIMA/Shimadzu system and the SARAMIS database with 186 clinical isolates (including 35 species and subspecies) and 47 *Staphylococcus* species type strains.[48] The clinical isolates were also identified by *tuf* gene sequencing as the reference method. While *tuf* sequencing provided the identification of all but one of the isolates, MALDI-TOF MS correctly identified only 138 (74.2%) of the isolates. The reason for the poor performance of MS in this evaluation was likely due to the absence of Superspectra for many of the animal and environmental staphylococcal species included in this particular evaluation. MALDI-TOF MS is a reliable and relatively inexpensive bacterial identification method that is faster and superior to most phenotypic panels and has accuracy approaching those of molecular methods. As more isolates are examined by MS techniques, the databases of MALDI-MS instrumentation and the applications of a variety of spectral databases will revolutionize microbial identification, characterization, and systematics.

Identification of Micrococcus *and Related Species*

Micrococci and related species are not generally identified to species level in clinical laboratories because they are rarely clinically significant. Using the tests described above and in Table 12-2, laboratories may issue reports of "*Micrococcus* species" without further testing. However, with the recognition of these agents as opportunistic pathogens, it may be necessary to identify these organisms to species on occasion. Identification criteria for the newly reclassified members of the former genus *Micrococcus* are presented in Table 12-7. On Gram's stains, these organisms generally occur in pairs, tetrads, and clusters. All members of the micrococcal species are aerobic, catalase-positive, furazolidone-resistant, and bacitracin-susceptible. The micrococcal species that have been isolated from human clinical specimens and infections include *Kytococcus species (K. schroeteri*, *K. sedentarius)*, *Kocuria* species (*K. kristinae*, *K. rhizophila*, *K. varians*, *K. rosea*, *K. marina),* and *M. luteus.* Colonies of *K. schroeteri* can be smooth and shiny or rough, dry, and "volcano-like"

with irregular edges. Colonies also can vary in pigmentation from a dark, muddy yellow color to creamy white. *K sedentarius* colonies vary in pigmentation from a creamy white to a deep buttercup yellow. Colonies of *K. kristinae* are smooth or rough, with colony pigmentation ranging from pale cream to pale orange, with pigmentation becoming more intense with time. *K. rhizophila* and *K. varians* form pale yellow-pigmented, smooth, shiny colonies, although rough variants of *K. varians* have been described. *K. marina* colonies are smooth and orange in color, while those of *K. rosea* are cream to pale pink. Some species are included in the databases of commercial automated and manual identification systems used in laboratories and may be misidentified as *Staphylococcus* species and vice versa. For example, the Vitek 2 system erroneously identified 20 isolates as *Kocuria* species that were confirmed as coagulase-negative staphylococcal species by 16S rRNA gene sequencing.[41]

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TABLE 12-7 Phenotypic Characteristics for Identification of *Micrococcus* and Related Genera

								Hydrolysis of:			Acid Produced Aerobically From:					
Species	PIGM	OX	Cell Arrangements	MOT	URE	ADH	NO3 RED	ESC	GEL	STA	GLU	GLYC	MNTL	MANN	LAC	XYL
A. agilis	Rose red	+	Pairs, tetrads, clusters	+	–	–		+	+	+	–	–	–	–	–	
D. nishinomiyaensis	Orange	+	Pairs, tetrads, clusters	–	V+	–	V	–	+	V	V	–	–	–	–	–
K. kristanae	Cream to pale orange	+	Tetrads, clusters	–	V	–	–	+	–	–	+	+	–	+	–	–
K. marina		–	NA		+	–	+		+	–	–	–	–	NA	–	–
K. polaris	Orange	+	Pairs, tetrads, cubes	–	–	–	+	–	–	+	+	NA	+	–	NA	+
K. palustris	Pale yellow	–	Pairs, tetrads, cubes	–	+	–	+	–	+	–	+	–	–	–	–	–
K. rhizophila	Yellow	–	Pairs, tetrads, cubes	–	–	–	–	–	+	–	+	–	–	+	+	–
K. rosea	Orange, pink, red	V–	Pairs, tetrads, clusters	–	NA	–	+	–	–	+	+	–	+	–	–	–
K. schroeteri	Dull yellow	–	Pairs, tetrads	–	–	+	–	–	+	–	–	–	–	–	–	–
K. sedentarius	Cream to deep yellow	–	Tetrads, cubes	–	–	+	V	–	+	–	–	–	–	–	–	–
K. varians	Deep yellow	–	Tetrads, clusters	–	+	–	+	–	+w	–	+	–	–	–	+	–
M. antarcticus	Yellow, mucoid	+	Pairs, cubes, clusters	–	–	–	+	–	–	+	–	–	–	–	–	–
M. endophyticus	Yellow, slimy	+		–			+	NA	+	–	+	–	–	+	–	–
M. luteus	Yellow, yellow-green	+	Pairs, tetrads, cubes, clusters	–	–	–	–	–	+	–	–	–	–	–	–	–
M. lylae	White	+	Pairs, tetrads, cubes, clusters	–	–	–	–	–	+	–	–	–	–	–	–	–
N. halobia	None	+	Pairs, clusters	–	–	–	–	NA	NA	+	+	+	+	+	+	+
N. halotolerans	Deep orange to yellow	–	Single cells	+	+	NA	–		+	–	–	NA	–	NA	–	–
N. lacusekhoensis	Yellow	–	Pairs, clusters	–	–	NA	–	NA	NA	–	–	NA	–	NA	–	–
N. xinjiang	Light yellow	–	Diphtheroidal irregular rods	–	+	NA	–	NA	+	–	–	NA	–	NA	–	–

+, positive reaction; –, negative reaction; V, variable reaction; V+, variable reaction, most strains positive; V–, variable reaction, most strains negative; +w, slow or delayed positive reaction; NA, data not available; OX, Modified oxidase; MOT, Motility; URE, Urease; ADH, arginine dihydrolase; NO_3 RED, Nitrate reduced to nitrite; ESC, Esculin; GEL, Gelatin; STA, Starch; GLU, Glucose; GLYC, Glycerol; MNTL, Mannitol; MANN, Mannose; LAC, Lactose; XYL, Xylose.

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Identification of Rothia mucilaginosa

R. mucilaginosa colonies are sticky or mucoid, clear to gray–white in color, and adherent to the agar surface. They appear on Gram-stained smears as large gram-positive cocci arranged in pairs or clusters. They are weakly catalase-positive, although some strains are catalase-negative. *R. mucilaginosa* can be differentiated from *Micrococcus* and *Staphylococcus* species by failure to grow on nutrient agar medium containing 5% NaCl and by the presence of a capsule. These organisms are also included in the database of the API Staph-Ident, the API Staph, and ID32 Staph systems. Other biochemical characteristics are shown in Table 12-8.

Laboratory Approach to the Identification of Staphylococci

Because staphylococci are among the most frequently isolated organisms in the clinical laboratory, decisions must be made on "how far to go" in identifying them. This is especially true regarding the coagulase-negative organisms. Many laboratories have adopted rapid coagulase procedures (i.e., latex tests), so these tests may quickly be performed on colonies that appear to represent staphylococci and are catalase-positive. If the colonies are coagulase-positive, the organism is identified as *S. aureus*. For isolates that are coagulase-negative, a furazolidone or bacitracin disk test, or the modified oxidase test, may be performed to differentiate coagulase-negative staphylococci from *Micrococcus* and related species. Significant staphylococcal isolates from urine cultures should also be tested for susceptibility to novobiocin for presumptive identification of *S. saprophyticus*. Complete species identification using a reference procedure, a kit, or a molecular approach should be reserved for clinically significant isolates, including those isolates that have been recovered from multiple sets of blood cultures, from infected intravenous catheters (where the patient may have the same isolate in multiple blood cultures), or from other normally sterile sites where the same coagulase-negative *Staphylococcus* sp. has been repeatedly isolated. Decisions involving further identification of these organisms should be made on a case-by-case basis with inputs from both the laboratory director and the physicians caring for the patient.

TABLE 12-8 Identification of *Rothia mucilaginosa*

Test	Reaction
CAT	V

Growth, anaerobic conditions	+
Coagulase	−
Growth, nutrient agar with 5% NaCl	−
GEL	+
PAL	−
ACET	+
Acid produced from:	
GLU	+
FRU	+
SUC	+
MNTL	−
MANN	V
SBTL	−
SAL	+
TREH	V

+, positive reaction; −, negative reaction; V, variable reaction; CAT, Catalase; GEL, Gelatin hydrolysis; PAL, Alkaline phosphatase; ACET, Acetoin (VP); GLU, Glucose; FRU, Fructose; SUC, Sucrose; MNTL, Mannitol; MANN, Mannose; SBTL, Sorbitol; SAL, Salicin; TREH, Trehalose.

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CHAPTER 13

Gram-Positive Cocci

Part II: Streptococci, Enterococci, and the "*Streptococcus* -Like" Bacteria

thePoint For additional ancillary materials related to this chapter, please visit the point.

General Characteristics of the Streptococci

Group A β-Hemolytic Streptococci (*Streptococcus pyogenes*)

- Virulence Factors
- Clinical Spectrum of Group A Streptococcal Disease

Group B β-Hemolytic Streptococci (*Streptococcus agalactiae*)

- Virulence Factors
- Clinical Spectrum of Group B Streptococcal Disease
- Prevention of Group B Streptococcal Disease
- Other Infections Caused by Group B Streptococci
- Antimicrobial Susceptibility of Group B Streptococci

Group C and Group G β-Hemolytic Streptococci

Group F β-Hemolytic Streptococci

Emerging Pathogens Among the Streptococci

Streptococcus suis

Streptococcus porcinus and *Streptococcus pseudoporcinus*

Streptococcus iniae

Streptococcus pneumoniae

Virulence Factors

Pneumococcal Vaccines

Clinical Spectrum of *S. pneumoniae*

Antimicrobial Susceptibility of *S. pneumoniae*

Viridans Streptococci

The Anginosus Group: Streptococcus anginosus, Streptococcus constellatus, and Streptococcus intermedius

Group D Streptococci: The "*Streptococcus bovis*" Group

***Enterococcus* Species**

Taxonomy

Virulence Factors

Clinical Spectrum of Enterococcal Infections

Antimicrobial Susceptibility of Enterococci

Genus *Melissococcus* and Genus *Catellicoccus*

The "*Streptococcus-Like*" Bacteria

Abiotrophia and *Granulicatella* Species

Aerococcus and *Helcococcus* Species

Leuconostoc Species

Pediococcus and *Tetragenococcus* Species

Gemella Species

Vagococcus Species

Alloiococcus Species

Globicatella Species

Facklamia Species

Dolosigranulum, Ignavigranum, Dolosicoccus, and *Eremococcus* Species

Lactococcus Species

Isolation and Identification of Streptococci and the "*Streptococcus-Like*"

Bacteria

Direct Gram-Stained Smears

Culture Media

Hemolysis on Blood Agar

Nonculture, Direct Detection Techniques for Group A β -Hemolytic Streptococci in Pharyngeal Specimens

Nonculture, Direct Detection Techniques for Group B β -Hemolytic Streptococci

Nonculture, Direct Detection Techniques for *Streptococcus pneumoniae*

Nonculture Techniques for the Detection of Enterococci in Blood Cultures

Colony Morphology and Catalase Testing

Recognition and Preliminary Characterization of Streptococci and the "*Streptococcus*-Like" Bacteria

Presumptive Identification of Streptococci and Enterococci

- Susceptibility to Bacitracin
- Susceptibility to Sulfamethoxazole-Trimethoprim (SXT)
- CAMP Test and Pigment Production
- Hydrolysis of Sodium Hippurate
- Bile Esculin Test
- Salt Tolerance Test (6.5% NaCl Broth)
- Leucine Aminopeptidase (LAP) Test
- Pyrrolidonyl Arylamidase (PYR) Test
- Commercial Presumptive Identification Tests

Serologic Identification of β -Hemolytic Streptococci

- Capillary Precipitin Test
- Coagglutination
- Latex Agglutination

Phenotypic Characteristics for Identification of Groupable Streptococci

Identification of *S. pneumoniae*: Susceptibility to Optochin, the Bile Solubility Test, and AccuProbe *Pneumococcus* Test

Serologic Identification of *Streptococcus pneumoniae*

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Identification of the Viridans Streptococci

- Mitis/Sanguinis Group
- Mutans Group

Salivarius Group
Anginosus Group
Bovis Group
Identification of *Streptococcus suis* and Other Streptococci Isolated From Animals
Detection of Vancomycin-Resistant Enterococci
Identification of *Enterococcus* Species
Identification of *Abiotriophia* and *Granulicatella* Species
Identification of *Aerococcus* and *Helcococcus* Species
Identification of *Leuconostoc*, *Pediococcus*, and *Tetragenococcus* Species
Identification of *Gemella* Species
Identification of *Vagococcus* Species
Identification of *Alloiococcus* Species, *Globicatella* Species, *Facklamia* Species, *Dolosigranulum* Species, *Ignavigranum* Species, and *Dolosicoccus* Species
Identification of *Lactococcus* Species
Commercial Systems for Identification of Streptococci, Enterococci, and Selected "Streptococcus-Like" Bacteria
Vitek 2
Phoenix (Becton-Dickinson Diagnostic Systems, Sparks, MD)

The streptococci, enterococci, and *Streptococcus*-like bacteria are gram-positive, catalase-negative bacteria that tend to grow in pairs and chains (Image Plate 13-1B). The detection of cytochrome enzymes with the catalase test distinguishes members of the various micrococcal and staphylococcal species groups (catalase-positive) from the streptococci, enterococci, and "*Streptococcus*-like" bacteria, which are catalase-negative. As with other microbial groups, the classification and taxonomy of the streptococci and *Streptococcus*-like bacteria have changed radically with the descriptions of several new genera of catalase-negative cocci. These changes have more than academic significance for clinical microbiologists in the 21st century because previously uncommon organisms belonging to these groups are being recovered from human infections with increasing regularity, and the pathogenic potentials of both old and newly described species are still not understood and are being actively investigated.

The application of molecular taxonomic methods and the description of several new genera of catalase-negative, gram-positive cocci have resulted in a complete reorganization of streptococcal taxonomy compared with that published in the 1984

edition of *Bergey's Manual of Systematic Bacteriology*. Molecular methods such as DNA–DNA hybridization, DNA–ribosomal RNA (rRNA) hybridization, and small-subunit (16S) rRNA sequencing were initially used to validate the division of the family *Streptococcaceae* into the genera *Streptococcus*, *Enterococcus*, and *Lactococcus*, and are now being applied to newly described viridans streptococci, enterococci, and other catalase-negative isolates to determine their relationships to these three genera.[71] In the new edition of *Bergey's Manual*, catalase-negative, gram-positive cocci of human origin are classified among six families in the proposed order "*Lactobacillales*" and one family in the order *Bacillales*, both of which are in the proposed class "*Bacilli*" in phylum *Firmicutes* (Box 13-1). The genus *Streptococcus*, which contains the most important human pathogens, can be operationally divided into seven groups, as shown in Box 13-2.

BOX 13-1

Current Classification of the Streptococci, Enterococci, and the "*Streptococcus-Like*" Bacteria

Order	Family	Genus
"Lactobacillales"	*Streptococcaceae*	*Streptococcus, Lactococcus*
	Enterococcaceae	*Atopobacter, Bavaricoccus, Catellicoccus, Enterococcus, Melissococcus, Pilibacter, Tetragenococcus, Vagococcus*
	"Aerococcaceae"	*Abiotrophia, Aerococcus, Dolosicoccus, Eremococcus, Facklamia, Globicatella, Ignavigranum*
	Leuconostocaceae	*Leuconostoc, Oenococcus, Weissella*
	Lactobacillaceae	*Pediococcus*
	"Carnobacteriaceae"	*Alloiococcus, Dolosigranulum, Granulicatella*
Bacillales	*Staphylococcaceae*	*Gemella, Macrococcus*

Organisms listed in this Table belong to: Domain Bacteria, Phylum *Firmicutes*, Class "Bacilli"

BOX 13-2

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Classification ofStreptococcusSpecies Groups Based on Small-Subunit rRNA Sequence Analysis[71]

Group Members	Habitat and Comments
Group I. Pyogenic Group	
S. pyogenes (Group A β-hemolytic *Streptococcus*)	This species is a well-known human pathogen (see text).
S. agalactiae (Group B β-hemolytic *Streptococcus*)	*S. agalactiae* is a well-known agent of neonatal disease, in particular (see text).
S. dysgalactiae subsp. *dysgalactiae* (Groups C and L β-hemolytic streptococci)	This streptococcal species causes infections in several animal species, including pigs, cattle, and humans (see text).
S. dysgalactiae subsp. *equisimilis* (Groups C and G β-hemolytic streptococci)	This streptococcal species causes infections in several animal species, including pigs, cattle, and humans (see text).
S. equi subsp. *equi* (Group C β-hemolytic *Streptococcus*)	This species causes disease primarily in animals, with occasional infections being seen in humans (see text).
S. equi subsp. *ruminatorum*[336]	This fairly recently described subspecies causes mastitis in sheep and goats (see text).
S. equi subsp. *zooepidemicus* (Group C β-hemolytic *Streptococcus*)	Many animal species (see text)
S. canis (Groups G, L, and M streptococci)[284]	This β-hemolytic species has been isolated from a variety of animal species, especially dogs (see text).
S. castoreus (reacts with Lancefield group A antisera)[616]	*S. castoreus* was isolated from wound, liver, and spleen specimens from a European beaver carcass.
S. didelphis (No Lancefield group)[877]	*S. didelphis* are β-hemolytic streptococci that were isolated from skin lesions, splenic, liver, and lung tissues of opossums that developed skin bruising followed by sudden death.
S. halichoeri (reacts with Lancefield group B antisera)[615]	This species was described in postmortem and clinical specimens from several gray seals in the United Kingdom.
S. hongkongensis[599]	This newly described species has been isolated from marine flatfish and from a thumb wound in a 44-year-old fishmonger following a puncture injury from a fish fin. This new species is most closely related to *S. iniae*, *S. pseudoporcinus*, *S. uberis*, and *S. paruberis*.
S. ictaluri (No Lancefield group)[919]	This species was isolated during an investigation of suspected bacterial disease in Channel Catfish (*Ictaluris punctatus*) broodstock. The isolates were associated with meningitis, myositis, osteolysis, and arthritis in the fish.
S. iniae (No Lancefield group designation)[830]	This emerging pathogen causes disease in many species of fish and is also pathogenic for humans (see text).
S. phocae (Groups C, F, or nongroupable streptococci)[942]	*S. phocae* was originally described as a cause of pneumonia in seals and has subsequently been isolated from respiratory tract infections in porpoises. *S. phocae* also causes septicemic infections in Atlantic salmon.[871]
S. porcinus (Groups E, P, U, and V streptococci)[216]	This β-hemolytic streptococcal species found in both swine and humans (the latter isolates have been re-named *S. pseudoporcinus*, see text).
S. pseudoporcinus (No Lancefield group)[67]	This novel *Streptococcus* species was described in 2006. It is found in the human female genitourinary tract and has been isolated from bloodstream, placental, and wound infections (see text).[923] Phenotypic methods do not adequately differentiate *S. pseudoporcinus* from *S. porcinus*, and sequencing of 16S rRNA or the *rpoB* gene is required for definitive identification.[371,923] Isolation of an organism from the female genitourinary tract that is phenotypically *S. porcinus* is likely to be *S. pseudoporcinus*. Some evidence suggests that presence of *S. pseudoporcinus* in the genitourinary tract of reproductive-aged women may contribute to the pathogenesis of premature rupture of fetal membranes and endocervical insufficiency.[820] Shewmaker and colleagues[923] have proposed that *S. pseudoporcinus* be divided into two subspecies. *S. pseudoporcinus* subsp. *hominis* grows in 6.5% NaCl, hydrolyzes hippurate, utilizes pyruvate, and is lactose-negative, while *S. pseudoporcinus* subsp. *lactis* does not grow in 6.5% NaCl, is negative

	for hippurate hydrolysis and acidification of pyruvate, and produces acid from lactose. Human isolates belong to the former subspecies, while dairy and animal isolates comprise the latter subspecies.
S. urinalis (No Lancefield group)[223]	This novel *streptococcus* was first isolated from a urine culture of a patient with cystitis and lower abdominal pain, and, subsequently, *S. urinalis* was isolated from a blood culture of a 60-year-old man with a history of urethral stricture.[817] Even though *S. urinalis* is an α-hemolytic *streptococcus*, it is considered as a member of the pyogenic streptococcal group, with *S. pyogenes* and *S. canis* as its nearest phylogenetic relatives.
Group II. Mitis/Sanguinis Group	
S. australis[1131]	*S. australis* was first were isolated from the saliva of children attending a dental hospital in Sydney, Australia. Initially thought to be *S. mitis,* additional biochemical and hybridization studies demonstrated that these isolates were a separate species. In 2011, this organism was the cause of bacterial meningitis in a 77-year-old man.[449]
S. cristatus[422]	*S. cristatus* is oral *streptococcus* that is unusual in possessing tufts of short fibrils located in a lateral position on the cell surface that help the organism to co-aggregate with other organisms in dental plaque. *S. cristatus* is a rare cause of endocarditis.[694]
S. danieliae[202]	This species was isolated from the caecum of a mouse. The organism is genotypically related to *S. alactolyticus*, *S. gordonii*, *S. sanguinis*, and *S. intermedius*. It does not react with any Lancefield antisera and it is β-hemolytic.
S. gordonii[556]	This viridans streptococcal species is found in the oral cavity of humans and has been isolated from bloodstream infections in cancer and endocarditis patients, joint fluid in two cases of septic arthritis, and peritoneal fluid from a patient on continuous ambulatory peritoneal dialysis.[183,421,1158]
S. infantis[545]	*S. infantis* is a member of the Mitis Group and is found on the teeth and in the nasopharynx of humans.
S. lactarius[681]	This recently described species was isolated from breast milk of healthy women.
S. massiliensis[383]	This species is based on a single isolate from a blood culture. On 16S rRNA gene sequencing, this isolate clustered with the Mutans Group streptococci, but *rpoB* and *sodA* gene sequence comparisons placed the organism in the Mitis/Sanguinis Group of streptococci.
S. mitis[556]	*S. mitis* is an α-hemolytic *Streptococcus* that is found in the oral cavity, the gastrointestinal tract, the female genital tract, and on skin (Image Plate 13-4F, 13-4G and 13-4H). *S. mitis* is a cause of infective endocarditis and meningitis, particularly in patients with risk factors that include prior valvular damage, poor dentition/oral hygiene, malignancies, stem cell transplantation, other underlying disease (e.g., hemolytic anemia, alcoholism, hypertension, diabetes), immunosuppressive therapies (e.g., corticosteroids, chemotherapy agents), head and neck infections (e.g., maxillary sinusitis) and neurosurgical procedures.[290,585,659,691] Complications of endocarditis and bacteremia include embolic phenomena, endophthalmitis, and osteomyelitis.[290,771] *S. mitis* is also a cause of viridans streptococcal shock/respiratory distress that occurs in profoundly immunosuppressed patients with malignancies and hematopoietic stem cell transplant recipients 1–28 days posttransplant.[179,666] *S. mitis* is one of the more frequently isolated species from blood cultures and isolates with reduced susceptibility or frank resistance to β-lactam agents are increasing in prevalence.[507,577,691] Matsui and colleagues[691] reported a case of *S. mitis* infective endocarditis in which the isolate was resistant to all penicillins, cephalosporins (all generations), all carbapenems, and erythromycin, and susceptible only to vancomycin, levofloxacin, and clindamycin. Levofloxacin resistance was documented in an otherwise pan-susceptible *S. mitis* strain recovered from blood and vitreous cultures in a patient with endocarditis and endogenous endophthalmitis.[290] Linezolid has been used for treatment of *S. mitis* endocarditis with success.[759] Fulminant *S. mitis* peritonitis in a CAPD recipient has also been reported.[720] Some isolates of *S. mitis* may be optochin-susceptible, but they are not soluble in bile or desoxycholate.[259]
S. oligofermentans[1033]	*S. oligofermentans* was first described in 2003 when it was isolated from dental plaque and saliva of nasopharyngeal carcinoma patients. In 2009, *S. oligofermentans* was documented as the cause of a left forearm abscess complicated by endocarditis in a 43-year-old woman.[693]

S. oralis[556]	*S. oralis* has been isolated from cases of native- and prosthetic valve endocarditis.[860,1057] *S. oralis* is phenotypically similar to *S. mitis*, and can be differentiated by certain enzymatic tests (e.g., β-GLU and NAGA).
S. parasanguinis[1125]	This species has been isolated from respiratory tract, blood, and urine specimens from humans and has also been associated with asymptomatic mastitis in sheep.[337] Endocarditis due to penicillin-resistant *S. parasanguinis* was reported in 2008 and peritoneal dialysis-associated peritonitis due to *S. parasanguinis* was reported in 2011.[361,882]
S. peroris[545]	*S. peroris* is a newer member of the Mitis Group and is found on the teeth and in the nasopharynx of humans.
S. pneumoniae	See text
S. pseudopneumoniae	See text
S. sanguinis[556]	This species is found in the human oral cavity and has been isolated from patients with endocarditis and as a cause of mycotic aneurysm.[61,175,539] Complications of hematogenous dissemination during endocardial and bloodstream infections include brain abscess and septic arthritis.[539,800] This organism also caused purulent meningitis in a patient with cirrhosis who underwent endoscopic ligation of bleeding esophageal varices.[653] Molecular methods (e.g., PCR-generated, species-specific DNA probes) have been designed to facilitate study of the interaction of *S. sanguinis* and other oral streptococci with the cariogenic species, *S. mutans*.[639] Resistance to β-lactam antibiotics has been noted for both *S. mitis* and *S. sanguinis*.[61]
S. sinensis[1142]	This species is part of the bacterial flora of the human oral cavity and was first isolated from blood cultures of patients with endocarditis.[326,1060,1142–1144] This organism may carry the Lancefield group F antigen.[1143]
S. tigurinus[1169]	This streptococcal species was described in 2012 and has been isolated from blood cultures of patients with endocarditis, meningitis, and spondylodiscitis.[1169] This species is most closely related to *S. mitis*, *S. pneumoniae*, *S. pseudopneumoniae*, *S. oralis*, and *S. infantis*.[1168]
S. troglodytidis[1173]	This species was isolated from a foot abscess of a chimpanzee (*Pan troglodytes*).
Group III. Mutans Group	
S. cricetus	This species is found in the oral cavities of hamsters, rats, and, rarely, humans.
S. dentaprl[992]	This species, described in 2010, was isolated from the oral cavities of wild boars.
S. dentirousetti[994]	This species was isolated from the oral microflora of bats.
S. devriesei	This species is found in the oral cavity of horses and cows.
S. downei	This species is found in the oral cavity of monkeys.
S. ferus	This species is found in the oral cavity of rats.
S. macacae	This species is found in the oral cavity of monkeys.
S. mutans[1124]	This species is the principal cause of dental caries in humans. Isolates of *S. mutans* produce glucosyltransferase enzymes that synthesize the polysaccharides forming the matrix of dental plaque from dietary sucrose (see text).
S. orisratti[1176]	This species is found in the oral cavities of Sprague-Dawley rats. Phylogenetic studies of the 35 serotypes of *S. suis* revealed that types 32 and 34 were distant from other *S. suis* serotypes and, when additional sequencing studies were completed, these two "*S. suis*" serotypes were actually *S. orisratti*.[455]
S. orisuis[993]	This *Streptococcus* species was recovered from the oral cavities of swine.
S. ratti	This species is found in the oral cavity of rats (and occasionally humans).
S. sobrinus	This species is constitutes a portion of the cariogenic streptococci found in the human oral cavity.
S. ursoris[926]	This streptococcal species was cultured from the oral cavities of bears.
Group IV. Salivarius Group	
S. hyointestinalis[285]	This species was isolated from the intestines of pigs and characterized in 1988.
S. infantarius[901]	*S. infantarius* has been isolated from humans and food products. *S. infantarius* strains are also included in the Bovis Group because some strains are bile esculin–positive.

S. salivarius[1124]	This species is found in the oral cavity and saliva of humans. "*S. thermophilus*" is related *to S. salivarius*, and may be a subspecies of *S. salivarius* or a distinct species.
S. thermophilus	This species is found in dairy products.
S. trolodytae[781]	This species was isolated from the oral cavity of a chimpanzee. *S. mutans* was shown to be the most closely related species.
S. vestibularis[1128]	This species was first isolated from the vestibular mucosa of the oral cavity of humans. In 2010, this species was isolated from a 65-year-old woman as a cause of native aortic valve endocarditis and spondylodiscitis.[1050]
Group V. Anginosus Group	
S. anginosus	Humans (see text)
S. constellatus subspecies	Humans (see text)
S. intermedius	Humans (see text)
Group VI. Bovis Group	
S. bovis/S. equinus	These isolates are found in the genitourinary and gastrointestinal tracts of cattle and horses.
S. caballi[714]	This species and *S. henryii* were isolated from horses with experimentally-induced laminitis, a serious disease of the equine foot that is associated with dietary intact of excess carbohydrate.
S. gallolyticus subsp. *gallolyticus* (*S. bovis* I)	See text
S. gallolyticus subsp. *macedonicus*[675,1048] (formerly "*S. macedonicus*," "*S. waius*")	*S. gallolyticus* subsp. *macedonicus* is found in cheeses and other dairy products. *S. macedonicus* was originally called *S. waius* and then *S. macedonicus* before being incorporated as a subspecies of *S. gallolyticus* in the *Bovis* group (see text).
S. gallolyticus subsp. *pasteurianus* (*S. bovis* II.2)	See text
S. henryi[714]	*S. henryi* and *S. caballi* were isolated from horses with experimentally-induced laminitis, a serious disease of the equine foot that is associated with dietary intact of excess carbohydrate. This species reacts with Lancefield group D antisera.[714]
S. infantarius subsp. c*oli*	Swine, dogs, chickens
S. infantarius subsp. *infantarius* (*S. bovis* II.1)	Humans, cattle
Group VII. Miscellaneous Streptococci	
S. acidominimus	*S. acidominimus* strains have been poorly characterized since the original species description in 1922. Reexamination of *S. acidominimus* strains recovered from humans established the true identity of these isolates as *Facklamia sourekii*.[220] This organism has been isolated from cattle, poultry, and humans. Infections with *S. acidominimus* in humans have included pneumonia with empyema, pericarditis, meningitis, endocarditis, otitis media, and brain abscess.[48,115,236,342,991]
S. entericus[1088]	This α-hemolytic species was recovered from intestinal specimens obtained from a cow with catarrhal enteritis, a relentless wasting diarrheal syndrome in calves.
S. gallinaceus[222]	This species was first isolated from blood cultures of a broiler chicken with sepsis and has subsequently been documented as a major cause of sepsis and endocarditis in chicken flocks in Denmark.[164,222] In 2006, *S. gallinaceus* was isolated from a blood culture of an abattoir worker with a febrile illness in New Zealand.[50]
S. hyointestinalis	This species was isolated from the intestines of swine.
S. hyovaginalis[286]	This species was recovered from vaginal specimens of swine.
S. marimammalium[614]	This species was recovered from respiratory specimens obtained from gray seals and common seals.
S. merionis[1010]	This species was isolated from parasite vesicles inoculated into the peritoneal cavities of Mongolian jirds to study the larval stage of *Echinococcus multilocularis*.
S. minor[1072]	This α-hemolytic species was isolated from tonsils, anal swabs, and feces of dogs and from tonsillar tissues of a cat and a calf.

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S. ovis[224]	This species is based on seven isolates obtained from chest wall, navel, abdominal, lung abscesses and joint fluid specimens from sheep.
S. pluranimalium[287]	This species is unusual in that isolates have been recovered from a variety of animals, including cattle (genital tract and mastitis isolates, lung isolates), lungs and crops of canaries, and tonsillar tissues of cattle, a goat, and a cat. This species was the cause of an outbreak of endocarditis and septicemia in hens.[438]
S. plurextorum[1087]	This species was isolated from lung and kidney tissues of swine that had died of pneumonia and septicemia.
S. porci[1091]	This organism was isolated from pericardial tissues and bronchial lymph node specimens from swine with pericarditis and pneumonia, respectively. This species reacts with Lancefield group B antisera.
S. porcorum[1092]	This species, described in 2011, was isolated from wild and domestic pigs. It is most closely related to *S. suis*.
S. rupicaprae[1090]	This streptococcal species was isolated from clinical samples from a Pyrenean chamois.
S. suis (Lancefield groups R, S, and T)	This novel species is a cause of disease in piglets, cattle, and is an emerging agent in human disease (see text).
S. thoraltensis[286]	This species was isolated from the intestinal contents of a pig.
S. uberis/S. paruberis[761]	*S. uberis* and *S. parauberis* are causes of bovine mastitis and have not been isolated from humans. These two species are indistinguishable from one another using phenotypic criteria, and species-specific molecular probes for these two organisms have been developed. Human isolates that were phenotypically identified as *S. uberis* have been reidentified as *Globicatella sanguinis*.[761] *S. parauberis* is a cause of streptococcosis in olive flounder. Some isolates carry the Lancefield group E antigen, while others are nongroupable.

General Characteristics of the Streptococci

Streptococci are facultative anaerobes, although some strains grow better under anaerobic conditions. Although most species will grow in air, the growth of most species is stimulated by increased CO_2. Medically important streptococci, enterococci, and aerococci are **homofermentative**, meaning that the sole product of glucose fermentation is lactic acid with no gas formation. Streptococci are also catalase-negative and oxidase-negative, a property that, together with the Gram's stain, differentiates streptococci from *Neisseria* species. Members of the genus *Streptococcus* characteristically grow in chains (or chains of diplococci) when grown in broth media (Image Plate 13-1B and13-1C). This characteristic is shared with the enterococci, the lactococci, and some of the newly recognized genera (i.e., *Leuconostoc* species, *Vagococcus* species, and *Globicatella* species). Other *Streptococcus*-like bacteria (i.e., aerococci, *Alloiococcus* species, *Gemella* species, *Pediococcus* species, *Tetragenococcus* species, and *Helcococcus* species) grow as pairs or tetrads in broth. Assessment of the cellular arrangement is best made by performing a Gram's stain from a culture of the organism growing in thioglycolate

broth. When thioglycolate broth is used, the smear preparation should be fixed in methanol after air-drying, rather than heat-fixed, to prevent the bacteria from "washing off" the slide during the staining process.

The cell wall composition of the streptococci is similar to that of other gram-positive bacteria, being composed primarily of peptidoglycan, in which are embedded a variety of carbohydrates, teichoic acids, lipoproteins, and surface protein antigens (see Chapter 5). Some streptococcal species may be serologically classified on the basis of cell surface carbohydrate antigens. The pioneering work of Rebecca Lancefield established the Lancefield-grouping system for the β-hemolytic streptococci (Image Plate 13-3A). The antigens detected in the Lancefield-grouping system are either cell wall polysaccharides (as in the human group A, B, C, F, and G streptococci) or are cell wall lipoteichoic acids (group D streptococci and *Enterococcus* species). Originally, these cell wall grouping antigens were extracted with dilute hydrochloric or nitrous acid, formamide, or by autoclaving, and groups were determined by capillary precipitin reactions. Commercially available streptococcal grouping kits use enzymatic extraction techniques and either coagglutination or latex particle agglutination for antigen detection (Image Plate 13-1G). Other streptococci, particularly members of the viridans streptococcal groups, do not possess any of the recognized Lancefield cell wall grouping antigens, although some strains may possess similar antigens that will cross-react with β-hemolytic streptococcal group-specific antisera. Well-studied viridans streptococci, such as the cariogenic organism *S. mutans*, have been divided into serotypes based on their own cell wall carbohydrate antigens. The various serotypes of *S. mutans* have subsequently been elevated to species status and now comprise the "*Mutans* group" of oral streptococci.

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Group A β-Hemolytic Streptococci (*Streptococcus pyogenes*)

The pathogenic streptococci have several characteristics that contribute to their virulence. The virulence mechanisms of the group A, β-hemolytic streptococci (*S. pyogenes*), in particular, have been studied most extensively. This organism remains an extremely important human pathogen, and the only known reservoirs of group A streptococci in nature are the skin and mucous membranes of humans. The World Health Organization estimates that over 500,000 people die each year from severe group A streptococcal infections, particularly invasive disease and the sequelae of acute rheumatic fever and consequent rheumatic heart disease.[135,276] Twenty-five to 35 million cases of group A streptococcal pharyngitis occur each year in the United

States.[739] In the United States, group A streptococcal infections occur at an annual rate of about 3.5 cases per 100,000 population, resulting in over 9,600 cases and 1,100 to 1,300 fatalities per year.[775] In addition to acute infections, the group A *Streptococcus* is also associated with two nonsupperative sequelae—acute rheumatic fever and acute poststreptococcal glomerulonephritis—that continue to occur particularly in developing countries. More than 95% of the estimated 294,000 fatal cases of rheumatic heart disease worldwide occur in developing countries, which also bear the overwhelming number of other invasive group A streptococcal diseases.[135,964]

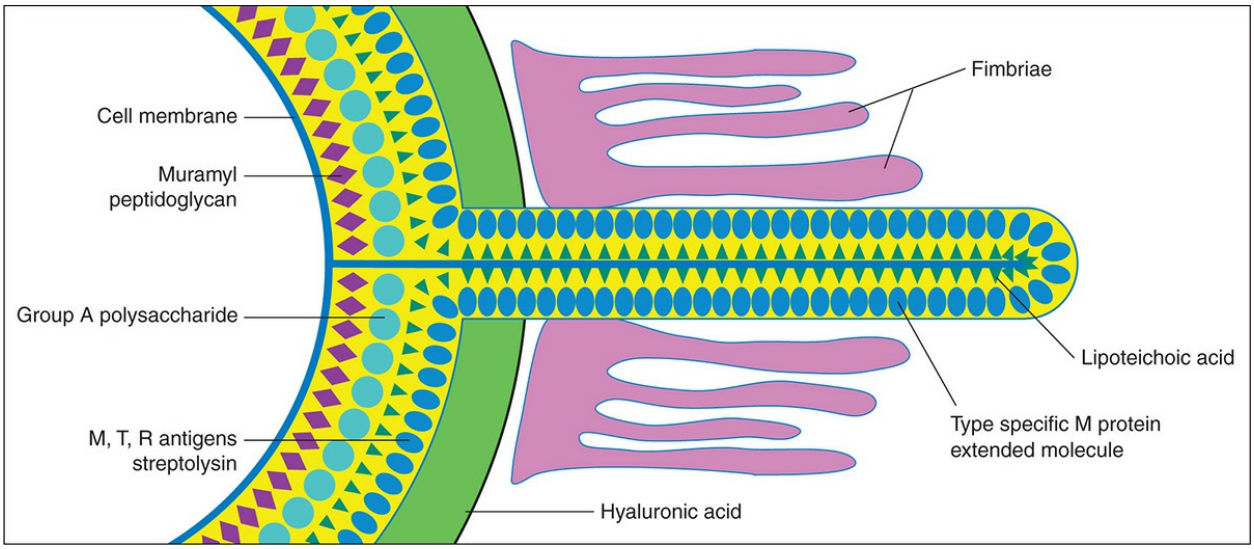
Virulence Factors

The cell wall of group A β-hemolytic streptococci consists of a thick peptidoglycan along with integral **lipoteichoic acids (LTAs**) and other surface associated molecules, as described in Chapter 5. LTAs are believed to play a central role in promoting initial adherence of group A streptococci to pharyngeal epithelial cells, to other cell types, and to host proteins such as fibronectin.[247] In addition to LTAs, several other group A streptococcal adhesins have been described, including several fibronectin-binding proteins (e.g., protein F1 [SfbI, streptococcal fibronectin-binding protein], protein F2 [SbfII], FPB54, and PFBP).[247,424,504,866] These surface-binding proteins promote adherence to both pharyngeal and cutaneous cell types. M proteins play a role in adherence to keratinocytes in skin via interaction with the keratinocyte membrane cofactor CD46.[780] The major group A cell wall antigen is a complex polysaccharide consisting of L-rhamnose and *N*-acetyl-D-glucosamine in a 2:1 ratio.[90] The antigen is covalently attached to the peptidoglycan. The role of the cell wall grouping antigen as a virulence factor is not known, although the peptidoglycan material itself has biologic activity, including the induction of fever, dermal and cardiac necrosis in animals, lysis of erythrocytes and platelets, and enhancement of nonspecific resistance.

Some group A strains possess a **capsule** composed of hyaluronic acid, which is a high molecular weight linear polymer composed of β(1-4)-linked disaccharide repeating units of D-glucuronic acid and (1-3)-β-D- *N*- acetylglucosamine.[970,1119] This material is the product of enzymes encoded by a three-gene cluster consisting of *hasA*, *hasB*, and *hasC*. These three genes encode a hyaluronic acid synthase, a UDP-glucose dehydrogenase, and a glucose pyrophosphorylase, respectively.[19] These genes are highly conserved among group A streptococcal strains, and variations in the degree of capsular gene expression likely reflect differences in regulation of gene transcription. Strains that maximally express these genes appear mucoid when grown on sheep blood agar (SBA).[1121] Two gene products, **CrsS** and **CrsR**, appear to function as a two-component regulatory system that is able to increase or decrease the degree of encapsulation by up- or downregulation of *has* gene expression.[634] Chemically, this

capsular hyaluronate material is indistinguishable from the ground substance of connective tissue, which may explain the lack of immunogenicity of this substance in the infected host. In vitro, capsule production is maximal during logarithmic growth, and the organisms shed their capsules as they enter the stationary phase of growth; this loss is probably due to the elaboration of **hyaluronidase** during the latter stages of the logarithmic growth phase. The hyaluronic acid capsule functions to help the organisms resist complement-dependent killing by phagocytic cells. In animal models, the hyaluronic acid capsule has been shown to contribute to the capacity of group A streptococci to produce invasive soft tissue infections.[38] The capsule also influences the ability of group A streptococci to adhere to epithelial cells by modulating the interaction of M protein and other surface molecules and by serving as a ligand for binding to the CD44 receptor on epithelial cell surfaces.[907,1119]

The major virulence factor of the group A *Streptococcus* is a cell surface antigen designated **M protein.**[90,709,777] M proteins are acid- and heat-stable, trypsin-labile, fibrillar proteins associated with the outer surface of the cell wall. M proteins are composed of two polypeptide chains complexed together in an α-helical coiled-coil configuration.[343] The M protein is anchored in the cell membrane, extends through the peptidoglycan layer, and projects from the surface of the bacterial cell (Fig. 13-1). The amino acid sequence and structure of the carboxy-terminal end of the molecule is located within the cell membrane and cell wall of the organism and is highly conserved among group A strains. The *N*-terminus extends beyond the cell surface and terminates with a sequence of about 11 amino acid residues. This terminal sequence varies among clinical isolates and constitutes the basis for the Lancefield serologic classification of group A streptococci. Strains that are rich in M protein are resistant to phagocytosis and intracellular killing by polymorphonuclear cells, thereby allowing the organisms to persist in infected tissues; cells lacking demonstrable M protein are readily phagocytosed and killed.[90] M protein apparently exerts its antiphagocytic effects by interfering with opsonization of the bacterial cells via inhibition of both the classical and the alternate complement pathways. M proteins are also able to form complexes with fibrinogen that consequently bind to β2 integrins of neutrophils. Binding triggers release of inflammatory mediators that induce vascular leakage, a pathologic component of streptococcal toxic shock.[447] Some M proteins can act as superantigens, causing T-cell proliferation and release of cytokines, and M proteins from classically "rheumatogenic" M types elicit the formation of antibodies that cross-react with several mammalian host cell proteins, including myosin, laminen, and



■ **FIGURE 13-1** The major known antigenic determinants on the surface of virulent, encapsulated group A streptococci.

keratin.[709] Eventually, antibodies against the more prevalent M types emerge, and with development of herd immunity, these types disappear and new M types appear and expand through increased transmission. Serologic M typing is usually performed on hot acid extracts of group A streptococci using capillary precipitin or agarose gel immunodiffusion techniques. Only a single M-type antigen is expressed by group A strains, and 93 different M serotypes have been identified using these methods.[318] Cloning of the M protein gene, called the *emm* gene, has resulted in the expansion and standardization of *emm* typing to replace the conventional serologic method. Consequently, there are now over 200 known *emm* types. The *emm* typing system is performed by sequence analysis of the NH_2-terminal nucleotide residues and has resulted in the identification of over 124 recognized *emm* genotypes.[323] The definition of a new *emm* type sequence is based on identification of more than 160 nucleotide bases at the 5′ terminus of the hypervariable region. Different *emm* types will have less than 80% sequence homology with other *emm* types.

Since its introduction, *emm* typing has been used in several large population-based studies in the United States, Canada, and other countries. These studies have shown differences in *emm* type distribution worldwide. Similarities in *emm* types were noted in higher-income countries (i.e., Europe, North America, urban Australia, New Zealand, Japan), with the greatest diversity in *emm* type occurring in Africa, indigenous Australian populations, and the Pacific Island region.[963,964] Amino acid sequence analysis of M proteins of many types has also identified portions of the M protein molecule that are common among several M protein types; that is, this sequence of amino acids is highly conserved. Sera from individuals living in different

geographic areas with high rates of streptococcal infection reacted with this conserved M peptide in an enzyme-linked immunosorbent assay (ELISA).[963] Furthermore, antibodies directed against this peptide were able to opsonize streptococci belonging to a variety of M types. This highly conserved part of the M protein molecule is being investigated further as a possible candidate vaccine antigen against group A streptococcal infection and rheumatic fever.[963,964] Use of M proteins as vaccine candidates is challenged by the realization that, even within established *emm* types, various subtypes also exist. For example, analysis of over 300 *emm3* strains from three separate Canadian group A streptococcal epidemics showed that each epidemic was caused by emergence of distinct *emm3* subtypes within previously immune populations.[74]

In addition to M protein, several other M-related cell surface proteins have been identified on group A streptococci, and the genes encoding these molecules (e.g., *enn*, *mrp*, *arp*, *fcrA*, *protH*) have been grouped together as members of the "*emm* gene superfamily."[517] In addition to M protein, some streptococci have other "M-like" proteins (e.g., the Spa protein of M-type 18 group A streptococci) that have been shown to contribute to and augment group A streptococcal virulence.[704] These molecules apparently work in concert with M proteins to help the organisms resist phagocytosis. M-protein-like molecules are also able to bind to several host proteins such as plasminogen and fibrinogen, and, via this interaction, also exert anti-opsonic effects. The sequences of the recognized *emm* types may be found online at http://www.cdc.gov/nci-dod/biotech/infotech_hp.html.

Opacity factor (OF) is another M-protein-associated cell surface antigen of group A streptococci that is a virulence factor. OF is an α-lipoproteinase that is able to opacify media containing mammalian serum. Antibodies directed against OF are specific in inhibiting the opacity reaction of the M-type producing it; so OF typing can be used as a supplementary or complementary typing reaction. OF is produced by strains that belong to 29 different M types and can be detected in those M types even if M-type-specific reactivity is lost or undetectable (i.e., the presence of OF is associated only with specific M types). Therefore, OF-positive and OF-negative reactions are consistently associated with specific M types. OF is primarily associated with strains of group A streptococci isolated from skin infections.[79]

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Group A streptococci produce two hemolysins: streptolysin O and streptolysin S. **Streptolysin O** (SLO) is oxygen-labile, antigenic, inhibited by cholesterol, and toxic to a variety of cell types, including leukocytes, monocytes, and cultured cells. Because of its oxygen lability, streptolysin O is primarily responsible for the β-hemolysis seen

around subsurface colonies of group A streptococci in pour plates or in the stabbed regions of surface-inoculated SBA plates. Streptolysin O is also produced by some group C and group G streptococci. SLO appears to exist in two active forms, with molecular weights of between 50,000 and 70,000 Da; cleavage of these molecules during secretion results in the fully active form of SLO having a molecular weight of about 57,000 Da. SLO causes the formation of pores in the membrane of susceptible cells by initial binding of SLO monomers to cholesterol in the cell membrane. This binding results in a conformational change in the molecule that causes the co-aggregation of additional SLO monomers on the membrane. These aggregates initially form arc-shaped structures on the membrane that eventually become fully formed pores, resulting in osmotic lysis of the affected cell. SLO also induces degranulation and lysis of PMNs, inhibits phagocytosis by macrophages, and impairs the response of lymphocytes to mitogens. SLO may also stimulate the production of cytokines. Measurement of antibodies against streptolysin O (antistreptolysin O [ASO] titers) in serum is useful for retrospective diagnosis of recent pharyngeal streptococcal infections. The ASO response following skin infections is poor, presumably because of inactivation of the antigen by cholesterol present in skin. In such cases, anti-DNase B titers are more reliable (see later discussion).

Streptolysin S (SLS) is oxygen-stable, nonantigenic, and, like SLO, is toxic to a variety of cell types. SLS exists in intracellular, cell surface bound, and intracellular forms and is usually associated with some type of carrier molecule, such as serum albumin, RNA, or α-lipoprotein. SLS, a small molecule having a molecular weight of about 1,800 Da, is maximally produced during the late logarithmic and early stationary phases of growth, and requires iron for maximal production. SLS is believed to interact with membrane phospholipids in exerting its toxic effects. Erythrocytes exposed to SLS undergo swelling, followed by lysis due to the disruption of the osmotic barrier and leakage of ions from the cell. Unlike SLO, no slits or pores are observed on affected erythrocyte cell membranes by electron microscopy. SLS is active in both surface and subsurface hemolysis when the organisms are grown on SBA. The hemolytic activity of SLS is inhibited by serum lipoproteins and other simple phospholipids. Like SLO, SLS is able to damage the membranes of polymorphonuclear cells, platelets, and internal subcellular organelles.

Group A streptococci also produce several extracellular products; many of these play real or theoretical roles in virulence of group A streptococci. **Streptococcal pyrogenic exotoxins (SPEs)** (particularly SPE A and SPE B) are responsible for the rash of scarlet fever and are also the principal virulence determinants in the pathogenesis of the streptococcal toxic shock-like syndrome. Three immunologically distinct SPEs, designated SPE types A, B, and C, have been well described, and the genes that encode them have been identified and characterized. The genes for SPEs A and C (*speA* and *speC*) are encoded on a streptococcal lysogenic bacteriophage, whereas the gene for the type B exotoxin (*speB*) is chromosomal. The *speB* gene is

found in all group A streptococci, whereas the other two genes may or may not be present. SPE B, the product of the *speB* gene, is actually a **cysteine protease** enzyme that is able to cleave human immunoglobulin, fibronectin, vitronectin, and other host cell proteins, resulting in the formation of small, biologically active peptides, including interleukin-1, histamine, and kinins.[1109] Expression of cysteine protease SPE B and other virulence determinants is regulated by the ***CovR/S* gene system**, which mediates the group A streptococcal stress response and regulates the repression and derepression of major virulence genes.[87,200] Following colonization with group A streptococci, mutations in *CovR/S* genes cause upregulation of several virulence factors, including capsule production and the secretion of several virulence factors, including the newly described serine protease SpyCEP.[1054] The latter virulence factor mediates the cleavage of various cytokines (e.g., interleukin 8), enabling dissemination of the organisms within soft tissues and the respiratory tract in vivo.[584] Pyrogenic exotoxins such as SPE A and SPE C not only induce fever, as their name implies, but they also function as superantigens. **Superantigens** are molecules that are able to induce proliferation of host T-lymphocytes, regardless of their antigenic specificity, by binding to class II major histocompatibility complex (MHC) molecules.[357] Activation of T cells by superantigens results in the massive release of cytokines by human monocytes and lymphocytes (e.g., tumor necrosis factor-α [TNFα], interleukin 1β, interleukin-2, interferon-γ).[357,414,415] These cytokines activate the complement, coagulation, and fibrinolytic cascades and cause capillary leakage with resulting hypotension and shock, the most severe manifestations of streptococcal toxic shock syndrome. Superantigens also potentiate the host response to minute amounts of gram-negative endotoxin by a factor greater than 100,000-fold, and have direct toxic effects on endothelial cells lining the capillaries. SPEs have molecular weights of about 25 to 28 kDa and contain T-cell receptor binding and class II MHC binding sites, plus a zinc binding site that functions in mediating the binding of the class II molecules to the exotoxin. With the complete sequencing of several group A streptococcal genomes, at least 11 streptococcal superantigens have been described and include SPE A, SPE C, SPE G, SPE H, SPE I, SPE J, SPE K, SPE L, SPE M, SSA (streptococcal superantigen), and the polymorphic SMEZ.[357] Production of superantigens varies among streptococcal strains belonging to different M or *emm* types In addition to group A streptococci, various streptococcal superantigen genes (e.g., *ssa*, *speM*, *smeZ*) are also found in some group C and G β-hemolytic streptococci (*S. dysgalactiae* subsp. *equisimilis* and *S. canis*).[489]

Group A β-hemolytic streptococci also produce several other products that contribute to virulence. As mentioned, SPE B is actually a **C5a peptidase** that is bound to the cell surface. This molecule contributes to disease because of its peptidase activity rather than as a superantigen. C5a peptidase inactivates C5a, the chemotactic

complement component, resulting in limiting recruitment and chemotaxis of polymorphonuclear leukocytes. This peptidase also cleaves immunoglobulins, fibronectin, vitronectin, and other proteins with the generation of biologically active peptides (e.g.,

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histamine, kinins).[1109] These organisms also produce four immunologically and electrophoretically distinct **deoxyribonucleases**, designated DNase A, B, C, and D. Antibodies against DNase B (anti-DNase B) are helpful, along with ASO titers, for serologic documentation of prior group A streptococcal pharyngeal or skin infections. **Hyaluronidase** produced by group A streptococci depolymerizes the ground substance of connective tissue, resulting in contiguous spread of the organism. **Streptokinases** produced by group A streptococci hydrolyze fibrin clots and may function in virulence by preventing the formation of fibrin barriers at the periphery of spreading streptococcal lesions. The contribution of these enzymes and toxins to infection is uncertain. Many of these factors are produced by other β-hemolytic streptococci as well.

Clinical Spectrum of Group A Streptococcal Disease

Humans are the natural reservoir for group A, β-hemolytic streptococci, and the organism is transmitted from person to person by the respiratory route. The most common infection caused by group A streptococci is **streptococcal pharyngitis**.[929,1120]Group A streptococci are responsible for 5% to 15% of pharyngitis in adults and 20% to 30% of cases in children. Most cases of pharyngitis are seen in school-aged children (5 to 15 years old) during the winter or spring. Following an initial incubation period of 2 to 4 days, onset is generally abrupt, with fever, sore throat, headache, malaise, and abdominal pain. The posterior pharynx is usually inflamed and swollen, and a grayish white exudate may be present on the tonsils. The anterior cervical lymph nodes are usually tender and swollen. The presence of rhinorrhea, hoarseness, cough, or diarrhea speaks against group A streptococcal infection, instead suggesting a viral or mycoplasmal etiology. Infection with strains that elaborate pyrogenic exotoxins A, B, or C may also cause a scarlatiniform rash (i.e., classic scarlet fever). Complications of group A streptococcal pharyngitis may be suppurative (i.e., peritonsillar abscess, retropharyngeal abscess, suppurative cervical adenitis, otitis media, sinusitis, mastoiditis, bacteremia), nonsuppurative (i.e., acute and chronic rheumatic fever, glomerulonephritis), or toxin-mediated (streptococcal toxic shock-like syndrome). In the absence of complications, streptococcal pharyngitis is self-limited. Fever usually resolves in 3 to 5 days and throat pain resolves in 7 to 10 days without therapy. However, treatment (ideally, culture followed by antimicrobial

therapy) is usually sought. About 10% to 15% of individuals with streptococcal pharyngitis may become asymptomatic carriers of the organisms following treatment.[90] Treatment guidelines are published by the FDA, the American College of Physicians, the Centers for Disease Control, the American Heart Association, and the Infectious Diseases Society of America.[88,374,929,1120] Current recommendations for treatment of streptococcal pharyngitis include oral penicillin V (children < 27 kg, 250 mg two or three times/day; adolescents and adults ≥ 27 kg, 500 mg two or three times/day for 10 days), or intramuscular benzathine penicillin G (children < 27 kg, 600,000 units IM; weight ≥ 27 kg, 1.2 million units IM).[90,374] Amoxicillin (50 mg/kg once daily to a maximum of 1 g) may also be used in place of either penicillin V or benzathine penicillin. Erythromycin was the standard alternative agent for penicillin-allergic patients, but, largely due to the gastrointestinal side effects experienced by some patients with erythromycin, alternative agents (e.g., clindamycin, azithromycin, cephalexin, and cefadroxil) have been approved. Clindamycin (7 mg/kg/dose PO, twice a day to a maximum of 300 mg/dose for 10 days) is an approved alternate drug, and the FDA has also approved a 5-day azithromycin course (12 mg/kg PO once per day to maximal dose of 500 mg for 5 days).[1120] Group A β-hemolytic streptococci remain highly susceptible to penicillin G.

The "nonsuppurative" complications of group A streptococcal infections include acute rheumatic fever and glomerulonephritis. **Acute rheumatic fever (ARF)** is associated with prior group A streptococcal pharyngitis, while glomerulonephritis usually occurs after prior pharyngeal or skin infections. ARF is a multisystem disease characterized by major manifestations of carditis, polyarthritis, subcutaneous nodules, erythema marginatum, and chorea.[86,87,374,1148] Onset usually occurs 2 to 5 weeks after streptococcal pharyngitis and is not usually initiated by cutaneous group A streptococcal infections. Cardiac pathology involves the endocardium, myocardium, pericardium and, oftentimes, the mitral valves. Clinically, the patient develops characteristic heart murmurs, cardiac enlargement, congestive heart failure, or intractable cardiac arrest and death.[668] ARF-associated arthritis is migratory, involves multiple joints, and usually resolves spontaneously. Painless, firm, subcutaneous nodules appear simultaneously with carditis and occur around the bony areas of the hands and feet. Erythema marginatum appears as inflamed eruptions with raised, serpiginous borders and central areas of clearing that usually appear on the trunk, arms, and legs. Chorea is a neurologic condition characterized by muscle spasms, incoordination, and weakness that develop simultaneously with or several months after the appearance of ARF. Attacks of ARF generally last 3 to 6 months. Because of the protean clinical manifestations of ARF, the differential diagnosis is diverse and includes rheumatoid arthritis, systemic lupus erythematosus, sickle cell disease, rubella, septic arthritis, disseminated gonococcal infection, Lyme disease, bacterial endocarditis, and myocarditis. Laboratory findings of ARF include elevated sedimentation rates and C-

reactive protein, and evidence of antecedent streptococcal infection, as determined by a positive throat culture, a positive direct antigen test for group A streptococci, and/or elevated anti-streptolysin O (ASO), anti-DNase B, and anti-hyaluronidase titers. All three of these antibody tests should be performed if ARF is suspected. Therapy for ARF includes analgesics, salicylates, and corticosteroids for treatment of fever and inflammation, in addition to supportive therapy for prevention of cardiac failure.

Acute glomerulonephritis (AGN) is associated with glomerular lesions, hypertension, hematuria, and proteinuria. Glomerular lesions contain deposits of complement component C3, properdin, and immunoglobulin, and these deposits can be demonstrated by immunofluorescence techniques. Glomerulonephritis may occur as soon as 10 days following pharyngitis or 3 to 6 weeks after cutaneous infections. Disease manifestations include malaise, weakness, anorexia, headache, edema, and circulatory congestion, as evidenced by hypertension and encephalopathy. Anemia, elevated

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sedimentation rate, decreased C3 and total complement, hematuria, and proteinuria are pertinent laboratory findings. Urinalysis usually reveals red blood cells, leukocytes, and casts. Prior streptococcal infection is demonstrable by isolation of the organisms from the throat or from skin lesions or by elevation of anti-streptococcal antibodies. Anti-DNaseB and anti-hyaluronidase assays should be performed because ASO antibody titers are not reliably elevated following skin infections.

The mechanism by which group A streptococci induce ARF and AGN is not clear, but the prevailing theories are that streptococcal infection results in production of antibodies against various streptococcal components (e.g., capsular materials, cell wall carbohydrate and protein antigens, cell membrane antigens) that cross-react with antigenic epitopes of heart tissues, including myocardial, endocardial, and valvular heart tissues, myocardial sarcolemma, skeletal muscle, and joints.[405] Studies with monoclonal antibodies have shown that certain streptococcal M protein antigens and group A streptococcal carbohydrate antigens (e.g., *N*-acetyl-β-D-glucosamine) cross-react with cardiac myosin and several other proteins (e.g., tropomyosin, vimentin) found in heart muscle and heart valve tissue.[253,334,405,663] In this model, antibodies developed during acute streptococcal pharyngitis subsequently bind to these cross-reactive epitopes, activating the complement cascade and resulting in immunologically-mediated damage to cardiac muscle and adjacent tissues and the development of ARF.[668] Group A β-hemolytic streptococci that are responsible for ARF are usually rich in M protein, and M types M1, M3, M5, M16, M18, M19, M24, and a few others are known to be "rheumatogenic"; they have an increased capacity to trigger nonsupperative sequelae. Isolates with these M types have a mucoid colony

morphology, are usually OF negative, are associated with pharyngitis, and evoke a strong type-specific immune response. These M types share antigenic determinants with cardiac muscle, sarcolemma membrane proteins, and synovial membranes. A similar mechanism is believed to operate in the pathogenesis of poststreptococcal glomerulonephritis. Antibodies elicited various non–M-protein antigens of "nephritogenic" group A streptococcal (e.g., cytoplasmic endostreptosin, extracellular "nephritis strain-associated protein [NSAP]," streptococcal pyrogenic exotoxin B [speB]) may react with renal tissues to produce glomerular injury.[305,868] Similarly, some group A streptococcal strains have been deemed as "nephritogenic," (i.e., M types M-2, M-49, M-55, M-57, M-59, M-60, and M-61) and have been disproportionately associated with glomerulonephritis following cutaneous infections, while others (i.e., M-1, M-4, M-12, and M-25) have been associated with glomerulonephritis after pharyngeal infection. Glomerular pathology may result from deposition of preformed immune complexes containing streptococcal antigens and host antibodies or from binding of streptococcal products to the glomerulus, with subsequent antibody binding to create immune complexes. Recent evidence suggests that group A streptococcal pharyngitis may not always be required for development of ARF and that skin infections (pyoderma and impetigo, see below) may play an antecedent role as well.[699,808] Areas of the world with the highest prevalence rates of ARF (and AGN) are tropical regions (i.e., Australia, Fiji, Samoa Pacific Islands) that also have the highest prevalence rates of group A streptococcal impetigo and low rates of pharyngitis. During 2001 to 2005, when prevalence of ARF in indigenous Australians was 254 per 100,000, 80% of impetigo and pyoderma cases were due to group A streptococci while pharyngeal infection remained low (3.7%) during the same period.[136,700]

In addition to pharyngitis, group A β-hemolytic streptococci cause a variety of cutaneous infections, puerperal sepsis, and post-partum infections. **Impetigo** is usually seen in children and constitutes the most common skin infection in this age group worldwide, particularly in developing countries with tropical climates.[76,89] **Pyoderma** usually occurs in children ages 2 to 15 years, with peak incidence in the 2- to 5-year age group. This skin infection is characterized by the development of papules that develop into vesicular lesions that evolve into pustules. These pustules break down over the next 5 to 7 days to form thick scabs. These lesions are usually on the lower extremities and may also involve other pathogens, such as *Staphylococcus aureus*. Interestingly, the *emm* types of strains causing pyoderma are distinct from pharyngitis strains. As mentioned, cutaneous infection with nephritogenic group A streptococcal strains may give rise to poststreptococcal glomerulonephritis. In pyoderma patients, the ASO titer may not be elevated, but DNase B titers will be elevated.[89] **Erysipelas** is an acute infection that is associated with soft tissue involvement and cutaneous lymphatics, resulting in systemic evidence of infection (i.e., fever) (Image Plate 13-

1A).[149] The lesions present as areas of edema and erythema that spread rapidly and have a raised, well-demarcated margin. Lesions of erysipelas are frequently on the face and patients often have accompanying streptococcal pharyngitis.[101] Lesions of erysipelas on other body sites start as areas of redness and swelling, with rapid spread and a raised, well-demarcated border. Complications of erysipelas include necrotizing fasciitis, abscess formation, and septicemia.[149] **Cellulitis** usually results from streptococcal infection of preexisting lesions, such as wounds, burns, or surgical incisions. This infection presents as a spreading inflammatory process that may involve larges areas of the skin and subcutaneous tissues, along with fever, chills, lymphangitis and, occasionally, bacteremia. Cellulitis is usually seen in intravenous drug users and may be complicated by bacteremia and other sequelae (e.g., osteomyelitis, deep tissue infection, endocarditis).[304] **Puerperal sepsis** is seen in women following delivery (either vaginal or abdominal) or abortion.[73,669] Organisms colonizing the genital tract or from obstetrical personnel invade the upper genital tract, causing endometritis, lymphangitis, bacteremia, necrotizing fasciitis, and streptococcal toxic shock syndrome.[55,124,388] Genital tract infection may be complicated by pelvic cellulitis, peritonitis, and abscess formation. Intrapartum transmission of group A streptococci, leading to severe and often fatal group A streptococcal disease in the neonate has also been observed. Manifestations in the neonate include stillbirth, septicemia, jaundice, and cellulitis. **Necrotizing fasciitis** describes infection of deep subcutaneous tissues that results in progressive devitalization and destruction of the fascia.[508,884] This infection sometimes presents with erythema at a site of localized trauma or previous surgery, or via hematogenous seeding of subcutaneous muscles and soft tissue. The infection spreads very rapidly, resulting in cutaneous bullous lesions containing

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serosanguinous fluid. The tissues become gangrenous, with sloughing of devitalized tissues and extensive subcutaneous tissue necrosis. Spread along the fascial planes occurs to involve adjacent soft tissue and muscle. Imaging studies such as MRI can help to define the extent of tissue involvement prior to clinically apparent necrosis. Despite appropriate antimicrobial coverage and surgical debridement of devitalized tissues, mortality rates may be 30% to 60%; deaths may occur as rapidly as 48 hours after the onset of signs and symptoms. Patients with extensive muscle involvement may have fatality rates as high as 80% to 100%. Aggressive medical and surgical interventions are necessary and lifesaving in many cases.

Streptococcal toxic shock syndrome (STSS) first appeared in the United States during the late 1980s and is now a well-recognized clinical entity associated with group

A β-hemolytic streptococci.[967–969] (The attack rate has been estimated to be approximately 3.5 cases per 100,000 population in the United States, with higher rates among the very young (5.3 case per 100,000) and the elderly (9.4 cases per 100,000).[785,968] Patients who develop invasive group A streptococcal infections often have comorbid diseases, especially diabetes mellitus, congestive heart failure, malignancies, and immunosuppression.[68] Some patients present with a viral-like prodrome of fever, chills, and myalgias; diarrhea and vomiting may also be prominent features. Soft tissue infection, indicated by swelling, tenderness, erythema and/or pain, may be evident if the primary infection is cutaneous, but infection may not be apparent in up to 50% of cases and may already have progressed to severe cellulitis or necrotizing fasciitis by the time the patient seeks emergency medical care.[969] These individuals may develop several severe complications and disease manifestations, including myocarditis, hepatitis, peritonitis, septic arthritis, endophthalmitis, puerperal sepsis, meningitis, and overwhelming toxemia.[148,567,785,1029] Pulmonary symptoms include cyanosis, tachypnea, and respiratory failure. Renal involvement occurs in over 80% of patients and often persists despite aggressive treatment with antibiotics and intravenous fluids. The disease is characterized by the sudden onset of overwhelming shock and organ system failure. Patients become hypotensive with little or no response to albumin and electrolyte administration. The acute respiratory distress syndrome develops in more than half of patients, requiring intubation and mechanical ventilation. In fulminant, ultimately fatal infections, the Waterhouse–Friderichsen syndrome and disseminated intravascular coagulation may occur.[535] Almost 80% of these patients have bacteremia with group A streptococci, and the organism may also be isolated from surgical specimens, tissue, peritoneal fluid, pleural fluid, and rarely CSF. If soft tissue infection is present, surgical procedures may be necessary to remove infected, devitalized and necrotic tissues. This syndrome characteristically results in shock and multiorgan system failure shortly after the onset of symptoms and may have a mortality rate of 30% to over 80%.[62] Management requires aggressive antimicrobial chemotherapy, volume resuscitation, and intensive supportive care. Even with these interventions, case fatality rates are 36% and 24% for STSS and necrotizing fasciitis, respectively.[785]

SPEs appear to play a major role in STSS pathogenesis. In a study of 34 STSS group A streptococcal isolates, 74% were either type M-1 or M-3, 53% produced SPE A and 85% contained the *speA* gene, 100% contained the gene coding for SPE B (*speB*), and 21% contained the SPE C gene (*speC*).[433] In a study of TSLS isolates from nine states, more than half of the isolates belonging to types M-1 or M-3 contained the gene for SPE A. SPE A and other pyrogenic exotoxins have been shown to be superantigens that are capable of inducing the release of various cytokines and lymphokines These cytokines and lymphokines trigger and mediate the events that

promote streptococcal dissemination and precipitate the profound multiorgan system involvement that characterizes the syndrome.[845] Combination therapy with penicillin and clindamycin is recommended. Clindamycin has greater efficacy than penicillin, and this drug is often used for therapy along with high-dose penicillin.[53]Clindamycin inhibits protein synthesis at the ribosomal level and, thereby, is able to inhibit the synthesis of the pyrogenic exotoxins more efficiently than cell wall–active agents such as penicillin and other β-lactams. Inhibition of protein synthesis also inhibits expression of M proteins and the synthesis and export of toxins including SPE A, SPE B, and enzymes (e.g., DNase, SLO, and SLS).[121,685,958]

Group A β-hemolytic streptococci cause a variety of other infections, including pneumonia, meningitis, osteomyelitis, endocarditis, peritonitis, and nosocomial infections. Pneumonia due to group A streptococci usually occurs in debilitated hosts with other conditions, including influenza or other intercurrent respiratory virus infections, chronic obstructive pulmonary disease (COPD), alcoholism, and neoplastic disease.[56] Pneumonia may also be a part of the clinical presentation of invasive group A streptococcal diseases, such as STSS. Streptococcal pneumonia often presents with an abrupt onset of fever, chills, malaise, dyspnea, and chest pain. Chest films usually show basilar infiltrates with pleural effusions. Diagnosis is usually made by culture of sputum, pleural fluid, empyema fluid, and/or blood.[731,874] Sputum is usually purulent and streaked with blood. Complications of pulmonary group A streptococcal infections include sepsis, pleural effusions, empyema, pneumothorax, pericarditis, mediastinitis, shock, pulmonary cavitation, bronchiectasis, metastatic abscesses, and osteomyelitis.[56] Mortality may be as high as 38%.[731] Osteomyelitis due to group A streptococci is seen as a complication of varicella infections in children; in adults, streptococcal osteomyelitis usually occurs as a complication of bacteremia.[17] Meningitis due to group A streptococci occurs in about 2% of all patients with systemic group A streptococcal infection and accounts for anywhere from 0.2% to 1% of all cases of meningitis.[327,814] This infection is similar in presentation to other acute bacterial meningitides, with headache, fever, nuchal rigidity, and focal neurologic deficits. Patients with meningitis usually have a focus of infection (e.g., otitis media) that results in bacteremia, with subsequent invasion of the meninges.[327] In a review of 41 adult patients with this infection, 60% had a history of otitis media, sinusitis, pneumonia, recent head injury, recent neurosurgery, or the presence of a neurosurgical device.[1077] Group A streptococcal endocarditis is a rare infection that occurs in infants, children, and adults. Pediatric endocarditis may be associated with bacteremia and varicella infection.[721,1134] Endocarditis in adults usually occurs in patients with no antecedent cardiac abnormalities and is associated with a

high rate of complications, including cerebral or other systemic emboli, arthritis, diskitis, and osteomyelitis. Group A streptococci have also joined the growing list of other microorganisms implicated in continuous ambulatory peritoneal dialysis-associated peritonitis.[148]

Group B β-Hemolytic Streptococci (*Streptococcus agalactiae*)

Virulence Factors

Group B β-hemolytic streptococci (*S. agalactiae*) (GBS) contain a Lancefield-grouping antigen, a type-specific cell surface polysaccharide and protein antigens. The group B antigen is composed of a rhamnose–glucosamine polymer attached to the peptidoglycan layer. Type specificity is provided by both capsular polysaccharide and protein antigens. Group B streptococci are invariably encapsulated and belong to 1 of 10 recognized capsular serotypes. The capsular types are composed of glucose, galactose, *N*-acetylglucosamine, and *N*-acetyl-neuraminic acid (sialic acid); serotype specificity is determined by differing arrangements of these four components in each of the nine capsular types. The **polysaccharide capsular antigens** are designated Ia, Ib, and II through IX; in the United States, serotypes Ia, Ib, III and V are the predominant serotypes.[495,946] About 4% to 7% of isolates may be nontypeable. The lower-numbered serotypes were detected using Lancefield extraction methods, but the newer capsular serotypes were delineated by detection of serotype-specific genes in the capsular region of the group B streptococcal genome.[201,576,852] Kong and colleagues[576] developed primers and PCR-based sequencing assays for the capsular *cps* genes of all serotypes that correlated 100% with serologic approaches. The **protein antigen** is designated by the single letter c, which exists in two forms, designated cα and cβ. This c antigen is found in all Ib strains (type Ia strains lack the c protein), in 60% of type II strains, and is rarely found in type III strains (not enough serotype IV-VIII strains have been examined for the presence of the c antigen). Therefore, serotype designations for c-antigen-containing strains are expressed as Ib/c and II/c. Amplification and sequencing of the genes for cα (*bca* gene), cα-like (*alp2*, *alp3*, and *alp4* genes), Rib (*rib* gene), and cβ (*bac* gene) cell surface proteins have also been developed to further subtype group B streptococcal isolates.[576] The importance of group B streptococcal capsular components as virulence factors is demonstrated by both in vivo and in vitro data. Opsonizing antibodies against group B

streptococci are serotype-specific as demonstrated by studies using PMNs and macrophages, and lack of maternal antibodies to these type-specific antigens is a recognized risk factor for development of group B streptococcal disease in the neonate.

The prevalence of the various GBS capsular serotypes varies over time and may differ from place to place. Prior to the 1990s, most group B streptococcal disease was caused by serotypes Ia, Ib, II, III; serotypes IV through VIII were relatively uncommon, and serotype IX isolates were likely among those strains designated as nontypeable. During the early- to mid-1990s, serotype V strains began to emerge, with the percentage of isolates in this group increasing from 2.6% in 1992 to 20% in 1994.[306] More recent studies conducted in the United States indicate that serotype prevalence still varies widely from place to place, but, in most regions, serotype Ia is predominant (26.8% of isolates), followed by serotypes III (24.8%), V (14.97%), II (10.86%), and Ib (8.12%).[495] In some regions of the country (e.g., Ohio), serotype V emerged between 2001–2002 and 2003 to become the predominant serotype, accounting for 27% of the 349 isolates.[250,701] In most regions of Europe, serotypes III, Ia, V, and II account for 85% to 100% of isolates. During the 1990s, serotypes VI and VIII appeared as the predominant serotypes in Japan, and neonatal early-onset disease due to serotype VIII has also been reported in that country.[588,688] While serotype VIII still accounts for 3.16% of isolates in Asia, in other parts of the world, serotype VIII represents only 0.0% to 0.56% of isolates). Serotypes III, V, Ia, and Ib are currently the most prevalent serotypes in Asia.[495] In eastern Europe and the Middle East, serotypes II, III, Ia, and V predominate, and data from United Arab Emirates indicates that, at least during the late 1990s, serotypes IV and Ia accounted for 26% and 21% of isolates, respectively.[28] Data from Africa indicate that serotypes II and V account for 42% to 62% of group B streptococcal isolates and 24% to 43% of group B streptococcal isolates, respectively.[495]

Type III strains of group B streptococci account for 60% of isolates from cases of neonatal sepsis and over 80% of isolates from infants with meningitis, suggesting that this group B streptococcal serotype possesses enhanced virulence. The type III capsular polysaccharide is composed of a repeating structural backbone consisting of galactose, glucose, and *N*-acetyl-glucosamine with side chains consisting of galactose and a terminal *N*-acetyl-neuraminic acid moiety. The structural component of the type III capsule that appears to be associated with augmented virulence is the presence of *N*-acetyl-neuraminic acid (sialic acid). The presence of this molecule on the surface of the organism inhibits activation of the alternative complement cascade and prevents phagocytosis. Removal of sialic acid residues with neuraminidase leads to complement activation, phagocytosis, and intracellular killing of the organisms and diminished virulence on intravenous challenge in a rat model.[303] Like the group A streptococci,

Group B streptococci also produce **C5a peptidase**. C5a is a complement component cleavage product that is produced by alveolar epithelial cells, acts as an attractant for inflammatory cells, and is involved in the process of pulmonary inflammation. The C5a peptidase produced by the streptococci cleave this molecule and thereby interfere with C5a-mediated neutrophil chemotaxis.[97] This peptidase also binds to fibronectin and serves as a bacterial adhesin and invasin.[65,182] This enzyme exists in a cell-associated form and also requires the presence of the group B capsule for maximal activity. Opsonization of Group B streptococci belonging to serotypes Ia, Ib, II, III, and V with anti-C5a peptidase antibodies results in increased killing by both macrophages and polymorphonuclear cells.[181] C5a peptidase is also produced by group A and group G β-hemolytic streptococci, and the genes encoding the C5a peptidase in group B (*scpB*) and group A strain (*scpA*) demonstrate 98% sequence homology with each other. The **β-hemolysin/cytolysin** of group B

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streptococci is a pore-forming hemolysin that is able to lyse pulmonary alveolar epithelial and endothelial cells in vitro and augments the ability of the organism to invade endothelial cells, suggesting a role for the hemolysin as a virulence factor in neonatal lung infection and subsequent sepsis.[768,769] **Lipoteichoic acid** is also found in group B streptococci and may participate in facilitating adherence as a first step in infection. Several **cell surface proteins** (e.g., c, R, BPS, and Rib antigens) are also found in various combinations in different group B streptococcal serotypes.[312] The **c antigen**, in particular, may act to mediate internalization of organisms within human cervical epithelial cells following attachment and to protect organisms from intracellular killing following phagocytosis.[98] Jones and colleagues[523] identified a **cell surface penicillin-binding protein** (PBP1a) that enabled streptococcal cells to resist intracellular killing by phagocytic cells. Group B streptococci also produce a **hyaluronic acid lyase**, which may act to spread infection by breakdown of hyaluronic acid in the extracellular matrix, and may also act on the hyaluronic acid present in high concentrations in placental tissues, fetal tissues, and amniotic fluids. The presence of CAMP factor, protease enzymes, and various nucleases have also been identified in some group B streptococci, but the role of these molecules in disease pathogenesis is uncertain.

Clinical Spectrum of Group B Streptococcal Disease

Group B β-hemolytic streptococci are a major cause of disease in the neonatal and perinatal periods.[707] Women become colonized in the gastrointestinal tract, which is the source for vaginal colonization. Among pregnant women, rectovaginal colonization

is found in 10% to 37% of pregnant women; up to 60% of the colonized women will carry the organism intermittently.[423,855,1093] Vaginal colonization with group B streptococci is usually asymptomatic, although reports have appeared that document vaginitis associated with heavy colonization and resolution of vaginal symptoms with treatment.[467,468] Vaginal colonization may be transient, intermittent, or persistent. Colonization generates a serotype-specific immune response, with cumulative increases of antibodies with increasing age; lower antibody levels in teenaged girls may translate to increased risk of group B streptococcal disease in babies born to these younger women.[130] The presence of group B streptococci in the female genital tract at the time of birth can lead to infection of the neonate. Of neonates born to women who are colonized with group B streptococci, 30% to 70% become transiently colonized on the skin or mucosal surfaces by vertical transmission from the colonized mother either in utero or during delivery; 1% to 4% of these babies may develop systemic disease. Among colonized infants, disease may occur in one to four infants per 1,000 live births.[303,1093] In addition, the neonate may become colonized by nosocomial exposure to the organism after birth. Neonatal disease with group B streptococci follows two patterns, termed **early-onset disease** and **late-onset disease**.

In the United States, early-onset group B streptococcal disease occurs with an incidence of 0.7:1,000 to 3.7:1,000 live births and is associated with in utero or perinatal organism acquisition.[303] This incidence is probably an underestimate; a study conducted in the United Kingdom on early-onset disease reported an incidence of three to four infections per 1,000 live births based on the number of neonates requiring screening for possible sepsis during the first 72 hours of life.[661] The incidence of early-onset disease varies from country to country and likely reflects differences in group B streptococcal carriage rates, ethnic/racial susceptibility to infection, variations in diagnostic criteria, differences in virulence among streptococcal strains, and the type/availability of prenatal, natal, and postnatal care.[707] The organism is acquired either by ascending infection in utero before delivery, through ruptured fetal membranes, or during passage through a birth canal that is colonized with group B streptococci. Although a substantial proportion of these infants (approximately 50%) will be colonized with group B streptococci, only 1% to 2% of them become infected.[303] Mucous membrane colonization and aspiration of infected amniotic fluid introduce the streptococci into the lung alveoli of the neonate, where they adhere, replicate, and produce various virulence factors (e.g., capsular polysaccharides, β-hemolysin) The bacteria then invade pulmonary endothelial and epithelial cells, thereby gaining access to the bloodstream.[766–769] Onset of disease occurs during the first 5 to 6 days of life; in more than half the cases, infants become ill within the first 12 to 20 hours after birth. The disease spectrum includes bacteremia, pneumonia, meningitis, septic shock, and neutropenia. Although more than 50% of cases occur in

term infants, a higher attack rate and greater morbidity are associated with preterm infants. Preterm neonates are at 3- to 30-fold increased risk of developing early-onset disease than full-term infants, and this may be related to relative immunologic immaturity and incomplete receipt of transplacental maternal antibodies against the capsular polysaccharide of the infecting group B strain. Mortality owing to early-onset disease in term infants ranges from 2% to 8%; higher mortality rates are seen in premature infants and are inversely proportional to the birth weight of the neonate.[1153] Maternal factors that increase the risk for early-onset infection of the neonate include preterm labor with delivery before 37 weeks, rupture of the fetal membranes >18 hours before delivery, maternal chorioamnionitis, heavy vaginal colonization with group B streptococci, group B streptococcal bacteriuria, and postpartum bacteremia.[156,303,707] During the 1970s, about half of the infants who developed early-onset disease died of the infection. Among those children who survive group B streptococcal meningitis, about half will have some type of neurologic deficit and up to 30% will have severe neurologic sequelae.[1093]

Late-onset disease occurs with an incidence of 0.5:1,000 to 1.8:1,000 live births.[303] Disease becomes clinically evident 7 days to 3 months (average 3 to 4 weeks) after birth. Whereas about half of the late-onset infections are acquired from the birth canal of colonized mothers, the remaining cases result from postnatal organism acquisition from the mother, other caregivers, or nosocomially; breast milk has also been implicated as a source of the organism.[386] Bacteremia is the predominant clinical presentation, and about 25% of these infants also develop group B streptococcal meningitis.[351,842] A pediatric case report described late-onset disease presenting as necrotizing fasciitis of the face and thigh in a 5-month-old preterm infant.[596] Mortality associated with late-onset disease is about 10% to 15%. Up to 50% of children

with late-onset meningitis will have permanent neurologic complications and sequelae.[1093] The distribution of group B streptococcal serotypes varies with early-onset versus late-onset disease.[303] Among neonates with early-onset disease without meningitis, the serotype distribution is equally divided among types Ic, II, and III. Among similarly infected neonates with meningitis, serotype III strains predominate. On the other hand, group B streptococcal meningitis in adults is primarily associated with serotype II organisms.

Prevention of Group B Streptococcal Disease

By the mid-1980s, increased knowledge about group B streptococcal infections and recognition of symptoms in at-risk patients lead to improvements in neonatal care that resulted in reduction of the fatality rate to about 15%. Since infants born to heavily colonized mothers are more likely to develop early-onset disease and because infants who acquire a large bacterial inoculum during birth have significantly increased likelihoods of developing both early- and late-onset disease, identification of colonized mothers became a central focus for prevention strategies. Investigators examined various interventions to prevent group B streptococcal disease, and clinical trials demonstrated that intrapartum administration of antimicrobial agents interrupted transmission of group B streptococci from the mother to the neonate and reduced the incidence of early-onset infections.[114,707] This approach prevented about 70% to 75% of early-onset disease, but had no effect on the development of late-onset disease. Commercial vendors began developing products for rapid direct detection of group B streptococci in vaginal swab specimens similar to those used for detection of group A streptococci in throat swab specimens. Theoretically, these tests could be performed in the immediate prepartum period to determine colonization status, and antimicrobial chemoprophylaxis could be administered prior to and during delivery if the assays were positive. Commercial rapid tests for direct detection of group B streptococci varied significantly in sensitivity (i.e., 11% to 88%) when compared with overnight broth techniques and identified only those women who were heavily colonized. The validity of the assumption that the risk of neonatal infection is greater for infants born to heavily colonized women was confounded by clinical studies. In an evaluation of one rapid EIA kit, Towers and coworkers[1037] reported that two of nine infants born to mothers with light colonization and negative rapid EIA results developed fatal early-onset disease. In another study of a rapid method for detection of group B streptococcal colonization, Morales and Lim reported that, among 37 women with light colonization and negative rapid screening tests, six delivered babies with early-onset sepsis.[726] The timing of antepartum testing for group B streptococcal colonization was also an issue, since 60% to 70% of women with positive vaginal cultures in the second trimester will be colonized at term, and up to 30% of women with negative cultures during the second trimester will be culture-positive at delivery. Consequently, the development of methods for rapid, sensitive, and specific detection of group B streptococcal colonization in women at or near the time of delivery became a central focus of clinical and microbiologic research.

In 1996, the Centers for Disease Control and Prevention (CDC), together with the American Academy of Pediatrics (AAP) and the American College of Obstetrics and Gynecology (ACOG) issued consensus recommendations on a prevention strategy for group B streptococcal disease.[151,1093] These guidelines recommended that obstetrical healthcare providers adopt either a culture-based or a risk-based strategy for the prevention of early-onset group B streptococcal disease. Subsequent implementation

of and compliance with these guidelines has resulted in a significant decrease in the incidence of early-onset neonatal group B streptococcal disease. A critical component of these guidelines is the use of maternal antimicrobial prophylaxis during labor and delivery. This raised concerns regarding the adverse effects of increased antibiotic usage and the increased risk of anaphylactic reactions to penicillin, the drug of choice for chemoprophylaxis. Another concern has been the potential emergence of antimicrobial resistance in group B streptococci. To date, no penicillin-resistant strains of group B streptococci have been isolated, although clindamycin and erythromycin resistance has become relatively common. A third concern was that increased penicillin administration in the newborn period may provide sufficient selective pressure to result in neonatal infections due to penicillin-resistant organisms like *E. coli*, which is the second most common agent of sepsis in the newborn period. This scenario has not developed to date, and continued surveillance will be required to detect and contain this possibility. In 2002, the Committee on Obstetrical Practice issued a statement supporting the use of culture-based prevention strategies based on data from the Active Bacterial Core Surveillance/Emerging Infections Program network suggesting that the culture-based approach was superior to the risk-based approach.[26] New guidelines were issued by the CDC in 2002, replacing the 1996 guidelines and recommending universal, prenatal, culture-based screening for vaginal and rectal colonization of all pregnant women at 35 to 37 weeks' gestation.[156] The culture protocol recommended by the CDC, ACOG, and the FDA involved collection of vaginal and rectal swab specimens during the 35th to 37th week of gestation, followed by 18- to 24-hour growth enrichment, subculture to SBA, and identification of group B streptococci by phenotypic (e.g., CAMP test, hippurate hydrolysis, API Rapid Strep), serologic (i.e., serogrouping by coagglutination or latex agglutination), or chemiluminescent hybridization (i.e., Gen-Probe Accuprobe) methods. The entire screening procedure could take 2 to 3 days.

In 2010, the CDC updated and revised the guidelines for prevention of group B streptococcal disease (Box 13-3).[27,157] Screening for group B streptococci is still recommended at 35 to 37 weeks of gestation. Rectovaginal swab specimens are collected from the lower third of the vagina and from the anal canal (a single swab from the vagina, then the anal canal, or one swab collected from each site). The swab(s) are placed in Trans-Vag, LIM broth (Todd–Hewitt broth with 10 μg/mL nalidixic acid, 15μg/mL colistin, and 10 mg/mL yeast extract), or chromogenic broth (e.g., Carrott Broth, Hardy Diagnostics, Santa Maria, CA), and incubated for 18 to 24 hours. In the 2010 document, group B streptococcal identification options were expanded to include use of the chromogenic medium and nucleic acid amplification tests (NAATs) performed on the Lim or Trans-Vag enrichment broth for group B streptococcal detection in addition to DNA probe or latex agglutination as in the previous document. The previous guideline also recommended that laboratories

identify and report group B streptococci in urine specimens in any concentration, while the 2010 guidelines clarified this to require that laboratories identify and report group B streptococci in urine in colony counts of $\geq 10^4$/cfu/mL urine.[27,157] Use of NAAT tests directly on rectovaginal specimens is not recommended, but in settings where NAAT testing is available, an intrapartum NAAT test may be done when the colonization status of the patient is unknown, when there are no intrapartum risk factors, and when the patient is at term.[157] In the 2010 guidelines, penicillin remains the drug of choice, with ampicillin as an alternative, and cefazolin remains the drug of choice in penicillin-allergic patients if penicillin-allergy is doubtful (i.e., no urticaria, angioedema, or respiratory distress). Group B streptococcal isolates from women at high risk for penicillin anaphylaxis should be tested for susceptibility to clindamycin, erythromycin, and vancomycin. Erythromycin is no longer recommended for treatment and testing is performed along with the D-zone test to determine inducible clindamycin resistance in isolates that are erythromycin-resistant. Group B streptococcal early-onset infection in newborns due to clindamycin-resistant isolates has been reported.[93] Even with timing of screening between 35 and 37 weeks' gestation, up to 6% of women colonized with group B streptococci are not detected at this time (94% predictive value of a negative test).[1070] Prevention of group B streptococcal disease in the newborn is a practical reality. Globally, only five group B serotypes (i.e., Ia, Ib, II, III, and V) cause more than 85% of disease in infants less than 3 months of age, and a conjugate vaccine directed against these serotypes could conceivably prevent infection.[302,495,906] Such a vaccine would be administered to pregnant women and could potentially also reduce other conditions associated with group B streptococci (i.e., late-onset disease, premature birth, intra-amniotic infections, puerperal sepsis, and peri-/postpartum bacteremia).[437] Several vaccines against group B streptococci are in clinical trials at the present time, and perhaps soon, group B streptococcal disease of the newborn will go the way of type b *Haemophilus influenzae* in neonates.[278,906]

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BOX 13-3

Procedures for Collecting and Processing Rectovaginal Specimens for Perinatal Detection of Group B Streptococci[26,27,156,157]

Procedure for Collecting Clinical Specimens for Culture of Group B

Streptococci (GBS) at 35 to 37 Weeks' Gestation

- Swab the lower vagina (vaginal introitus) followed by the rectum (i.e., insert swab through the anal sphincter) using the same swab or two different swabs. Culture should be collected in the out-patient setting by the health care provider or the patient, with appropriate instruction. Cervical cultures are not recommended and a speculum should not be used for culture collection.
- Place the swab(s) into a nonnutritive transport medium. Appropriate transport systems (e.g., Amies of Stuart's without charcoal) are commercially available. If vaginal and rectal swabs were collected separately, both swabs can be placed in the same container of medium. Transport media will maintain GBS viability for up to 4 days at room temperature or under refrigeration.
- Specimen labels should clearly identify that specimens are for group B streptococcal culture. If susceptibility testing is ordered for penicillin-allergic women, specimen labels should also identify the patient as penicillin-allergic and should specify that susceptibility testing for clindamycin should be performed if GBS is isolated. The isolates should be tested for inducible clindamycin resistance (e.g., D-zone test, see below).

Procedure for Processing Clinical Specimens for Culture of GBS

- Remove swab(s) from transport medium. Inoculate swab(s) into a recommended selective broth medium, such as Todd-Hewitt broth supplemented with either gentamicin (8 μg/mL) and nalidixic acid (15 μg/mL), or with colistin (10 μg/mL) and nalidixic acid (15 μg/mL). Examples of appropriate commercially available options include Trans-Vag broth or LIM broth. The addition of 5% sheep blood to these media can increase the recovery of GBS. Selective enrichment broth also can contain chromogenic substrates that provide for a change in color in the setting of β-hemolytic GBS (Carrot Broth, Hardy Diagnostics, Santa Maria, CA); however, nonhemolytic isolates will not be detected in this medium, so subculture of the broth in the case of no color change is required.[94]
- Incubate inoculated selective broth for 18–24 hours at 35°C–37°C in ambient air or 5% CO2. Subculture the broth to a SBA plate (e.g., tryptic soy agar with 5% defibrinated sheep blood).
- Inspect and identify organisms suggestive of GBS (i.e., narrow zone of β-hemolysis, gram-positive cocci, catalase-negative). Note that hemolysis may be difficult to observe, so typical colonies without hemolysis should also be further tested. If GBS is not identified after incubation for 18–24 hours, reincubate and inspect at 48 hours to identify suspected organisms.
- Various streptococcus grouping latex tests or other tests for GBS antigen detection (e.g., genetic probe, NAATs) may be used for specific identification, or the CAMP test may be employed for presumptive identification. The sensitivity of NAATs for GBS increases to 92.5%–100% with the use of an enrichment step before testing the specimen.[94,391,911] Data on the currently available NAATs do not support their use as a replacement for antenatal culture. The additional time required for

enrichment of samples makes it not feasible for intrapartum testing, and the sensitivity of NAATs in the absence of enrichment is not adequate in comparison with culture. Such tests may be of value for the circumstance of a woman at term with unknown colonization status and no other risk factors. Other rapid tests (e.g., EIAs) have been developed to detect GBS rapidly from nonenriched samples; however, none are sufficiently sensitive when used directly on a specimen for reliable detection of group B streptococcal colonization in the intrapartum setting (see text).

Procedure for Clindamycin and Erythromycin Disk Susceptibility Testing of Isolates, When Ordered for Penicillin-Allergic Patients

- CLSI recommends disk diffusion or broth microdilution testing for antimicrobial susceptibility testing of GBS
- Commercial systems that have been cleared or approved for testing of streptococci other that *S. pneumoniae* may also be used.
- To ensure accurate results, laboratories should include a test for detection of inducible clindamycin resistance. The double-disk diffusion method (D-zone test) is recommended for testing erythromycin-resistant and clindamycin-susceptible GBS. Other validated tests to detect inducible clindamycin resistance may be used in place of the D-zone test.[861,1004]
- CLSI disk diffusion/D-zone test procedure[203]
  - Use a cotton swab to make a suspension from 18- to 24-hour growth of the organism in saline or Mueller-Hinton broth to match a 0.5 McFarland turbidity standard.

  - Within 15 minutes of adjusting the turbidity, dip a sterile cotton swab into the adjusted suspension. The swab should be rotated several times and pressed firmly onto the side wall of the tube above the fluid level. Use the swab to inoculate the entire surface of a Mueller-Hinton SBA plate. After the plate is dry, use sterile forceps to place a clindamycin (2 μg) disk onto half of the plate and an erythromycin (15 μg) disk 12 mm apart for D-zone testing.
  - Incubate at 35°C in 5% CO2 for 20–24 hours.
  - Measure the diameter of the zone of inhibition using a ruler or calipers. Interpret according to CLSI guidelines for *Streptococcus species* β-hemolytic Group.[203]
- For disk diffusion
  - Clindamycin: ≥19 mm = Susceptible, 16–18 mm = Intermediate, and ≤15 mm =Resistant
  - Erythromycin: ≥21 mm = Susceptible, 16–20 mm = Intermediate, and ≤15 mm =Resistant
- For broth microdilution

- Clindamycin: ≤0.25 µg/mL = Susceptible, 0.5 µg/mL = Intermediate, and ≥1.0 µg/mL = Resistant
- Erythromycin: ≤0.25 µg/mL = Susceptible, 0.5 µg/mL = Intermediate, and ≥1.0 µg/mL = Resistant
- Isolates with blunting of the inhibition zone around the clindamycin disk adjacent to the erythromycin disk (D-zone positive) should be considered to have inducible clindamycin resistance and are presumed to be resistant (other validated tests to detect GBS with inducible clindamycin resistance may be used).

- The following comment should be included in patient reports for isolates that show inducible clindamycin resistance:
  - "This isolate is presumed to be resistant on the basis of detection of inducible clindamycin resistance. Clindamycin still might be effective clinically in some cases."

Other Infections Caused by Group B Streptococci

Group B streptococci are associated with about 20% of postpartum endometritis, 25% of bacteremias following cesarean section, 25% to 30% of cases of asymptomatic bacteriuria during and after pregnancy, and a variety of other infections in nonpregnant adults.[168,458, 687,689] With the decline in neonatal group B streptococcal disease, over two-third of infections now occur in adults and are not associated with pregnancy. Adults with group B disease usually have significant underlying disease, including diabetes, liver cirrhosis, stroke, neoplasia, or urinary tract abnormalities.[536,979] Skin and soft tissue infections are the most common clinical entities associated with invasive group B streptococci and include cellulitis, erysipelas, abscesses, infected decubitus ulcers, and invasive wound infections following surgical procedures.[45,718,914] Cellulitis and other cutaneous infections are often manifestations of bacteremia in both adults and children.[718,952] Group B streptococci may also cause necrotizing fasciitis and STSS that

are clinically similar to those condition involving group A streptococci. Up until 1998, only four cases of necrotizing fasciitis due to group B streptococci had been reported, but then Gardam and colleagues[369] reported three cases of group B streptococcal necrotizing fasciitis in adults that occurred in Quebec and Ontario over a 10-month period. In this report, all three patients were immunocompromised (i.e., chronic lymphocytic leukemia, type 2 diabetes mellitus, alcoholism). These patients were

treated with multiple antimicrobial agents and required wide excisional debridement of involved devitalized tissue (including a below-the-knee amputation) and one case resulted in a fatality. Several reports have subsequently appeared describing necrotizing fasciitis and toxic shock syndrome due to group B streptococci in both infants and adults, including three fatal cases of invasive serotype VI necrotizing fasciitis and toxic shock in Japan.[251,570,596,689] In one pediatric case, necrotizing fasciitis developed in a 5-month-old preterm infant as a manifestation of late-onset disease.[596] Toxic shock syndrome reminiscent of that caused by *Staphylococcus aureus* and group A streptococci has been reported primarily in immunocompromised adults. Patients are usually female, greater than 50 years of age, and have diabetes, hematologic malignancies, liver and/or heart disease, or splenectomy as their underlying condition; toxic shock syndrome in nonimmunocompromised patients has also been reported.[66,171,914,935] In Japan, isolates causing toxic shock syndrome belonged to a variety of serotypes (Ib, III, V, VII) and had different PFGE patterns.[171] Strains that cause this toxic shock-like syndrome display enhanced resistance to phagocytic clearance due to increased production of capsular material and induce higher levels of interleukin-8 than wild-type isolates.[914] This is of considerable importance since there is a known association between serum levels of interleukin-8 and severity of sepsis.[1136]

Several types of group B streptococcal osteoarticular infections have also been reported, including septic arthritis, osteomyelitis, spondylodiscitis and prosthetic hip and joint space infections.[199,244,466,625,751,770,1041] Most native bone, joint, and prostheses infections have been reported in elderly, nonpregnant patients with underlying diseases or chronic medical predisposing conditions (e.g., diabetes, malignancies, chronic liver disease).[244,625,1171] Pyogenic arthritis usually affects the knee or the shoulder and is monoarticular in 68% of cases and bacteremia can be documented in two-thirds of patients.[770] Septic arthritis with group B streptococci usually presents with fever and joint pain following or concomitant with bacteremia. Antimicrobial therapy with aspiration or open drainage of infected joints and removal of prosthetic devices, if present, are necessary to effect cure. Osteomyelitis may occur as a complication of cellulitis by contiguous spread, particularly in association with decubitus ulcers, or as a result of hematogenous seeding from another site of infection or from the gastrointestinal tract. Group B streptococci are also a cause of osteomyelitis in neonates, with hematogenous seeding of bone being the source of the organism; osteomyelitis may present as an uncommon manifestation of late-onset group B streptococcal disease.[466] Spondylodiscitis and vertebral osteomyelitis are complications of bacteremia and usually occur in adults.[199,751]

Orbital infections and endophalmitis due to group B streptococci are rare manifestations of infection and occur in both infants and adults. In infants,

endogenous endophthalmitis is generally a complication of late-onset bacteremic disease and usually occurs along with meningitis.[697,956,1151] Endophthalmitis in adults occurs in those with underlying disease and complications may include cataract formation, scleral perforation, and anterior-chamber neovascularization.[408,1151] Infection may be so severe that enucleation of the eye may be required.[697] Endophthalmitis in adults has also been reported as a complication of endocarditis.[187]

Group B streptococcal bacteremia can be complicated by endocarditis and meningitis. Endocarditis is a rare manifestation of group B streptococcal infection. Endocarditis occurs in both men and women, may present acutely or subacutely, and accounts for 2% to 18% of invasive group B disease in adults.[46] Patients usually have underlying predisposing conditions (diabetes, immunosuppression, renal transplantation), and the infection can involve native or prosthetic heart valves.[46,813,886] Preexisting cardiac abnormalities are usually present before disease onset, and once established, vegetations usually develop on the valves, with the mitral valve being the most commonly affected. Vegetations on the heart valves are often large, and embolic phenomena occur in about 50% of cases.[886] Complications caused by embolic phenomena or rapid destruction of valvular tissue may necessitate valve replacement. The mortality rate associated with group B streptococcal endocarditis can be up to 40%, but if combined medical/surgical intervention occurs, mortality decreases to about 20%. A mortality rate of almost 90% may be seen with cases of prothetic valve infection.[813,886] Endocarditis in neonates and infants occurs rarely but resembles adult cases with extensive valvular destruction and the occurrence of embolic phenomena. Group B streptococcal endocarditis has been diagnosed in a 15-year-old girl following an elective abortion.[129] Meningitis is rare, accounts for about 4% of bacterial meningitis in adults, and occurs most frequently in postpartum women and older adults with chronic underlying diseases, such as diabetes, cirrhosis, neurologic impairment, malignancy, renal failure, cardiovascular/pulmonary disease, and HIV infection.[404] Group B streptococcal meningitis following severe head trauma or associated with cerebrospinal rhinorrhea has also been reported.[1083]

Group B streptococci are also a cause of urinary tract infections (UTIs). The clinical spectrum of group B streptococcal UTIs include asymptomatic bactiuria, cystitis, pyelonephritis, urethritis, and urosepsis.[1063] UTIs usually occur in women greater than 50 years of age without underlying disease, but with prior histories of UTI. Bacteriuria with group B streptococci has also been associated with adverse pregnancy outcome, increased rates of premature labor, and premature rupture of fetal membranes.[29] Besides being a well-recognized cause of urinary tract infection in pregnant women, this organism is also a cause of cystitis and pyelonephritis in men, nonpregnant women, and children. Anywhere from 5% to over 20% of nonpregnant

adults with bacteremia will have group B streptococcal urinary tract infection.[733] Risk factors for urinary tract infection caused by group B streptococci include advanced age, underlying disease (especially diabetes), presence of an indwelling urinary catheter, prior UTIs, structural abnormalities of the urinary tract, and other comorbid conditions (e.g., prostatic disease). Pyelonephritis and renal abscess are potential complications

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of both ascending infection and hematogenous dissemination of group B streptococci.[450,537]

Antimicrobial Susceptibility of Group B Streptococci

Like the group A *Streptococcus*, isolates of group B streptococci remain susceptible to penicillin G, the drug of choice for treatment of infections and for perinatal chemoprophylaxis.[157,826] Ampicillin, cefotaxime, ceftriaxone, cefazolin, quinupristin-dalfopristin, and meropenem are also highly active, with most strains having MICs of ≤0.06 μg/mL.[103,145,189] Very rare isolates from Japan and the United States have decreased susceptibilities to penicillin due to a point mutation in the group B streptococcal *pbp2x* gene.[255,564,798] These isolates have penicillin MICs that are at the threshold of susceptibility (i.e., ≤0.12 μg/mL for penicillin and ≤0.25 μg/mL for ampicillin) These isolates also have elevated MICs to cefazolin (MIC, 1 μg/mL). During the last 15 to 20 years, resistance to macrolides and clindamycin among the group B streptococci has increased from less than 5% to between 20% and 30% depending on locality.[157,377] Most erythromycin resistance is due to a ribosomal modification due to erythromycin ribosome methylase, encoded by the *erm* gene. This also confers inducible or constitutive resistance to clindamycin (MLSB phenotype). Macrolide-lincosamide resistance genotypes that have been detected in group B streptococci include *ermA* (erythromycin-resistant, clindamycin-susceptible), *ermB* (erythromycin-resistant, clindamycin-resistant), and *mef* (erythromycin-resistant, clindamycin-susceptible).[289] All group B streptococcal strains tested to date have been susceptible to vancomycin.

Group C and Group G β-Hemolytic Streptococci

The group C β-hemolytic streptococci currently include *S. dysgalactiae* subsp. *equisimilis*, *S. dysgalactiae* subsp. *dysgalactiae, S. equi* subsp. *equi, S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *ruminatorum.* Former *S. equisimilis* strains

isolated from humans have been shown to carry the group C or group G Lancefield (and, rarely group A) antigens and are genetically similar enough to animal group C strains (subsp. *dysgalactiae)* to be placed in the same species, but different subspecies.[1076,1095] *S. dysgalactiae* subsp. *equisimilis* grows as large colonies on SBA and is usually β-hemolytic. Related strains that carry the C or the L grouping antigen and are isolated primarily from animals are now named *S. dysgalactiae* subsp. *dysgalactiae*.[1076] Isolates of this subspecies may be α-, β-, or nonhemolytic. Molecular genetic studies have demonstrated extensive similarities between the former species *S. equi* and *S. zooepidemicus* and these species are now called *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*, respectively. These subspecies carry the Lancefield group C carbohydrate cell wall antigen and produce hemolysins similar to those of group A streptococci. *S. equi* and *S. dysgalactiae* subspecies form large colonies on SBA, which differentiate them from the "minute" or "small-colony" α-, β-, or nonhemolytic streptococci of the Anginosus Group of viridans streptococci, which also may carry the group C polysaccharide antigen. *Anginosus* group organisms also differ from the "large-colony" group C streptococci genetically and phenotypically.[139]

S. dysgalactiae subsp. *equisimilis,* the most common human isolate, can colonize the human upper respiratory, gastrointestinal, and female genital tracts; colonization serves as a potential reservoir of infection to other humans.[118,995,996] This subspecies has been recovered from the pharynges of carriers and from cases of exudative pharyngitis and tonsillitis.[118,1055] In young adult populations, pharyngitis due to group C β-hemolytic streptococci has been associated with the same clinical features as group A streptococci, including headache, fever, exudates, and cervical lymphadenopathy.[1027] *S. dysgalactiae* subsp *equisimilis* can cause potentially severe infections in adult and pediatric age groups and usually presents as acute sepsis without a defined source.[118,853] Soft tissue infections and cellulitis, the other most common presentation, may serve as a nidus for bloodstream infection and can lead to hematogenous complications, including endocarditis, endophthalmitis, meningitis, and septic arthritis.[118,640,665,810,853,937,1130] *S. dysgalactiae* subsp. *equisimilis* carrying group C or G antigens have also been associated with STSS, necrotizing fasciitis, and ARF.[432,500,568] Patients with invasive disease are usually adult males and at least three-fourths of patients have underlying medical conditions.[123,995,996]

S. dysgalactiae subsp. *equisimilis* is one of several streptococcal species that is currently regarded as an emerging pathogen. Increasing prevalence of invasive *S. dysgalactiae* subsp. *equisimilis* disease has been noted in Japan, Europe, Asia, and the Americas.[116,123,568,640,996] In Japan, the prevalence of toxic shock syndrome and invasive disease (defined as isolation from a normally sterile site) caused by *S. dysgalactiae* subsp. *equisimilis* has been increasing gradually since 2003. A study performed there found that most patients with *S. dysgalactiae* subsp *equisimilis*

infections were male, elderly, and had underlying disease, including diabetes, malignancy or stroke.[995,996] Of interest was a report from France where a patients developed a near-fatal septic transfusion reaction following pooled platelet transfusion.[63] *S. dysgalactiae* subsp. *equisimilis* was isolated from the blood culture of the recipient and from the residual blood component container. Virulent strains of *S. dysgalactiae* subsp. *equisimilis* in Japan have caused invasive wound infections, toxic shock syndrome, peritonitis, pneumonia and gas gangrene requiring amputation.[469,470,690,1061] These invasive isolates possess virulence factors similar to *S. pyogenes*, including M proteins (encoded by *emm* gene) and production of cellular toxins and hemolysins (e.g., streptokinase A, C5a peptidase, hyaluronidase, streptolysin S, streptolysin O, hemolysin).[14,996]Several superantigen genes (i.e., *speA, speC, speM, ssa,* and *smeZ*) have been identified in *S. dysgalactiae* subsp. *equisimilis.*[118,844] Igwe and colleagues[489] examined 21 group C and group G β-hemolytic streptococcal isolates and found that 11 strains were PCR-positive for at least one of the *speM*, *ssa*, or *smeZ*, genes, which are members of the group A streptococcal superantigen family. In a study using a microarray containing 216 group A streptococcal virulence genes (including those for adhesins, fibronectin-binding proteins,

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toxins, extracellular proteins, and superantigens) tested with 58 isolates of *S. dysgalactiae* subsp *equisimilis,* each isolate possessed an average of 34% of the genes on the microarray, and 44 of these virulence genes were found in every isolate.[263]

S. equi subsp. *zooepidemicus* is a commensal organism found in the upper respiratory and urogenital tracts of horses and other animal species that acts as an opportunistic pathogen, usually causing respiratory tract or cutaneous infections. Infections in animals include bovine mastitis, equine respiratory infections, purulent arthritis in lambs and goats, and genital tract infections in poultry. *S. equi* subsp *zooepidemicus* is an infrequent cause of infections in dogs, but wound infections and bacteremia have been reported particularly in kennels.[595,819] The first outbreak of *S. equi* subsp. *zooepidemicus* respiratory tract infection ever described in cats was reported from a cattery in Israel in 2010.[95] *S. equis* subsp. *zooepidemicus* has rarely been implicated in human infections, and those who develop infections have had significant exposure to animals (especially horses) or have consumed unpasteurized milk products. Outbreaks of pharyngitis and bacteremia caused by *S. equi* subsp. *zooepidemicus* have been traced to consumption of unpasteurized cows' milk and to fresh unpasteurized goat cheese.[356,587] Transmission of *S. equi* subsp. *zooepidemicus*

from a dog with respiratory tract infections to his handler was reported in 2010.[2] The handler developed signs of sepsis and nose and throat cultures were positive for the organism. Infections in humans have included pneumonia, bacteremia, endocarditis, meningitis, septic arthritis, abdominal aortic aneurysm, deep vein thrombosis, nephritis, and cervical lymphadenitis.[208,317,840,850] In some of these individuals, poststreptococcal glomerulonephritis was observed.[356,1025] *S. equi* subsp. *equi* causes a respiratory tract infection in horses called "strangles." Strangles is characterized by a high fever, mucopurulent nasal discharge, and abscesses in the submandibular and retropharyngeal lymph nodes that eventually rupture and drain into the respiratory tract of the infected animal.[1031] This organism is extremely rare in humans and has been isolated from cases of bacteremia and meningitis.[307,838] *S. equi* subsp. *ruminatorum* is a recently described subspecies that is associated with mastitis in sheep and goats.[336]

Group G β-hemolytic streptococci constitute a part of the normal human gastrointestinal, vaginal, and skin flora. Human isolates that carry the group G antigen are also named *S. dysgalactiae* subspecies *equisimilis*, while animal isolates correspond to *S. canis*.[284] Infections caused by human-associated group G strains include pharyngitis, otitis media, pleuropulmonary infections, cellulitis, septic thrombophlebitis, bacteremia, endocarditis, and meningitis.[665,1140,1145,1167] Group G streptococcal cellulitis and septic arthritis at or near sites of parenteral injection, with bacteremia and subsequent hematogenous complications, have been reported in intravenous drug users.[249] Bacteremia also occurs in the clinical settings of underlying malignancies, puerperal sepsis, septic abortion, chronic pulmonary disease, and HIV infection.

S. canis is a species that was originally proposed in 1986 for β-hemolytic streptococci isolated from dogs and cows.[284] This species causes respiratory tract, cutaneous, genital tract infections, and UTIs in a variety of animals, including dogs and cats.[980]This bacterium has also been recovered from milk specimens from dairy cows with subclinical mastitis. *S. canis* was first isolated from a human with sepsis and leg ulcers in 1997.[78] It was subsequently recovered from blood cultures of a 76-year-old male with leukemia and from an ear wound culture of a 50-year-old female.[1123] Sepsis with cellulitis due to *S. canis* was also reported in a 75-year-old female who has been bitten on the hand by her dog.[998] Analysis by restriction fragment length polymorphism showed that the isolate from the patient's blood and bite wound were identical to that isolated from the oral cavity of her dog. A 5-year retrospective review of *S. canis* infections in 54 patients from Bordeaux, France reported that isolates of *S. canis* had been recovered from cutaneous infections, blood cultures, ENT specimens, vaginal specimens, urine, joint biopsies, and

bronchoalveolar lavage (BAL) specimens.[364] *S. canis* has been isolated and identified in nonhealing or gangrenous ulcers of compromised patients who lived in close companionship with their dogs.[594] In one case, the organism was isolated from blood cultures of a paraplegic patient with multiple open, draining skin lesions and chronic osteomyelitis. The authors of this report point out that the true incidence of *S. canis* infection is not known since most laboratories do not set up phenotypic identification tests on β-hemolytic streptococci that possess the group G Lancefield antigen.[594]

Group C and group G β-hemolytic streptococci are generally susceptible to penicillin, ampicillin, and third-generation cephalosporins like ceftriaxone.[52] Occasional isolates may show intermediate susceptibility to penicillin G, but these are susceptible to the third- and fourth- generation cephalosporins. Some strains may be resistant to erythromycin and other macrolides (e.g., clindamycin, clarithromycin, and azithromycin). Analysis of the susceptibility of β-hemolytic streptococci to fluoroquinolones in the Sentry Antimicrobial Surveillance Program, 1997 through 2004, identified 14 group C and G streptococci among 47 β-hemolytic streptococci with reduced susceptibility to the fluoroquinolones.[83] A study from Portugal found that 12% of 314 *S. dysgalactiae* subsp. *equisimilis* strains were levofloxacin resistant.[833] This latter study found that single mutations in either *parC* or *gyrA* were sufficient to raise the levofloxacin MIC, while higher-level resistance occurred in strains with mutations in both *parC* and *gyrA*. This study also found significant evidence for frequent horizontal gene transfer of *parC* gene sequences between *S. dysgalactiae* subsp. *equisimilis* and *S. pyogenes*.[833] All strains tested to date have been susceptible to vancomycin.

Group F β-Hemolytic Streptococci

Organisms of this group have been called *S. milleri* in the British taxonomic scheme and the **anginosus group** (i.e., *S. anginosus, S. constellatus,* and *S. intermedius*) in American taxonomic schemes. These organisms may be α-, β, or nonhemolytic and may possess the group F, C, G antigen or may be nongroupable. These streptococci characteristically grow as minute colonies on agar media. The colonies are pinpoints after 24 hours and, if β-hemolytic, have a large zone of hemolysis that extends well beyond the margin of the colony. Group F β-hemolytic streptococci are recognized causes of severe suppurative infections, including cellulitis, deep tissue

abscesses, bacteremia, osteomyelitis, and endocarditis (see section on the anginosus

group of the viridans streptococci). The anginosus group will be discussed under the viridans streptococci.

Emerging Pathogens Among the Streptococci

Streptococcus suis

Streptococcus suis deserves special mention because of its economic importance as an important swine pathogen and an occasional zoonotic pathogen for humans.[794,1046,1117] *S. suis* is also an uncommon isolate from dogs, cats, deer and horses. *S. suis* was officially recognized as an extant *Streptococcus* species in 1987.[557] Strains are groupable by Lancefield antisera into groups R, S, RS, and T or are ungroupable. Some strains also react with group D streptococcal antiserum, suggesting possession of the group D lipoteichoic antigen, but this reactivity is due to spurious cross-reactions between groups D and R. Other serotypes having Lancefield groups RS (reacts with both R and S antisera) and T reactivity may be found in both asymptomatic and diseased pigs. *S. suis* strains are encapsulated, and 33 different capsular serotypes, designated 1 through 31, 33, and 1/2 have been recognized; serotypes 32 and 34 have been shown to actually be *Streptococcus orisratti*.[455] Recent evidence from 16S sRNA sequencing showed that *S. suis* serotypes 20, 22, 26, and 33 shared only 94.08% to 97.4% relatedness with the type strain of *S. suis* and that these serotypes may represent different species.[959,960,1028] Sequence analysis of the superoxide dismutase gene (*sodA*) and the recombination/repair protein gene (*recN*) confirmed that serotypes 20, 22, 26, and 33 should be removed from the taxon of *S. suis*.[1028] Multilocus sequence typing (MLST) has been used to divide serotype 2 *S. suis* strains into at least 16 sequence types, with closely related STs being grouped into ST complexes. ST complexes 1, 27, and 87 are found most frequently in Japan, China, and Thailand.[565] Members of ST complex 1 cause the most invasive disease. In North America, most *S. suis* belong to ST28 (51%), ST25 (44%), and ST1 (5%).[345] *S. suis* serotype 1 is found in piglets younger than 6 weeks of age and includes strains bearing the Lancefield group S antigen.

S. suis causes a wide spectrum of severe disease in piglets and pigs, including pneumonia, meningitis, septicemia, and purulent arthritis.[664] Healthy pigs are colonized in the tonsils, nares, respiratory, and gastrointestinal tracts between 5 and 10 weeks of age; carriage rates among healthy animals may be as high as 80%. *S. suis* serotype 2 causes systemic disease in piglets 3 to 20 weeks of age and is also the predominant serotype isolated from infected humans. In infected animals, *S. suis* initially causes fever, anorexia, and other constitutional symptoms, with meningeal

involvement being manifested by rapid onset of seizures, paralysis, and death.[664] Human infection is caused mainly by direct contact with sick or carrier pigs, or raw/undercooked pork contaminated with *S. suis*. Consequently, infections with *S. suis* occur mostly in individuals who work directly with swine (e.g., abattoir and slaughterhouse workers, pig farmers, meat inspectors, veterinarians) or in the industrial processing and manufacture of pork products.[354,671] Slaughtering animals, cutting up carcasses, and handling sick or dead swine were identified as risk factors for *S. suis* infection.[1163] A study in Germany found that 5.3% of 132 persons with exposure to pigs (i.e., butchers, abattoir workers) were colonized with *S. suis* in the nasopharynx, while none of 130 control subjects, with no contact with pigs or pork products, were colonized.[972] Some human cases reported from Hong Kong and Vietnam suggest that processing, preparing, and consuming uncooked or partially cooked pork may also be a risk factor for disease.[494,671] The first human cases of *S. suis* infection were reported in 1968 in Denmark, and the total number of reported cases worldwide is now well over 700.[662] Most reports have come from Asia (China, Japan, Vietnam, Thailand), Europe (the UK, the Netherlands, France, Denmark, Norway, Germany, Spain, Belgium), and North and South America (the United States, Canada, Brazil).[662,1132] China, Thailand, and the Netherlands account for 69%, 11%, and 8% of the total number of cases reported worldwide, respectively. Many of the reported cases occurred during outbreaks of *S. suis* disease. Two large outbreaks of human infection occurred in rural Jianjsu Province during 1998 to 1999, with 25 cases and 14 deaths. In the summer of 2005, the largest outbreak of human *S. suis* infection occurred in the Sichuan Province of China, when 204 human infections and 38 deaths due to *S. suis* septic shock and meningitis were documented.[392,1162,1163] At the time of the human outbreak, a large porcine outbreak of *S. suis* serotype 2 was occurring simultaneously and almost all the patients had a history of contact with pigs or pork products from them. In addition, over 100 cases have been reported from Vietnam over the last 10 years.[671]

S. suis is believed to infect humans percutaneously through cuts, scratches, or abrasions in the skin. Entry via the nasopharynx and the gastrointestinal tract has also been documented, and acute gastroenteritis may be a prominent part of the septic presentation. After infection and following an incubation period of 2–3 to 14 days, disease onset is heralded by an "influenza-like" prodrome, followed by the rapid development of meningitis and bacteremia.[295,344,1117,1166] Initial symptoms include high fever, headache, nausea, vomiting, and nuchal rigidity. Overwhelming bacteremia without the development of purulent meningitis may also occur, and multiorgan system failure, pneumonia, peritonitis, and arthritis may also develop.[559] Toxic shock syndrome, with liver dysfunction, disseminated intravascular coagulation, acute renal failure, and acute respiratory distress syndrome may supervene over time. A common

feature of *S. suis* meningitis is hearing loss with cochlear–vestibular involvement resulting in ataxia and dizziness at presentation or after a few more days.[192,295] Involvement of the eighth cranial nerve is commonly seen and results in unilateral or bilateral hearing loss.[481] This hearing loss may be mild to severe in 4% to 66% of patients.[671] Patients may also develop pechial rashes that evolve into extensive purpura and ecchymoses resembling those of meningococcal disease.[671] Hemorrhagic bullous lesions, skin necrosis, and gangrene of the digits and extremities may result from extensive subcutaneous and skin involvement. A severe sepsis syndrome with a blanching rash reminiscent of toxic shock syndrome and high mortality has also been reported with substantial frequency.[1003,1162,1163]

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Sudden death due to *S. suis* sepsis was reported in a 49-year-old Thai male who had eaten a meal of raw pork 10 days before death.[425] Hematogeous complications of *S. suis* include arthritis, spondylodiscitis, endophthalmitis, peritonitis, pneumonia, and endocarditis.[559,790,1086,1097,1101] In fact, the first case of a human infection reported in North America was a case of *S. suis* endocarditis in a patient in Canada.[1045] Although disease in swine in the United States is not common, *S. suis* has been isolated from swine herds in the United States (e.g., Minnesota and Nebraska). Prior to 2011, only three cases of human infections with *S. suis* had been described in the United States.[345] These patients were diagnosed in New York and Hawaii, with the third case being acquired in the Philippines and diagnosed in California. In 2013, meningitis and bacteremia due to *S. suis* was described in a 60-year-old truck driver who transported swine across the Midwest.[354] He had been on a swine farm where the farmer reported pulmonary infections in some of the animals. A genetically confirmed isolate of *S. suis* was also isolated from the urine of a sick spayed Labrador retriever that had been fed commercial "pigs' ear" treats.[730] The animal responded to therapy with cephalexin.

S. suis strains are susceptible to penicillin, amoxicillin, cefazolin, ceftriaxone, ofloxacin, trimethoprim-sulfamethoxazole, and vancomycin. Some strains are resistant to tetracyclines, erythromycin, and clindamycin; in porcine populations, resistance to tetracyclines and macrolides exceeds 90% and 70%, respectively.[459,540,671,676,794] Sequencing studies have identified resistance determinants for tetracyclines, macrolides, and aminoglycosides, and genetic elements that carry these resistance genes include integrative/conjugative elements, transposons, genomic pathogenicity islands, and bacteriophages.[794] Ceftriaxone is recommended for empiric therapy with or without vancomycin until the bacterial identification is known. Penicillin is also effective as a front-line agent, but should be administered for at least 10 days, as

relapses of *S. suis* meningitis have occurred after 14 days treatment that responded to more prolonged courses of 4 to 6 weeks.

Streptococcus porcinus *and* Streptococcus pseudoporcinus

S. porcinus strains belong to Lancefield groups E, P, U, V, or are nongroupable with Lancefield antisera and cause infections in swine. In human infections, *S. porcinus* has been isolated from the female genital tract (both vaginal and cervical isolates), placental tissues, blood, skin, urine, and wound infections. In 2004, *S. porcinus* was identified as a cause of spontaneous preterm human stillbirth. The organism was isolated from amniotic fluid, endocervical swabs, and gastric fluid from the fetus.[680]Histopathologic examination of the expulsed placental tissues and the fetal viscera showed microabscesses consistent with bacterial chorioamnionitis. Isolates of *S. porcinus* were also shown to cross-react with group B streptococci using commercial reagents from 12 different manufacturers.[371,1024] An analysis of 25 human and 16 nonhuman isolates phenotypically identified as *S. porcinus* using randomly amplified polymorphic DNA-PCR and PFGE analysis revealed two clusters, with all human isolates belonging to one cluster, and all nonhuman isolates belonging to a second cluster.[298] Bekal and associates[67] in Quebec performed 16S rRNA gene sequencing on nine human and seven animal strains of *S. porcinus* and found the same type of clustering, with more than 2.1% dissimilarity between the human and the animal *S. porcinus* isolates. These workers proposed that human isolates be re-named *S. pseudoporcinus*. Like *S. porcinus*, S. *pseudoporcinus* produces small, smooth colonies on blood agar that are surrounded by very large zones of β-hemolysis. A study of 663 nonpregnant women in Pittsburgh enrolled in a group B *Streptococcus* conjugate vaccine trial determined a population prevalence of *S. pseudoporcinus* of 5.4%.[971] Higher rectovaginal colonization rates were associated with increased numbers of sexual partners and sexually transmitted infections. Pereira and colleagues[820] reported on two patients with positive vaginorectal cultures for *S. pseudoporcinus.* One patient lost a pregnancy with twins due to previable rupture of the fetal membranes. The following year during a second pregnancy, vaginorectal cultures were again positive for *S. pseudoporcinus*, and cervical shortening prompted cerclage and antimicrobial therapy. However, the patient delivered without incident 2 weeks later. The second patient delivered preterm after premature rupture of membranes and chorioamnionitis, but the infant did well subsequently. *S. pseudoporcinus* isolates are susceptible to penicillin, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, and vancomycin; resistance to tetracycline has been noted in many isolates.[371,680] Similar antimicrobial susceptibilities have been found for isolates of *S. porcinus*.[320] *S. pseudoporcinus* has also been isolated from a posttraumatic thumb wound.[670]

Streptococcus iniae

S. iniae is pathogen of fish and has been isolated from humans as a cause of bacteremia, meningitis, and endocarditis in individuals who handle contaminated fish. *S. iniae* infects at least 27 different species of fish, including tilapia, rainbow trout, Barramundi cod, Coho salmon, Japanese flounder, and snapper.[10] Infections can be severe and depend on the virulence of the isolate, the host species, the route and location of the infection, and the quality of the aqueous environment. *S. iniae* has become an important fish pathogen, with an estimated annual impact on the US aquaculture industry that exceeds $10 million.[10] The first reported case of human infection associated with *S. iniae* was reported in 1997, when Weinstein and coworkers[1113] from the Public Health Agency in Canada reported nine cases from 1995 to 1996 of invasive *S. iniae* infections. All nine individuals had positive blood cultures, eight had cellulitis of the hands, and one had meningitis, arthritis, and endocarditis. All patients had recently handled live or freshly killed fish, and infection usually followed penetrating injuries from bones or spines of the fish during the cleaning/deboning process. The fish most commonly implicated in these cases was tilapia (*Oreochromis* spp.) raised on aquaculture farms. These investigators reviewed their hospital records and found an additional four patients with *S. iniae* infection. Two additional cases were reported by the CDC retrospectively and included a patient in Texas with bacteremic cellulitis from 1991 and a patient with arthritis of the knee in Ottawa, Canada from 1994.[152] In 2003, two elderly male patients (79 and 81 years

old) in Hong Kong were diagnosed with bacteremic cellulitis and bacteremic osteomyelitis due to *S. iniae*, respectively.[602] Spinal discitis was diagnosed by culture of material from paravertebral abscesses in a 73-year-old Chinese female who regularly cleaned fish and sustained cuts on her fingers.[573] Both patients reported handling fresh fish recently, but had not sustained any penetrating injuries. Isolates of *S. iniae* have also been characterized by the CDC. Facklam and colleagues[321] examined seven clinical isolates; six of these were isolated from blood cultures and the seventh was from a thyroid cyst. Three of these patients have sepsis with cellulitis, while the others presented with an infected mass lesion, toxic shock, pneumonia, and cellulitis. As in previous reports, all patients were of Asian ethnicity, showed equal distribution among males and females, and were of similar ages (60 to 88 years) to the patients in previous reports. Invasive infections with *S. iniae* have been reported in patients with underlying diseases, including diabetes mellitus septic arthritis, gout, and hepatitis C-related liver cirrhosis.[601,978]Asian ethnicity may be important primarily because of the methods involved in handling and cleaning fish for the preparation of

various Asian foods.[601] Isolates of *S. iniae* produce a variety of virulence factors (e.g., M protein-like SiM proteins, C5a peptidase, interleukin-8 protease, streptolysin S, capsular polysaccharides) that are homologous to those found in *S. pyogenes* and that likely play major roles in the pathogenesis of severe *S. iniae* infections.[47] *S. iniae* is included in the database of the Biolog system; in a study comparing this system with amplification of *S. iniae*-specific 16S-23S spacer regions using two primer sets, the Biolog identified 70% of 25 US and Canadian *S. iniae* isolates.[864] Isolates of *S. iniae* are generally susceptible to β-lactams, macrolides, quinolones, and vancomycin.

Streptococcus pneumoniae

S. pneumoniae is the major cause of community-acquired bacterial pneumonia. The organism is transmitted from person to person by close contact. The organism may be harbored in the upper respiratory tract of 5% to 10% of adults, although carriage rates higher than 60% have been reported in closed populations.[738,774] Infants usually become colonized at about 3 to 4 months of age and remain colonized for about 4 months with a given serotype; the peak incidence of pneumococcal colonization occurs by age 1 to 3 years, when the rate may be 40% to 60%.[96,486] In adults, colonization and carriage persist from 2 to 4 weeks but may remain much longer, and carriage rates among adults usually range from 5% to 10%. The duration of carriage decreases with age, and, therefore, tends to be longer in pediatric populations than in adults. Colonization of infants with pneumococci is related to the absence of specific anticapsular antibody and the poorly immunogenic serotypes that are common in this age group.[774] The susceptibility of elderly persons to pneumococcal disease reflects aging of the immune system and consequent diminished production of antibodies, along with general changes in levels of activity, compromised mucociliary clearance mechanisms, malnutrition, or debilitation owing to other underlying chronic diseases such as diabetes and alcoholism. Prior to the availability of highly effective conjugate vaccines, the overall rate of disease due to *S. pneumoniae* was about 15 cases per 100,000 persons per year. The organism classically causes disease in newborns and infants up to 3 years of age, and in adults ages 65 and over.[712,738] Attack rates are four to five times greater among African-Americans than among Caucasians, and this difference appears to be real, although several other factors (e.g., access to health care, other underlying biological or environmental conditions) may also influence these rates. Attack rates are also higher among native Alaskan populations, native American Indian populations, and among Australian Aborigines.[260,431,1036,1111] Patients with underlying abrogations of various host defense mechanisms such as hypogammaglobulinemia, complement component deficiencies, and sickle cell disease,

are also at increased risk for invasive pneumococcal disease. Persons with functional asplenia or those who have had surgical splenectomies are at particularly high risk for development of invasive, often fatal, pneumococcal bacteremia.

Virulence Factors

The virulence of *S. pneumoniae* is primarily related to its ability to resist opsonization, phagocytosis, and intracellular killing by phagocytic cells.[717] This resistance is primarily related to the **polysaccharide capsule** of the organism. There are at least 91 capsular types of *S. pneumoniae*; 23 of these types account for over 88% of pneumococcal bacteremia and meningitis.[738] Capsular polysaccharides in *S. pneumoniae* are comprised of long polymers of repeating units comprised of two to seven monosaccharides, some of which may be in long or branching chains. These polymers are synthesized by addition of carbohydrate moieties to the proximal end of the chain, and most types are anchored to the peptidoglycan and the C polysaccharide of the cell wall. Certain pneumococcal capsular types (e.g., type 3) have biological properties that render these organisms more virulent than others, and pneumococci that belong to different capsular serotypes vary in their ability to elicit humoral antibody responses. These characteristics may account for the greater virulence associated with some capsular types over others. The capsule of these organisms is antiphagocytic and prevents C3b and the Fc region of immunoglobulin molecules from interacting with their receptors on the surface of bacterial cells. Initial adherence of pneumococci to the nasopharyngeal mucosa apparently is not mediated by capsular material but is related to bacterial adhesins that interact with receptors on pharyngeal epithelial cell surfaces. Interaction may occur with mucosal sialic acid via pneumococcal neuraminidase or via pneumococcal cell surface ligands that bind to *N*-acetyl-D-galactosamine-galactose disaccharide residues on respiratory tract cell surfaces. Pneumococci also possess both teichoic acids and LTAs. **Teichoic acids** are covalently linked to the peptidoglycan of the cell wall by phosphodiester linkages, while the **LTAs** are linked to the cell membrane. These molecules are structurally similar and are composed of repeating units containing glucose, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose, *N*-acetyl-glucosamine, phosphocholine, and ribitol-5-phosphate. Repeating units are linked to each other by phosphodiester bonds. Teichoic acids and LTAs of *S. pneumoniae* are also called C polysaccharide and

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F antigen, respectively, and all pneumococcal serotypes possess both of these antigens.

Other cellular products of *S. pneumoniae*, such as the pneumolysin, autolysin, and cell surface molecules, also play a role in pneumococcal virulence.[717] Mutants that are

defective in the production of these various components have diminished virulence in animal models, and immunization with purified products results in the production of specific antibodies that confer partial resistance to challenge infection. **Pneumolysin** is a 53-kDa thiol-activated cytotoxin protein that accumulates within the cells during growth and is released on cell lysis by the autolysin.[875] Pneumolysin interacts with cholesterol in the cell membranes of a variety of host cells, forms oligomers on and in the membrane that evolve into pores, and ultimately lyses the cells. Pneumolysin inhibits the bactericidal activity of phagocytic cells, arrests ciliary motility, stimulates cytokine production by macrophages (especially IL-1, IL-8 and TNF), and activates the classical complement pathway.[205,875] In animal models, pneumolysin has been shown to play a role the pathogenesis of pneumococcal bacteremia, pneumonia, and aspects of inflammation and deafness associated with otitis media and the sequelae of meningitis.[72,1114,1133] **Autolysin** is an *N*-acetyl-muramoyl-L-alanine amidase that, along with a glycosidase enzyme, functions during cell division to separate daughter cells, and at the end of exponential growth, to break down the organisms, causing lytic dispersal of both pneumolysin and α-hemolysin.[77]Autolysin-negative mutants have reduced virulence as compared to wild-type strains. Reduced virulence is likely due to failure to release toxic cell wall components and pneumolysin and, hence, the generation of a greatly reduced inflammatory response. Pneumococci also have several surface proteins that are termed LPXTG-anchored proteins. These proteins are covalently linked to the cell wall peptidoglycan and include hyaluronidase, neuraminidase, and serine protease PrtA. *S. pneumoniae* strains produce two different **neuraminidase enzymes (NanA and NanB**), which cleave terminal sialic acid from cell surfaces to expose *N*-acetyl-glucosamine-galactose moieties that mediate bacterial cell adherence.[128] The NanA enzyme plays an important role in biofilm formation by the organism, and the sialic acid that is release by neuraminidase activity in involved in cell-to-cell signaling, leading to enhanced colonization and invasion.[809,1040] Neuraminidase activity also plays a part in the pathogenesis of otitis media in animal models.[1034] **Hyaluronidase** hydrolyzes hyaluronic acid in the connective tissue and facilitates spread of the organisms. **PrtA serine protease** is produced by all *S. pneumoniae* isolates and antibodies directed against it are found in patients with pneumococcal infections and in healthy individuals.[1178] The exact role of this enzyme in disease pathogenesis is not known, but animal models have demonstrated a role in disease pathogenesis, as mutants lacking PrtA are less virulent and vaccination of animals with the enzyme engenders protective antibodies.[80] **PspA** (pneumococcal surface protein A) is a surface protein that is found on all pneumococci and comprises the immunodominant antigenic protein of pneumococci. PspA inhibits phagocytosis by blocking the deposition of complement on the bacterial cell surface.[738] Antibodies directed against PspA from a given pneumococcal strain protect experimental animals

against challenge with both the homologous strain and heterologous strains and mutant pneumococci that are defective in PspA production are avirulent. Intranasal inoculation of mice with PspA prevents both respiratory tract colonization and the development of invasive disease. For this reason, PspA has potential for use as a vaccine candidate antigen.[120]

Pneumococcal Vaccines

Two types of vaccines are now available for the prevention of disease due to *S. pneumoniae*: pure polysaccharide vaccines and polysaccharide-protein conjugate vaccines. The **pneumococcal polysaccharide vaccines** (PPSV23; Pneumovax23, Merck Sharpe and Dohme) is composed of a mixture of 23 pneumococcal capsular polysaccharides. The 23 capsular serotypes included in these vaccines are 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. The Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention (CDC) recommends vaccination of (a) persons who are aged 65 years or greater, (b) persons aged 19 to 64 years who have chronic illness (e.g., chronic cardiovascular disease, cardiomyopathies), chronic pulmonary disease (e.g., COPD, emphysema, but not asthma, smokers), diabetes mellitus, alcoholism, liver disease (e.g., cirrhosis), or CSF leaks, (c) those who have functional or anatomic asplenia (e.g., sickle cell disease, splenectomy), (d) persons aged 19 to 64 who live in environments or settings that render them at risk for invasive pneumococcal disease (i.e., Alaskan Natives, certain American Indian populations, residents of nursing homes and other long-term care facilities), (e) individuals 2 years of age or greater who are immunocompromised (e.g., those with HIV infection, leukemia, lymphoma, Hodgkin's disease, multiple myeloma, malignancies, chronic renal failure, nephrotic syndrome, other conditions associated with immunosuppression [e.g., bone marrow transplantation]), and (f) persons receiving immunosuppressive chemotherapy.[153,158,161] A second dose of PPSV23 is recommended 5 years after the first one for individuals aged 19 through 64 years for those who are immunocompromised or have functional or anatomic asplenia. The full immunization schedule for PPSV23 is found in Box 13-4. PPSV23 has been demonstrated to be safe and efficacious and is relatively inexpensive. Pure polysaccharide vaccines provoke a B-cell antibody response but are T-cell-independent antigens, so a protective immune response is not seen in children less than 2 years old. Other drawbacks to the pneumococcal polysaccharide vaccines include its inability to affect nasopharyngeal pneumococcal carriage and, therefore, the spread of organisms from person to person, and its limited efficacy in patients with underlying hematologic malignancies or immunodeficiency states.

In February of 2000, PCV7 (**P** neumococcal **C** onjugate **V** accine **7**), a new 7-valent pneumococcal conjugate vaccine (Prevnar, Wyeth Lederle Vaccines, Pearl

River, NY) was licensed for use in infants and children.[155] PCV7 was composed of an inert diphtheria toxin carrier protein (CRM197) covalently linked to the capsular polysaccharide antigens of seven *S. pneumoniae* serotypes. The capsular serotypes that were included in PCV7 were serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. At the time, these serotypes were associated with

about 80% of pneumococcal disease occurring in children less than 5 years of age.[155,824,832] The PCV7 conjugate vaccine provoked the brisk T-cell dependent antibody response that is required for optimal antibody production and ability to mount an anamnestic response via immunologic memory. Large studies have demonstrated that the pneumococcal heptavalent conjugate vaccine was efficacious in preventing invasive disease in young children due to vaccine serotypes of *S. pneumoniae*.[531,859] Following widespread use of PCV7, the reduction in invasive pneumococcal infections due to serotypes included in the vaccine was 100% in children less than 5 years of age and 94% when all ages were considered. Interestingly, no increases in infections due to nonvaccine serotypes were observed during the clinical efficacy trials, and the rates of nasopharyngeal carriage of *S. pneumoniae* were found to be decreased in vaccinated children, a phenomenon not seen in polysaccharide vaccine recipients. A small efficacy study found that PCV7 provoked protective antibody responses in children with sickle cell disease as well.[776] By the end of 2007, only about 2% of invasive pneumococcal disease in children less than 5 years of age was due to serotypes included in PCV7. During this post PCV7-vaccination period, some communities noted an increase of disease (e.g., meningitis) caused by nonvaccine serotypes (e.g., serotype 7F) in children less than 30 days of age.[783] A review of the literature on the global impact of the seven-valent conjugate vaccine found that, among vaccine eligible children in the postvaccination period, a reduction in invasive pneumococcal diseases ranging from 39.9% in Spain to 99.1% in the United States was documented.[740] Since the introduction of the PCV7 pneumococcal conjugate vaccine in 2000, hospitalizations for childhood pneumonia have declined, and this has been sustained over the subsequent 12 years. Less drastic but measurable declines in hospitalization of older adults with community-acquired pneumonias has also occurred over the same period.[400]

In 2010, a new pneumococcal polysaccharide conjugate vaccine—PCV 13—was licensed by the FDA and recommended for use in children by the CDC and the ACIP.[25,158] This vaccine includes the serotypes found in PCV7 plus six additional serotypes: 1, 3, 5, 6A, 7F, 19A. The additional serotypes included in PCV13 accounted for 64% of 4,600 cases of invasive pneumococcal disease documented in

2007 in children less than 5 years of age.[159] PCV13 is recommended for all children aged 2 to 59 months and children aged 60 to 71 months with underlying conditions that increase their risk for invasive pneumococcal disease. The complete immunization schedule for PCV13 includes recommendations that should be followed for children who may have received previous PCV7 vaccinations.[160,161] At the end of 2011, PCV13 was approved for adults aged 50 years and greater for the prevention of invasive *S. pneumoniae* disease.[162] Box 13-4 provides additional information on PPSV23 and PCV13 regarding target groups for receipt of vaccines and immunization schedules.

Clinical Spectrum of S. pneumoniae

In the appropriate host, *S. pneumoniae* gains access to the alveolar spaces by aspiration or inhalation and eventually may cause a lobar pneumonia, with consolidation and bacteremia.[490,738] Conditions that are recognized to predispose adults to pneumococcal disease include underlying bronchopulmonary disease and compromised humoral immunity. Conditions that abrogate the humoral immune response (e.g., myeloma, lymphoma, chronic lymphocytic leukemia, hepatic cirrhosis, complement component deficiencies) probably have the greatest influence on individual susceptibility to pneumococcal infection. The incidence and severity of pneumococcal disease are also increased among those persons with defects in upper respiratory tract clearance mechanisms, including smokers, and individuals with asthma, chronic bronchitis, COPD, or bronchogenic and squamous cell carcinoma of the lung. Viral respiratory tract infections also predispose to pneumococcal infection of the respiratory tract because these agents also damage the bronchial clearance mechanisms. The onset of pneumococcal pneumonia is generally abrupt, even in patients in whom a prior respiratory viral infection is the principal predisposing factor. In older children and young adults, symptoms include shaking chills, followed by sustained fever, cough, and production of purulent, often blood-tinged sputum. In elderly patients, the infection may present insidiously over several days.[655] Symptoms may vary from a minimal cough, with an actual decrease in temperature, to a fulminant presentation, leading rapidly to shock and death. Blood cultures are positive in 20% to 30% of patients with pneumococcal pneumonia. Older adults usually have additional conditions that put them at higher risk for serious disease, including malignancies, alcoholism, heart disease, COPD, and diabetes. Chest X-rays from patients with pneumococcal pneumonia demonstrate lobar consolidation in about 40% to 50%, with the remaining showing a patchy bronchopneumonic pattern. Infiltrates due to pneumococcal disease tend to be unilateral and involve the alveoli rather than the bronchioles and interstitial tissues. Complications of pneumococcal pneumonia include lung abscess, pericardial infections, empyema, pleural effusions, and

endocarditis.[315,402,465,645,729,746,1005] Pleural effusions and empyemas may be visualized on chest films, but are more accurately delineated by computed axial tomography (CT) and ultrasound diagnostic approaches. If these fluid collections are sufficiently large, drainage via chest tube placement may be necessary to effect cure, along with antimicrobial therapy. CT scans and ultrasound are also helpful for diagnosing necrotizing pneumonias and deep lung abscesses due to *S. pneumoniae*. Hemolytic uremic syndrome (HUS) may also occur as a complication of pediatric *S. pneumoniae* infection and accounts for 4.6% to 15% of all childhood HUS.[180,240,957,1085] This complication occurs with an incidence of 0.04% to 0.6% and is seen primarily in children less than 2 years of age. Of the described cases of HUS, 72% were associated with pneumonia with or without effusion/empyema, 29% occurred with pneumococcal meningitis, and 5% was seen in patients with both conditions.[240] HUS associated with pneumococcal pneumonia was recently reported in adults as well.[22] The case fatality rate for pneumococcal pneumonia is about 5% but may approach 20% to 30% when accompanied by bacteremia. Pneumococcal bacteremia without pulmonary involvement is an entity that occurs in immunologically compromised hosts. Underlying diseases or conditions that predispose patients to recurrent or relapsing pneumococcal bacteremia include splenectomy, acute myelogenous leukemia, bone marrow transplantation, sickle cell disease, HIV infection, and short bowel syndrome.[186]

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BOX 13-4

Pneumococcal Vaccines and Immunization Schedules for Infants and Children (see text as well)[25,153,155,158-162]

PPSV23—23 Valent Pneumococcal Polysaccharide Vaccine

Pneumovax 23 (Merck, Sharpe & Dohme) and Pneu-Immune 23 (Lederle)

Composed of a mixture of 23 pneumococcal capsular polysaccharides, including types 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F.

Recommended for (a) persons greater than or equal to 65 years of age, (b) persons aged 2–64 years who have chronic or advanced cardiovascular disease, chronic pulmonary disease (e.g., COPD, emphysema, asthma), diabetes mellitus, alcoholism, liver disease, chronic renal insufficiency, or CSF leaks, (c) those who have functional or anatomic asplenia (e.g., sickle cell disease, splenectomy), (d) individuals 2 years of age or greater who are immunocompromised, (e.g., HIV infection, leukemia, lymphoma, Hodgkin's disease, multiple myeloma, malignancies, chronic renal failure, nephrotic syndrome, bone marrow transplantation, and persons receiving chemotherapy) and (e) persons aged 2–64 who live in environments or settings that place them at risk for invasive pneumococcal disease (i.e., Alaskan Natives, certain American Indian populations, residents of nursing homes and other long-term care facilities).

Children aged more than 2 years with underlying medical conditions should receive PPSV23 after completing all recommended doses of PCV13. One dose should be given at ages 2 years or more and at least 8 weeks after the last PSV13 dose. Those children who received PPSV23 previously should receive the recommended PSV13 doses (at right).

Children aged 24–71 months with underlying medical conditions who received less than 3 doses of PCV7 before the age of 24 months should receive 2 doses of PCV13 followed by one dose of PPSV23 8 or more weeks later.

Children aged 24–71 months with underlying conditions and who received any incomplete schedule of 3 doses of PCV7 before age 24 months should receive one dose of PCV13 followed by one dose of PPSV23 after 8 weeks or more.

A second PPSV23 dose is recommended 5 years after the first PPSV23 dose for children with underlying medical conditions (i.e., sickle cell hemoglobinopathies, congenital/acquired/functional asplenia, congenital or acquired immunodeficiencies, immunosuppressive therapies, chronic illnesses, presence of cochlear implants or cerebrospinal fluid leaks).

PCV13—13 Valent Polysaccharide Conjugate Vaccine

Prevnar 13 (Wyeth Pharmaceuticals, a subsidiary of Pfizer, Inc.)

Composed of an inert diphtheria toxin carrier protein (CRM197) that is covalently linked to the capsular polysaccharide antigens of thirteen pneumococcal serotypes, including serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F.

PCV13 is recommended for children aged 2 through 59 months who have not received any previous PCV7. PCV13 is administered as a 4-dose series beginning at 2 (may be administered as early as 6 weeks of age), 4, 6 months, and 12–15 months of age. For infants aged 2–6 months, those children receiving the first dose at 6 months of age should receive 3 subsequent doses at intervals of 4–8 weeks. A fourth booster is recommended at age 12–15 months and at least 8 weeks after the third dose.

Healthy children aged 7–59 months who were not previously vaccinated with PCV7 or PCV13 should receive 1–3 doses of PCV13 depending on when vaccination begins and whether underlying conditions are present.

Children aged 24–71 months with underlying conditions should receive 2 doses of PCV13. Three doses of PCV13 are recommended for infants aged 7–11 months, with a 4 week interval between doses, with the third dose being given at age 12–15 months.

Two doses of PCV13 are recommended for children aged 1–23 months, with 8 weeks between the two doses. Unvaccinated healthy children aged more than 24–59 months should receive one dose of PCV13, but unvaccinated children with underlying medical conditions should receive 2 doses with 8 weeks between them.

In those children aged less than 24 months who have received one or more doses of PCV7 should complete the series with PCV13. Those children aged 12–23 months who have received 3 doses of PCV7 should receive a single dose of PCV13 at least 8 weeks after the last PSV7 dose (fourth and final PCV dose). No additional PCV13 doses are required for children aged 12–23 months who received 2–3 doses of PCV7 before age 12 months and at least one dose of PCV13 at an age more than 12 months.

For those children greater than 2 years of age, a single PCV13 dose if recommended for all children aged 24–59 months with any incomplete vaccination schedule. For children aged 24–71 months with underlying medical conditions who have received any incomplete schedule of less than three doses of either PCV7 or PCV13 before age 24 months, two doses of PCV13 are recommended. For children with underlying medical conditions who have received three doses of either PC7 or PCV13, a single dose of PCV13 is recommended through age 71 months.

A single PSV13 dose is recommended for all children aged 14–59 months who received 4 doses of PCV7. For children with underlying medical conditions, a single PCV13 dose is recommended through 71 months of age.

For children aged 6–18 years with certain at-risk conditions (i.e., children with sickle cell hemoglobinopathies, congenital/acquired/functional asplenia, congenital or acquired immunodeficiencies, immunosuppressive therapies, chronic illnesses, presence of cochlear implants or cerebrospinal fluid leaves) who have not received PCV13 previously, a single PCV13 dose should be given.

Pure polysaccharide vaccines provoke a B-cell antibody response but are T-cell-independent antigens. Protective immune responses are not seen in children less than 2 years old. The vaccine also has marginal effects on nasopharyngeal pneumococcal carriage has limited efficacy in patients with underlying hematologic malignancies or immunodeficiency states.	Provokes a T-cell-dependent antibody response that is required for optimal antibody production and ability to mount an anamnestic response. Studies have demonstrated efficacy in preventing invasive disease in young children due to vaccine serotypes, with prevention of greater than 90% of invasive pneumococcal disease due to vaccine serotypes. Rates of nasopharyngeal carriage of *S. pneumoniae* are also decreased in vaccinated children.

S. pneumoniae is also the most common cause of bacterial meningitis in older adults, followed by *N. meningitidis*, *S. aureus*, and other miscellaneous gram-positive organisms (e.g., *L. monocytogenes*).[462] With the increasing use of the conjugate vaccines for *Haemophilus influenzae* type b, a 2001 to 2004 multicenter study reported that the pneumococcus was the third most common agent in children 1 to 3 months of age (14%), with group B streptococci and *N. meningitidis* being the first and second most common agents (39% and 32%, respectively).[765] *S. pneumoniae* was the most common agent in children ages 3 months to 3 years (45%), followed by *N. meningitidis* (34%) and group B streptococci (11%). In the 3-year- to 10-year-old age group, *S. pneumoniae* (47%) and *N. meningitidis* (32%) were the most common etiologic agents, with *N. meningitidis* exceeding *S. pneumoniae* in the 10- to 19-year-old children.[765] A 2011 report found that, while group B streptococci remained the most common agent of meningitis in children less than 2 months of age (86%) and the meningococcus was the cause of meningitis in the 11- to 17-year-old age group, the pneumococcus prevailed in all other pediatric age groups.[1021] The introduction of the PCV7 vaccine in 2000 caused a significant decline in the incidence of pneumococcal meningitis in those countries where it is part of the national immunization schedule. This decline was most evident among children less than 2 years of age, but there was a notable increase in meningitis due to pneumococcal serotypes not included in the PCV7 vaccine (e.g., serotype 19A).[580] This serotype in included in the PVC13 vaccine formulation. In adults, *S. pneumoniae* accounts for about one-third of community-acquired meningitis in the United States and has an associated mortality of 20% to 25%. *S. pneumoniae* meningitis usually occurs as a result of seeding of the meninges during bacteremia, with the organisms probably entering via the choroid plexus. In adults, about 60% to 70% present with symptoms of fever, nuchal rigidity, and mental status changes. In infants, crying, irritability, malaise, failure to feed, vomiting, and seizures may dominate the clinical picture; only about one-third of these babies will develop a bulging fontanelle. In elderly and/or compromised adults, the presentation may be insidious, with lethargy, obtundation, and little or no fever being the norm.[462] However, meningitis along with ultimately fatal septic shock may occur in patients who are profoundly immunosuppressed (e.g., bone marrow transplant patients).[416] Pneumococcal endocarditis is an uncommon complication of

pneumococcal meningitis and is associated with an unfavorable clinical outcome.[660] *S. pneumoniae* is also the leading cause of meningitis following skull fractures. Head trauma resulting in basilar skull fracture with leakage of cerebrospinal fluid interrupts the integrity of the dura mater and may allow direct entry of organisms into the central nervous system from an adjacent site of infection (e.g., sinusitis, mastoiditis, otitis media).[738] Rare complications of pneumococcal meningitis include spinal epidural abscess, vertebral osteomyelitis, and paraspinal abcess.[81]

In pediatric populations, *S. pneumoniae* accounts for 40% to 50% of cases of acute otitis media and has been associated with sinusitis and mastoiditis.[829,953] Because of vaccination with conjugate pneumococcal vaccines, the etiology of acute otitis media continues to change over time. In patients with otitis media, the tympanic membrane appears inflamed, immobile, and bulging on examination with an otoscope. Most children with otitis media will also have rhinorrhea and nasal congestion, and about two-thirds will be febrile. Complications include tympanic membrane perforation, mastoiditis, facial nerve paralysis, bacteremia, and septic arthritis.[579] Specimens for diagnosis are best obtained via myringotomy or tympanocentesis. Pneumococci, along with nontypeable *H. influenzae*, are also responsible for about 70% of acute and subacute sinus infections.

S. pneumoniae is an infrequent cause of endocarditis, pericarditis, osteomyelitis, septic arthritis, peritonitis, female pelvic infections, neonatal infections, and skin/soft tissue infections. Most patients have underlying disease, such as diabetes, malignancies, alcoholism, systemic lupus erythematosus, or HIV infection, and have other foci of pneumococcal disease, such as meningitis or pneumonia. Pneumococci account for less than 3% of cases of bacterial endocarditis and 3% to 7% of all cases of pediatric endocarditis.[382] Pneumococcal endocarditis follows an acute course, is associated with valve destruction and formation of aortic perivalvular abscesses, and has a mortality rate of 24% up to over 50%.[529,621,931,1005] Treatment of pneumococcal endocarditis with medical therapy alone is associated with poor outcomes. Many patients actually have normal heart valves, however, in one study of pneumococcal endocarditis in children, congenital heart disease was the only identifiable risk factor.[190]Primary peritonitis caused by pneumococci was once a common clinical entity in children in association with nephrotic syndrome, but it now occurs primarily in adults with cirrhosis and other liver diseases. The pathogenensis of pneumococcal peritonitis is via the hematogenous route or local inoculation.[300] Osteoarticular infections, osteomyelitis and septic arthritis caused by *S. pneumoniae* often occur in children with sickle cell disease.[188,772,789,1160] Higher rates of pneumococcal septic arthritis have also been documented in

children with HIV infection.[865] In adults, pneumococcal septic arthritis usually occurs in patients with a focus of infection at another site (i.e., bloodstream infection, meningitis, sinusitis) and involves more than one joint in about one-quarter to one-third of patients.[413,848] Pneumococcal septic arthritis occurs more frequently in patients with underlying joint disease (e.g., rheumatoid arthritis) and usually involves native joints, although prosthetic joint involvement has been described.[848] In Japan, polyarticular pneumococcal septic arthritis was diagnosed in a 28-year-old man following a single infusion of infliximab for rheumatoid arthritis.[435] The patient had no evidence of pneumococcal disease elsewhere, except for positive joint fluid and blood cultures. Most cases can be managed with appropriate antimicrobial coverage and arthrocentesis. In some women, *S. pneumoniae* may be a transient part of the vaginal flora, and pelvic, obstetric, and gynecologic infections (e.g., bartholinitis, pelvic inflammatory disease) may occur, particularly with predisposing conditions, such as the presence of an intrauterine device or recent gynecologic surgery.[632,811] Tubo-ovarian abscesses due to *S. pneumoniae* may also be a source of pneumococcal peritoneal infections.[835] Neonatal pneumococcal infections have been reported and represent 1% to 11% of cases of neonatal sepsis.[461,698] These babies may present with meningitis, bacteremia, and/or pneumonia arthritis/osteomyelitis, or otitis media, and are usually 2 to 3 weeks of age and full-term at presentation. The likely source of infection in these babies is the mothers' genital tract.[390,698] *S. pneumoniae* has also been recognized as a rare but significant cause of soft tissue infections, including facial and periorbital cellulitis, necrotizing fasciitis, rhabdomyolysis, pyomyositis, and abscesses.[58,92,367,373,498,553,806,1137,1154,1165,] Facial and periorbital cellulitis occurs predominantly in children as a complication of upper respiratory tract infection, pneumococcal sinusitis, or trauma involving the eyelids.[373,818] In one report on 56 patients with soft tissue infections caused by *S. pneumoniae*, surgical wound infections were the most common, followed by burn wound infection, pyomyositis, cellulitis, and perineal or scrotal abscesses.[367] Most patients had underlying diseases or conditions, including burns, diabetes, neoplasms, psoriasis, or HIV infection. Necrotizing fasciitis due to *S. pneumoniae* occurs in elderly patients with multiple risk factors, including minor trauma, immunosuppression secondary to medication, alcoholism, underlying disease (e.g., diabetes, systemic lupus erythematosus), and cirrhosis.[264,498,806,1154] Pneumococcal rhabdomyolysis is a rare condition that occurs in elderly individuals with pneumococcal pneumonia, although other infected sites (e.g., meningitis, cellulitis, arthritis) may be present.[58,92,924] Disease presentation usually occurs at the onset of pneumococcal infection and includes limb weakness and generalized or localized muscle pain. Muscle biopsies may appear normal or may show focal fiber degeneration, muscle tissue breakdown, and necrosis, and peripheral leukocytosis,

elevated creatinine kinase, and myoglobinuria are usually present.

Antimicrobial Susceptibility of S. pneumoniae

A major concern surrounding *S. pneumoniae* is the emergence of resistance to antimicrobial agents, especially penicillin.[241,503,909,1129] Pneumococci with decreased susceptibility to penicillin were first reported in the 1960s. During the 70s and 80s, penicillin resistance among *S. pneumoniae* was rare in the United States, with only 0.2% of strains showing resistance. By the mid-1990s, 35% of the aggregate pneumococcal isolates from the United States demonstrated reduced susceptibility to penicillin.[49,909] A study published in 1998 on 845 pneumococcal isolates from US medical centers found that the percentages of strains with intermediate- and high-level resistance to penicillin were 21.8% and 16.0%, respectively.[294] Studies published at the beginning of the new millennium among populations in the US national surveillance system indicated that decreased susceptibility to penicillin among *S. pneumoniae* isolates ranged from 14.7% to 35.1% depending on the geographic region, and were more commonly seen in children less than 5 years of age.[1129] Pneumococcal serotypes included in PCV7 and PSV23 accounted for 78% and 88% of the penicillin-resistant strains, respectively. A study of 1,531 clinical isolates of *S. pneumoniae* collected from 33 US medical centers during the winter of 1999 to 2000 found that 34.2% of isolates were penicillin–nonsusceptible (MIC $\geq$ 0.12 μg/mL) and 21.5% demonstrate high-level penicillin resistance (MIC $\geq$ 2 μg/mL).[293] Among 10,103 community-acquired respiratory tract isolates of *S. pneumoniae* collected in 2000 to 2001 from 206 centers in 154 regions of the United States, 38.9% showed decreased susceptibility to penicillin.[292] During the same period, decreased susceptibility to penicillin was also documented in foreign isolates of *S. pneumoniae*. In a 2003 study, the percent of strains with diminished susceptibility to penicillin was 6.5% in the United Kingdom, 9.1% in Germany, 12.4% in Italy, 36.4% in Greece, 54.5% in Spain, and 56.7% in France.[524] International data collected on 3,778 *S. pneumoniae* isolates from 1999 to 2000, found that resistance to penicillin was very high in Middle eastern countries (65.5%), Africa (64%), and Asia (60.4%), but lower in North America (40.3%), Europe (36.9%), and the South Pacific (31.8%).[106] Data from the PROTEKT US longitudinal surveillance study on 39,495 *S. pneumoniae* isolates collected in the United States from 2004 through 2008 found that the percent of isolates resistant to penicillin increased from 12.5% in 2004 to 20% in 2008.[513] Again, marked regional differences in penicillin susceptibility were noted. Isolates of *S. pneumoniae* that showed decreased susceptibility or full resistance to penicillin G were also less susceptible to other penicillins and cephalosporins of all generations, and MICs to all β-lactam agents increased as the penicillin MICs increased.[293] As a corollary to this

observation and trend, treatment failures for serious pneumococcal infections were reported with previously useful agents such as cefotaxime, cefuroxime, and ceftriaxone.[134,146,503,520]

Penicillin resistance in *S. pneumoniae* is associated with altered penicillin-binding proteins (PBPs) that have a decreased affinity for binding penicillin to the bacterial cell wall.[477] PBPs are actually transpeptidase enzymes that are involved in peptidoglycan synthesis, and six PBPs—designated 1a, 1b, 2a, 2b, 2x, and 3—have been identified in *S. pneumoniae*. Strains that are resistant show mosaic arrangements in PBP-encoding genes 1a, 2b, and 2x.[858] When penicillin resistance determinants in *S. pneumoniae* were initially described, alterations in PBP1a were reported to be essential for high-level penicillin resistance, but additional work has

shown that changes in PBP1a along with simultaneous mutations in PBPs 2b and 2x are associated with high-level penicillin and ceftriaxone resistance, with such isolates having MICs of >4 μg/mL.[394,395,478,734,856] Mutations in these genes leading to high-level penicillin and ceftriaxone resistance are seen among a wide range of pneumococcal serotypes, including serotypes 14, 19F, and 23F. Accumulated mutations in the *pbp* genes are associated with the simultaneous increases in MICs of penicillin and third-generation cephalosporins. Other non–PBP-mediated β-lactam resistance determinants are also involved in penicillin and cephalosporin resistance in *S. pneumoniae*.[125] Breakpoints for pneumococci differ depending on the site of isolation. Isolates from the CSF (i.e., meningitis) with penicillin MICs of ≤0.06 μg/ml are susceptible, and those with MICs of ≥12 μg/ml are considered resistant. For nonmeningeal infections (i.e., isolates from sites other than CSF), isolates with penicillin MICs of ≤2.0 μg/mL are considered susceptible, those with MICs of 4.0 μg/mL are intermediate, and those with MICs ≥ 8 μg/mL are considered resistant.[204]

Besides the penicillins and cephalosporins, pneumococcal resistance to several other antimicrobial agents, including macrolides, sulfonamides, and tetracyclines, and fluoroquinolones has been reported.[241,293,294,525,526,1129] In an international study published in 2004, macrolide resistance among *S. pneumoniae* isolates was highest in Asia (51.7% resistant), followed by Europe (26.0% resistant), North America (21.6% resistant), the Middle East (13.7% resistant), the South Pacific (10.6% resistant), and Africa (10% resistant).[106] Prior to 1998, the percentages of US *S. pneumoniae* strains resistant to erythromycin, clarithromycin, and azithromycin were 14.3%, 12.7%, and 11.7%, respectively.[294] For the period from 1994 through 2000, pneumococcal resistance to erythromycin increased from 23.6% to as high as 34%.[293] Data from the PROTEKT US longitudinal surveillance study conducted between 2000 and 2004

found that resistance rates for macrolide antimicrobial agents (erythromycin, clarithromycin, and azithromycin) have remained fairly stable during this time period, ranging from 27.5% and 31%.[513] In the 2010 LEADER Surveillance study, 38.5% of 803 pneumococcal isolates were resistant to erythromycin and 20.4% were resistant to clindamycin.[347] Macrolide resistance in *S. pneumoniae* is mediated either by the *erm(B)* gene or the *mef(A)* gene.[619,990,1177] The former encodes a methylase enzyme that results in resistance to the macrolide-lincosamide-streptogramin B agents, while the latter gene encodes an antibiotic efflux pump. The *erm(A)* gene is not often found in pneumococci, but other mechanisms of macrolide resistance may involve mutations in the genes encoding ribosomal proteins (e.g., proteins L4 and L22) or mutations in the rRNA (i.e., 23S rRNA).[857] Resistance to sulfonamides, particularly trimethoprim-sulfamethozaxole (SXT) has also increased substantially. The 1997 SENTRY surveillance project found that 19.8% of 845 US and 15.8% of 202 Canadian pneumococcal isolates were resistant to SXT.[294] Of 1,531 *S. pneumoniae* collected during 1999 through 2000, 30.3% were SXT-resistant.[293] The data from the 2000 to 2004 PROTEKT US survey reported that SXT resistance slightly decreased between 2000 (33.9%) and 2004 (24.1%).[513] Data from the 2010 LEADER Surveillance program placed pneumococcal resistance to SXT at 23.8%.[347] Resistance to tetracycline in North America has remained fairly stable, ranging from about 9.0% to 10.9% in the mid-1990s to between 14.6% and 15.9% in the period 2000 through 2004. However, tetracycline resistance was observed in 22.6% of 803 *S. pneumoniae* tested in the 2010 surveillance study.[347] Interestingly, fluoroquinolone resistance has been slow to develop in *S. pneumoniae*.[106] Fluroquinolone resistance in pneumococci is due to chromosomal mutations involving the *parC* gene for topoisomerase IV and the *gyrA* gene of DNA gyrase.[503] In the period from 1994 through 2000, rates of ciprofloxacin resistance ranged from 1.4% to 1.8% in the United States.[293] An international surveillance study found that only 0.4% of 1,870 pneumococcal isolates were resistant to levofloxacin, moxifloxacin, or gatfloxacin.[524] In the 2003 SENTRY study, none of over 6,000 *S. pneumoniae* isolates were resistant to levofloxacin, and less than 1% were resistant to ciprofloxacin. The PROTECKT US data for 2000 through 2004 reported levofloxacin resistance rates of 0.8% to 1.1%.[513] Low rates of fluoroquinoline resistance in *S. pneumoniae* have been corroborated in many surveillance surveys.[106,347] In a study of 803 *S. pneumoniae* isolates tested as part of the LEADER Surveillance program, 99.9% and 99.6% were susceptible to linezolid and tigecycline, respectively.[347] Ceftaroline, a broad-spectrum parenteral cephalosporin with activity against MRSA, demonstrates good activity against *S. pneumoniae*. In multicenter studies, ceftaroline inhibited 98.7% of isolates at the FDA-susceptible breakpoint of ≤0.25 μg/mL and was 16-fold more active than ceftriaxone.

This agent also showed good activity against multidrug-resistant *S. pneumoniae*, with 90% of isolates having MICs of ≤0.25 μg/mL. The 44 nonsusceptible isolates in this group had ceftaroline MICs that were only one dilution higher (i.e., 0.50 μg/mL).[331] *S. pneumoniae* remains susceptible to vancomycin. Additional information regarding antimicrobial susceptibility of *S. pneumoniae* and methods for antimicrobial susceptibility testing are found in Chapter 17.

In 2004, Arbique and coworkers[36] isolated an unusual α-hemolytic *Streptococcus* that resembled *S. pneumoniae*. Because of certain phenotypic characteristics, this organism was named *Streptococcus pseudopneumoniae*. A subsequent study conducted by Keith and colleagues[550] in New Zealand evaluated the clinical, laboratory and treatment data on 33 patients whose respiratory tract specimens grew *S. pseudopneumoniae*. These patients ranged in age from 15 to 89 years and all patients had lower respiratory tract symptoms with cough. Over 75% of the patients presented with exacerbations of COPD. A case-control study suggested that COPD was significantly more common among those patients with *S. pseudopneumoniae* than among control patients who did not have the organism in respiratory tract specimens.[550] These workers stressed that subsequent studies were needed to determine the natural habitat of *S. pseudopneumoniae* and the role of this newly described organism in other infections, including pneumonia. A subsequent study by Harf-Monteil and colleagues[428] in France determined that the organism was quite uncommon, with only a single isolate recovered over a 7-month period during which 120 isolates of *S. pneumoniae* were isolated. These workers also showed that the type strain of *S. pseudopneumoniae* had pathogenic potential in a mouse peritonitis sepsis model, while the single clinical patient isolate was avirulent. A study performed

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in India found that the six isolates of *S. pseudopneumoniae* were the predominant or only isolate from purulent sputum specimens and all six patients had symptomatic lower respiratory tract infections.[892] In a study of the antimicrobial susceptibility profile of 95 sputum isolates of *S. pseudopneumoniae*, 32% showed reduced susceptibility to penicillin and ampicillin.[549] Among these isolates, resistance to erythromycin, azithromycin, clindamycin, and tetracycline was seen in 35%, 32%, 16%, and 45%, respectively. All isolates were susceptible to cefotaxime, ceftriaxone, cefepime, meropenem, levofloxacin, and vancomycin. Laurens and colleagues[603] also examined the susceptibility of 140 *S. pseudopneumoniae* isolates and found that 21% showed reduced susceptibility to penicillin, 57% were resistant to erythromycin, and 43% were resistant to tetracycline. All isolates were susceptible to vancomycin, levofloxacin, and gentamicin. In the study conducted by Sariya Mohammadi and

Dhanashree, all six *S. pseudopneumoniae* isolates were resistant to erythromycin and trimethoprim-sulfamethoxazole, and four had reduced susceptibility to penicillin, three were tetracycline-resistant, and two were ciprofloxacin-resistant.[892] Both *S. pneumoniae* and *S. pseudopneumoniae* belong to the Mitis Group of the viridans streptococci. Molecular genetic studies of *S. mitis* and *S. pseudopneumoniae* have identified homologues of virulence genes that are found in *S. pneumoniae*. In five *S. mitis* isolates, Johnston et al.[522] identified genes having 67% to 82% sequence homology with the pneumolysin (*ply*) and neuraminidase A (*nanA*) genes of *S. pneumoniae*, and that the *nanA* genes identified in these organisms shared 99.4% to 99.75% sequence homology with the corresponding genes of *S. pneumoniae*.

Viridans Streptococci

The viridans streptococci include several species of α- and nonhemolytic streptococci, and most of them constitute part of the normal upper respiratory and genitourinary tract microbiota. Before the 1980s, species of viridans streptococci were described entirely by their phenotypic characteristics and were placed into groups based on some of these characteristics. As shown in Box 13-2, these organisms are divided into the Mitis/Sanguinis Group, the Mutans Group, the Anginosus Group, and the Bovis Group. This section deals primarily with the Mitis/Sanguinis, Mutans, and Salivarius groups and their clinical significance. Because of certain unique characteristics and their clinical manifestations, the Anginosus and Bovis Groups will be described in subsequent sections. Analysis of the viridans streptococci by molecular and genetic techniques and expanded batteries of phenotypic tests has considerably altered the taxonomy of the viridans streptococci.[1017] Emended descriptions of well-recognized species (e.g., *S. mitis*, *S. sanguinis*) have been published over the years and several new species have emerged with the availability of molecular techniques such as 16S rRNA sequencing. The frequency of isolation and the roles of these newer species in infectious diseases await clarification as more data and case reports appear in the literature.

Viridans streptococci in Mitis/Sanguinis, Mutans, and Salivarius Groups are involved in about 20% of cases of subacute bacterial endocarditis, and in this setting cause a sustained bacteremia, leading to their recovery from multiple sets of blood cultures. Viridans streptococcal endocarditis occurs most frequently in individuals with preexisting native valvular disease due to rheumatic or congenital heart disease, in particular, or may also involve prosthetic valves. Sources of the organisms causing infective endocarditis are usually the oropharynx, the urogenital tract, and the gastrointestinal tract. Poor oral hygiene and/or periodontal disease are often noted in patients with infective endocarditis. Oral surgery and other invasive procedures (e.g.,

fiberoptic sigmoidoscopy, upper gastrointestinal endoscopy) can cause transient bacteremias that may initiate vegetations on previously damaged heart valves. Viridans streptococcal endocarditis presents insidiously, with fever, fatigue, and weight loss being the most common findings. Heart murmurs, peripheral stigmata of endocarditis (e.g., splinter and conjunctival hemorrhages, petechiae), and vegetations on the heart valves are frequently present. Complications of viridans streptococcal endocarditis include multi-valvular disease, mitral valve aneurysms, paravalvular abscesses and glomerulonephritis associated with circulating immune complexes. Reference identifications of viridans streptococcal species associated with subacute bacterial endocarditis have found that *S. mitis*, *S. sanguinis*, *S. parasanguinus, S. oralis*, *S. gordonii*, *S. mutans*, *S. salivarius, S. vestibularis*, and *S. sinensis* are the viridans streptococci most frequently found in endocarditis. The new viridans streptococcal species *S. tigurinus*, was first described in a 74 year old male with endocarditis complicated by meningitis and spondylodiscitis.[1169]

While transient bacteremias are usually cleared without any adverse effects, prolonged bacteremia with viridans streptococci in neutropenic patients undergoing cancer chemotherapy or human stem cell transplantation is a recognized clinical entity that occurs in both adult and pediatric patients.[15,144,257,420,677,792,1052] In this setting, bacteremia is associated with aggressive cytotoxic chemotherapy for treatment of leukemias (particularly acute myeloid leukemia), lymphomas, solid tumors, and bone marrow transplantation. Viridans streptococcal bacteremia generally occurs 8 to 21 day after preceding chemotherapy at a median of 6 to 7 days after onset of neutropenia. Risk factors for viridans streptococcal bacteremia include administration of high-dose cytotoxic agents (e.g., especially high-dose cytarabine), presence of oral mucositis secondary to cytotoxic chemotherapy or radiation, presence of indwelling lines (e.g., Hickman catheters), and profound neutropenia.[15,420,792] Oral mucositis, mucosal lesions in the gastrointestinal tract, and indwelling lines serve as portals of entry into the bloodstream.[35,257] In this regard, poor underlying dental health and periodontal disease are also risk factors for the development of viridans streptococcal bacteremia in neutropenic hosts.[393] Viridans streptococcal bacteremia in these patients may be complicated by the development of adult respiratory distress syndrome (ARDS), hypotension, shock, and bacterial endocarditis.[15,782] Streptococcal species associated with neutropenic bacteremia include *S. mitis*, *S. oralis*, *S. salivarius*, *S. sanguinis, S. gordonae; S. parasanguinis,* and *S. vestibularis,* with *S. mitis* being the predominant species isolated from blood cultures of patients with malignancies. (Box 13-2).[35,421] In the past, this syndrome was associated

with severe complications in 15% to 40% of patients and a high mortality rate of up to 20%, but recent studies have documented fewer complications (about 7%), with mortality attributable to viridans streptococcal bloodstream infection at less than 2%.[15,485,677,792] Improved outcomes in these patients are likely due to better diagnostic capabilities and supportive care for neutropenic patients in intensive care settings. Bacteremic infections with viridans streptococci have also been reported in low-birth-weight term and preterm neonates. Mothers often have risk factors that adversely affect pregnancy outcomes, including chorioamnionitis, premature rupture of fetal membranes, premature onset of labor, and urinary tract infection at the time of delivery.

Viridans streptococci may rarely be isolated from other serious infections, such as meningitis, pneumonia, and other miscellaneous infections, particularly in compromised hosts. Meningitis caused by viridans streptococci may occur in both adults and children, and the clinical presentation differs little from that of the other pyogenic meningitides (i.e., nuchal rigidity, seizures, meningeal inflammation, and altered mental status).[362,659,1169] The source of the organism is usually endogenous and associated with congenital structural abnormalities of the head and neck, head and neck infections (e.g., odontogenic maxillary sinusitis, mastoiditis), endocarditis, or extracranial infections.[539,843,1169] Previous head trauma or neurosurgical procedures (e.g., craniotomy) are risk factors for and have been associated with the development of viridans streptococcal meningitis.[173,659] Complications include intracranial suppuration and cerebral vasculitis.[659] Other CNS infections such as brain abscess may occur following cranial trauma or surgery, or secondary to a primary focus of infection elsewhere via direct extension from a contiguous site or via the hematogenous route.[539] Direct extension may occur from dental infections, paranasal sinus infections, or otogenic sources. Brain abscesses are usually singular, located in the frontal or temporal lobes, and may be polymicrobial. Predisposing conditions for viridans streptococcal brain abscess include congestive heart failure, chronic otitis media, otogenic infections, head injuries with CSF rhinorrhea, sinusectomy, immunosuppression, and craniotomy followed by ventriculoperitoneal shunt placement.[843,1094] Bacteremic viridans streptococcal pneumonia has been reported but is rare. Viridans streptococcal pneumonia occurs in older patients in the setting of oropharyngeal aspiration.[1018] These patients often have poor dentition, carious lesions, COPD, and underlying medical conditions such as diabetes. Complications (e.g., empyema, lung abscesses) associated with viridans streptococcal pneumonia are also rare. However, in one series describing organizing pneumonia that mimicked lung carcinoma, viridans streptococci and saprophytic *Neisseria* species were identified as the sole etiologic agents in 55.6% of 27 cases.[1150] Most of the patients were male and between 41 and 80 years of age. The resected tumors were variable in size (1.5 to 8.5

cm), sharply demarcated, showed inflammation with organization, and sometimes were associated with bronchial necrosis. Miscellaneous infections caused by various species of viridans streptococci have included septic arthritis, vertebral osteomyelitis secondary to bacteremia/endocarditis, pericarditis, Lemierre's syndrome, and infectious crystalline keratitis.[626,800,1047,1049,1066,1158]

Certain viridans streptococci in the oral cavity are associated with the initiation and pathogenesis of dental caries. *S. mutans*, *S. sobrinus*, and other members of the Mutans Group of oral streptococci produce enzymes called **glucosyltransferases**, which hydrolyze dietary sucrose (a disaccharide of glucose and fructose) and connect the glucose moieties together in α1,6 and α1,4 glycosidic linkages to form insoluble glucans.[51,472,582] Glucans enable bacteria to adhere to the tooth surfaces of the teeth and form the matrix of dental plaque. Attachment of *S. mutans* and other organisms to the insoluble, adherent glucans and subsequent formation of acid leads to demineralization of the tooth enamel and the initiation of carious lesions.[51] Other oral streptococci, including *S. sanguinis*, *S. salivarius*, and possibly *S. gordonii*, are able to synthesize similar polysaccharides, but only the streptococci within the Mutans Group display sucrose-induced enhancement of oral colonization. Molecular approaches for direct detection of *S. mutans* in plaque and gingival samples have been developed and monoclonal antibodies against *S. mutans* are used by workers in dental microbiology for identification and enumeration of these organisms in saliva and dental plaque specimens.[51,561] Epitopic regions of these glucosyltransferase enzymes are being identified in efforts to develop a component vaccine against dental caries.[473]

In the past, the viridans streptococci were generally susceptible to penicillin, ampicillin, and most other antimicrobial agents. However, antimicrobial susceptibility surveys of viridans streptococci have clearly shown that resistance to several classes of antimicrobial agents is increasing.[651] In 2001, Levy and colleagues[635] reported two patients with endocarditis caused by penicillin-resistant strains of *S. mitis* and *S. sanguinis*, respectively. Among 50 blood isolates from Memorial Sloan-Kettering Cancer Center, 44% were penicillin-resistant.[1052] In the 2010 LEADER Surveillance program, 29.9% of 411 viridans group streptococci had penicillin MICs of ≥0.12 μg/mL, and 53.5% and 9.7% were resistant to erythromycin and clindamycin, respectively.[347] In the same survey, resistance to levofloxacin increased from 5.9% to 8.5% in the period from 2006 through 2010. A 2011 study from Turkey documented penicillin and erythromycin resistance rates for 50 blood culture isolates at 30% and 36%, respectively.[313] Resistance to third- and fourth-generation cephalosporins (e.g., cefotaxime, cefepime) has also been reported, with rates as high as 30% to 41%.[420] Among the viridans group streptococci, *S. mitis* strains show the highest levels of antimicrobial resistance. A study published in 2004 from Finland found that *S. mitis* constituted 82% of the viridans streptococci isolated from neutropenic patients, and

that 5% and 4% of these *S. mitis* isolates exhibited high-level resistance to penicillin (MIC ≥ 4 μg/mL) and cefotaxime (MIC ≥ 4 μg/mL), respectively.[666] In a 2006 study of isolates recovered from blood cultures of cancer patients, 28% of 25 *S. mitis* isolates were resistant to penicillin, with MICs ranging from 4 to 12 μg/mL, while none of the 15 non- *S. mitis* isolates were penicillin-resistant.[421] *S. mitis* isolates also showed higher rates of resistance to the fluoroquinolones than non- *S. mitis* strains, although all 50 isolates tested in the study showed fluoroquinolone resistance rates ranging from 44% to 64% depending on the agent. The mechanism of penicillin resistance involves altered PBPs due to mutations in *pbp2b* and *pbp2x* genes; these genes were likely transferred

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to *S. pneumoniae* from viridans streptococci.[1177] Erythromycin intermediate and resistant phenotypes are mediated by mutations in *ermB*, *mefA,* and *mefB* genes, alone and in combinations, and the prevalence of strains carrying these mutations vary from place to place.[313,651,1177] Linezolid, daptomycin, tigecycline, and vancomycin remained highly active against the viridian streptococci, with rates of susceptibility ranging from 99.8% to 100%.[347,527,870]

The Anginosus Group: *Streptococcus anginosus, Streptococcus constellatus*, and *Streptococcus intermedius*

Organisms belonging to the Anginosus group have gone through repeated taxonomic revisions. British taxonomy refers to these species as the "*Streptococcus milleri*" group, and this pneumonic is still seen in the literature. Anginosus group streptococci correspond to three species—*S. intermedius, S. constellatus,* and *S. anginosus*—that form small, "minute" colonies on agar media. These organisms can be nonhemolytic, α-, or β-hemolytic; β-hemolytic isolates may carry Lancefield group antigens (group A, C, F, or G) or may be nongroupable. The small-colony size and the matte appearance of the colonies distinguish these group C and G Anginosus Group organisms from the "large-colony" β-hemolytic streptococci (*S. dysgalactiae* subspecies). Most *S. anginosus* isolates are nonhemolytic, while more than half of *S. constellatus* strains may be β-hemolytic; isolates of *S. intermedius* are rarely β-hemolytic. These organisms are part of the human oropharyngeal microbiome and may be isolated from the throat, the nasopharynx, and the gingival crevices. They are also found in the

gastrointestinal tract and in the vagina. A subspecies of *S. constellatus*—*S. constellatus* subsp. *pharyngis*—was described that expresses the group C antigen and is associated with phayngitis.[1127] In 2012, a molecular taxonomic study of this group used MLSA, 16S rRNA sequencing, and phenotypic tests to divide the Anginosus Group into seven distinct clusters, including two clusters of β-hemolytic, Lancefield group C-bearing members that suggested two new subspecies. *S. anginosus* includes two subspecies —*S. anginosus* subsp. *anginosus* and *S. anginosus* subsp. *whileyi*. *S. constellatus* includes three subspecies: subsp. *constellatus,* subsp. *pharyngis*, and subsp. *viborgensis*.[515] Virulence of this group may be related to the presence of a capsule on some strains, production of a partially characterized immunosuppressive protein, and the production of a variety of hydrolytic and glycosaminoglycan-degrading enzymes (e.g., neuraminidase, DNase, chondroitin sulfate depolymerase, and hyaluronidase).[716,1126] In vitro studies have shown that the Anginosus Group streptococci inhibit polymorphonuclear leukocyte chemotaxis and are ingested by PMNs more readily than *S. aureus*; once ingested, however, they are killed more slowly than *S. aureus*.[1105] *S. intermedius* strains also produce a staphylococcal leukocidin-like toxin called **intermedilysin** that may be virulence-associated, although neither the toxin nor its genes are found in *S. constellatus* or *S. anginosus.*[401,667,743]

S. intermedius, *S. constellatus*, and *S. anginosus* are recognized for their propensity to cause purulent tissue abscesses, intra-abdominal infections, pulmonary infections, bacteremia, CNS infections, oral infections, and endocarditis. Bacteremia is usually attributable to a focus of infection within the gastrointestinal or upper respiratory tract. Supperative metastatic abscesses are a significant complication of bacteremia, with *S. intermedius* and *S. constellatus* being more often associated with abscess formation than *S. anginosus*.[174] *S. anginosus* is involved in mixed infections having gastrointestinal or genitourinary sources. Up to 40% of infections caused by Anginosus Group streptococci are intra-abdominal infections that develop following gastrointestinal surgery or as a result of colonic perforation caused by trauma or other gastrointestinal lesions (e.g., colonic carcinoma). Complications include appendicular, subphrenic and pancreatic abscesses, pyogenic liver abscesses, and peritonitis.[150,296,427,673,702,735,756,891] Mucosal bowel trauma and disease apparently facilitate bloodstream invasion. *S. anginosus* and *S. constellatus* bacteremias have occurred in patients with vascular graft infections secondary to vasculo-digestive fistulas, sigmoid diverticulitis, severe postsurgical wound infections, peritonitis, and chorioamnionitis following gynecologic surgical procedures (e.g., cesarean delivery).[100,606,735,1042] Anginosus Group bacteria are significant pathogens in solid-organ transplant recipients, particularly liver, kidney, combined kidney/liver, pancreas, and small bowel transplants, causing intra-abdominal abscesses, pleural empyemas, and recurrent cholangitis due to biliary stricture and inflammation.[932,965] Potentially

lethal complications related to bacteremia include septic shock, pulmonary infections, central nervous system infections, and endocarditis. These organisms are causative agents in 8% to over 30% of skin and subcutaneous infections, and may cause severe soft tissue infections in patients with underlying conditions such as HIV infection, uncontrolled diabetes, and intravenous drug use.[1157] Osteomyelitis due to the Anginosus Group can arise from hematogenous dissemination, but more frequently occurs due to a contiguous infected site (e.g., decubitus ulcers).[399,562] Osteolytic lesions caused by these bacteria have been documented in infected foot wounds, and mandibular, cranial, and vertebral infections, and required aggressive management. Like other viridans streptococci, Anginosus Group streptococci cause bacteremia in neutropenic patients with underlying diseases who are being treated with cytotoxic regimens, although fulminant bacteremia in previously healthy individuals may also occur. These pathogens are also emerging as veterinary pathogens. In 2012, the first report appeared describing *S. constellatus* as a cause of deep chronic pyoderma in a 4-year-old male hound.[271]

Anginosus Group organisms resident in the oral cavity may cause a variety of supperative periodontal, maxillofacial, and head/neck infections, including periapical abscess, lymphadenitis, endophthalmitis, and brain abscesses.[131,185,453,716,773,802,1149]Pleuropulmonary and head and neck infections often occur as mixed-flora infections that can spread contiguously into the soft tissues via fascial planes and that can serve as a source for hematogenous dissemination and its complications. Organisms from infected dental sites, paranasal sinuses, soft tissues, and peritonsillar regions may spread by direct extension to involve the cranium, orbit,

deep tissue spaces in the neck, and the central nervous system. A review of cases in Japan from 2002 to 2003 described 17 patients with head and neck infections due to the Anginosus Group.[453] Orofacial infections included maxillary sinusitis, peritonsillar, subcutaneous, submandibular, and retropharyngeal space infections, with deep neck, mediastinal, parotid, tonsillar, and masseter muscle involvement.[453] Anaerobic bacteria were co-isolated from one-third of the patients' lesions. Descending, fatal, deep neck space infection due to *S. anginosus* occurred in a liver transplant recipient.[131] The patient developed a crepitant neck mass involving the tonsillar region and extending down into the mediastinum. Park et al.[802] described necrotizing fasciitis due to *S. anginosus* and *Bacteroides* species in a patient due to periapical abscesses surrounding several molars and pyomyositis of the masseter muscles. *S. constellatus* was isolated from a man who developed septic shock immediately following extraction of an infected lower molar.[760] The clinical course was further complicated by septic emboli

to the brain. *S. anginosus* thyroiditis with bacteremia and abscess formation were described in two patients who presented with space-occupying neck lesions on CT and MRI accompanied by changes in thyroid function tests.[1149] Lemierre's disease associated with *S. intermedius* bacteremia was described in a middle-aged woman who presented with pharyngitis following a tooth cleaning.[407] Scans revealed a neck mass with a thrombus in the left jugular vein extending into the sigmoid sinus and chest roentgenogram showed bilateral infiltrates and pleural effusions. Pulmonary infections can result from aspiration of oropharyngeal contents, leading to pneumonia, which may be complicated by empyema and lung abscesses. Anginosus Group bacteria have also been isolated from sputum specimens of cystic fibrosis patients in association with disease exacerbations and clinical deterioration.[401,807,930] Brain abscesses caused by all three Anginosus group members have been described in young previously healthy children, middle-aged adults, and the elderly.[110,185,316,566,716,843,883] Diagnosis and treatment often required brain biopsy and drainage. In one case, a young, previously healthy male initially presented with an abdominal pain due to a splenic abscess; *S. intermedius* was isolated from blood and pus drained from the abscess.[673] Two weeks later, the patient returned with a recurrence of the splenic abscess plus three abscesses in the frontal and occipital lobes of the brain. Aspirates from the splenic abscess and brain lesions failed to grow the organism, but the lesions resolved on ertapenem therapy. *S. constellatus*, along with *Actinomyces viscosus*, was isolated from subdural empyema fluid from a 7-year-old girl with poor oral hygiene and multiple dental caries. Multiple brain abscesses subsequently developed despite therapy, leading to a fatal outcome.[110] Multiple brain abscesses due to *S. constellatus* were diagnosed in a middle-aged male who initially presented with endogenous endophthalmitis.[185]

S. constellatus, S. intermedius, and S. anginosus have been involved as causes of rare but severe endocarditis and septic shock.[235,252,793,1039,1146] Tran and colleagues[1039] described a patient with mitral valve endocarditis where the organism originated from liver multiple liver abscesses that required chemotherapy and several percutaneous drainage procedures to clear the bacteremia. *S. constellatus* endocarditis involving the aortic bicuspid valve and the mitral valve and requiring replacement of both was described in a patient in Italy. The isolate was broadly susceptible, but resistant to penicillin G. Woo and colleagues reported 6 cases of Anginosus Group endocarditis out of 377 cases of endocarditis that occurred in Hong Kong over a 5-year period. Five patients had underlying diseases (i.e., rheumatic heart disease, ischemic heart disease, COPD, supraventricular tachycardia) and one was an IV-drug user. All isolates from these patients were identified as *S. anginosus*. *S. anginosus* endocarditis was complicated by endogenous endophthalmitis in a 60-year-old male patient; the source of the isolate was likely the oral cavity, and the patient required

total dental extraction to clear his bacteremia.[499]

Anginosus Group isolates are generally susceptible to a variety of antimicrobial agents, but resistance to some agents is emerging. A 1999 examination of 180 Anginosus Group isolates found that 94.4%, 92.8%, 87.1%, 97.3%, and 97.3% of isolates were susceptible to penicillin, ampicillin, cefuroxime, cefazolin, and cefotaxime, respectively.[646] Individual case reports have cited resistance to penicillin in otherwise susceptible isolates.[235,453] A study of 44 genotypically characterized strains found no penicillin resistance among 12 *S. intermedius*, 16 *S. constellatus*, and 16 *S. anginosus* isolates, although four isolates showed decreased susceptibility to penicillin and four isolates were intermediate to ampicillin; all isolates were ceftriaxone susceptible.[1038] A study of 22 β-hemolytic group C *S. anginosus* and 5 *S. constellatus* isolates found that all were susceptible to penicillin, cefotaxime, and vancomycin; one *S. constellatus* was resistant to levofloxacin and 10% to 20% of all isolates were resistant to erythromycin and clindamycin.[174] Other studies and case reports have documented emerging MLS (macrolide–lincosamide–streptogramin) resistance.[646,1039] A 1999 study found that 17.7% and 18.3% of 180 strains tested were resistant to erythromycin and clindamycin, respectively.[646] Surveillance studies indicate that erythromycin resistance may be found in 3.2% to 17.7% of isolates, depending on geographic location, and only slight differences have been noted in MLS resistance among the three anginosus group species.[502,1038] Genetic analysis of some MLS-resistant strains has documented the presence of *erm* (B), *erm* (TR), and *mef* (A)- *mef* (E) genes that result in constitutive erythromycin resistance, inducible macrolide resistance, and macrolide efflux-mediated resistance, respectively.[174] Most Anginosus Group isolates are resistant to aminoglycosides and about 30% of isolates may be resistant to fluoroquinolones.[756,965] A daptomycin-resistant *S. anginosus* isolate (MIC, 4 μg/mL) caused breakthrough bacteremia in a 47-year-old male patient after 21 days of daptomycin therapy.[793] The isolate was otherwise susceptible to all agents including the macrolides.

Group D Streptococci: The "*Streptococcus bovis*" Group

Group D streptococci are found among the intestinal flora of vertebrates. These streptococci have the group D cell wall antigen that is composed of lipoteichoic acid. Formerly, group D species that were predominant normal inhabitants of the human gastrointestinal tract were called group

D enterococci on the basis of a growth on bile esculin agar and growth in 6.5% NaCl broth, whereas other streptococci that possessed the group D antigen and grew on bile esculin agar but failed to grow in salt broth were termed "nonenterococcal Group D streptococci." The practical consideration behind this division was that the enterococci are more resistant to penicillins, cephalosporins, and aminoglycosides than nonenterococcal group D streptococci. In the mid- to late 1980s, the enterococcal group D streptococci were reclassified in the new genus *Enterococcus*, while the group D nonenterococcal species remained in the genus *Streptococcus*. Nonenterococcal group D streptococci were designated as *S. bovis* or *S. equinus*. *S. bovis* strains were divided into two biotypes based on mannitol fermentation: *S. bovis* biotype I strains were mannitol-positive, while biotype II (or *S. bovis* variant) strains were mannitol-negative. *S. bovis* biotype II was further divided by β-glucuronidase (β-GUR): *S. bovis* II.1 were β-GUR-negative, while *S. bovis* biotype II.2 were β-GUR-positive.

Beginning in 1984, extensive taxonomic changes were proposed for organisms in the "*S. bovis*" group, and several new species designations were proposed and rejected over the subsequent 20 years. Because the name "*Streptococcus gallolyticus*" had taxonomic precedence over *S. bovis* and related organisms prior to the species reorganization, this name has been appropriated for members of the former *S. bovis* group.[902,947] Clinically important species in the group D streptococci are *S. gallolyticus* subsp *gallolyticus* (formerly *S. bovis* I), *S. gallolyticus* subsp. *pasteurianus* (formerly *S. bovis* II.2), and *S. infantarius* (formerly *S. bovis* II.1), which includes two subspecies, subsp. *coli* and subsp. i *nfantarius*.[64,900] *S. gallolyticus* subspecies are also found in several animals as inhabitants of the gastrointestinal tract, and strains of *S. gallolyticus* subsp. *gallolyticus* have been isolated from the feces of cows, horses, pigs, dogs, guinea pigs, and the rumen of sheep.[64] The type strain of *S. bovis* was originally recovered from cow dung and differs phenotypically from human isolates. Some strains also cause septicemia and meningitis in pigeons, ducks, broiler chicken flocks, and other animals.[165,636] Sequencing of the genome of *S. gallolyticus* subsp. *gallolyticus* has revealed several unique features that may enable this organism to adapt to the colonic environment of a wide spectrum of animals, including humans.[878] These characteristics include its ability to synthesize all 20 amino acids and several vitamins, its ability to degrade a variety of carbohydrates of plant origin, and the production of a variety of enzymes (e.g., tannase, phenolic decarboxylases, and bile-acid hydrolases). *S. bovis*-like strains isolated from the gastrointestinal tracts of pigs and chickens have been identified as *S. alactolyticus*. *S. equinus* corresponds to certain equine "*S. bovis*" strains and human isolates of "*S. equinus"* were actually *S. bovis*. These studies confirmed that *S. alactolyticus* and *S. intestinalis* are identical as well. In 2008, two new animal

streptococcal species, *S. henryi* and *S. caballi*, were isolated from the hindgut of horses with equine laminitis.[714] Although these two species are genotypically most closely related to *S. suis*, certain phenotypic characteristics are similar to those for *S. gallolyticus* (e.g., bile esculin–positive, no growth in 6.5% NaCl, positive reaction with group D antisera [*S. henryi* only]).

S. gallolyticus (S. bovis) causes bacteremia, meningitis, and native and prosthetic valve endocarditis.[444,575,617,1084] An important clinical correlate of *S. gallolyticus* bacteremia is its association with colorectal carcinoma.[99,243,563] In a case–control study done in Spain, colonoscopy was performed on 98 of 109 patients with *S. gallolyticus* subsp. *gallolyticus* bacteremia. Sixty-nine cases of colorectal neoplasia were detected, including 12 invasive carcinomas and 57 adenomas (including 39 advanced lesions).[243] The prevalence of bacteremia with this organism in 196 matched control patients with other underlying disease or malignancies (32%) was lower than those patients with colorectal neoplasia (70%). *S. gallolyticus* endocarditis may also occur as a complication of bacteremia.[563,575] In some cases, the isolation of this organism in association with endocarditis often prompts a search for colorectal neoplasms. In a 2012 case report, a 76-year-old man presented with a prosthetic knee joint infection with *S. gallolyticus*. The patient underwent colonoscopy and an adenocarcinoma of the ascending colon was detected.[642] *S. gallolyticus* is found significantly more often in the stool of patients with colorectal malignancies than in that of healthy controls. Associations have also been noted between *S. gallolyticus* bacteremia, liver disease, inflammatory bowel disease, idiopathic ulcerative colitis, and chronic radiation enterocolitis.[3] Underlying colonic disease may affect the colonic microbiota, leading to the accumulation of different, novel materials (e.g., plant-derived carbohydrates and fibers) in the vicinity of colonic dysplasia and creating an environment for the proliferation of *S. gallolyticus*.[878] Changes in hepatic secretion of bile or immunoglobulins into the intestinal lumen may promote the overgrowth of *S. gallolyticus* and the movement of the organisms from the intestine into the portal venous circulation.[3] In vitro studies have also established that *S. gallolyticus* is able to adhere to and subsequently invade human endothelial cells.[1099] Failure of the hepatic reticuloendothelial system to contain the organisms results in bacteremia and endocarditis. Patients with *S. gallolyticus* bacteremia, endocarditis, or meningitis should have a colonoscopy to detect occult gastrointestinal lesions. *S. gallolyticus* meningitis and bacteremia may also occur in patients with no underlying malignancies. Some evidence suggests that *S. infantarius* subspecies may be associated with noncolonic cancers.[242] In a retrospective study of all "*S. bovis*" group bacteremias in Spain from 1988 through 2007, *S. gallolyticus* was isolated from 48.5% of 105 bacteremias associated with colon tumors, while *S. infantarius* was isolated from

11%.[242] For 28 bacteremias occurring in noncolonic cancer patients, *S. infantarius* was isolated from 57%, while *S. gallolyticus* was isolated from only 6%. The 16 noncolonic cancers associated with *S. infantarius* bacteremia included biliary tract and pancreatic cancer (12 patients) and cholangitis. Laboratories should report the isolation of *S. gallolyticus* subspecies to clinicians, but should also report the former species names (e.g., *S. bovis* I, *S. bovis* II.1, and *S. bovis* II.2) in the same report in order to avoid confusion and the omission of diagnostic steps (e.g., colonoscopy) that may be lifesaving for the patient.[1081]

Other *S. gallolyticus* subspecies are also emerging as causes of serious human infections. *S. gallolyticus* subsp. *pasteurianus* is increasingly being reported as an agent of bacteremia and meningitis in children and adults.[350,569,742,787,846,1019]

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Children with meningitis due to this organism have included preterm infants and neonates from 5 to 13 days old. In one case report, the infant was born at home to a mother with a history of exposure to farm animals (i.e., chickens, horses, dogs, cats, dairy cattle) and 1 week prior to delivery, 70 baby chicks died of an unknown illness.[569] *S. gallolyticus* subsp *pasteurianus* was also the cause of a cluster of bloodstream infections in five infants in a neonatal intensive care unit.[350] Although no source was identified for the organism, it was surmised that one of the first three neonatal cases was the index case and that caregivers likely infected the other infants via transient hand carriage. The first case of adult meningitis and bacteremia due to *S. gallolyticus* subsp. *pasteurianus* was described in a 75-year-old man with a history of prostate cancer that was treated by radiation; the patient subsequently developed radiation proctitis, which was believed to be the portal of entry for the organism.[973] *S. gallolyticus* subsp *pasteurianus* meningitis was also described in a 61-year-old patient who had no underlying history of colonic lesions or other malignancies, but had experienced painful hemorrhoids during the previous 2 weeks that were associated with intermittent low-level rectal bleeding on defecation.[948] In 2008, *S. gallolyticus* subsp. *macedonicus* was detected as the etiologic agent of multivalve infective endocarditis in a 61-year-old man.[674] A retrospective review of endocarditis isolates at the Mayo Clinic revealed a case of *S. gallolyticus* subsp. *macedonicus* native mitral valve endocarditis that occurred in 1978 in a 70-year-old-man.[446] This patient also had documented gastrointestinal pathology (i.e., a cecal polyp). *S. gallolyticus* subsp. *macedonicus* (formerly *S. macedonicus* and *S. waius*) is an α-hemolytic species that was originally isolated from Greek cheese and skim milk biofilms.[675,900,1048] Bacteremia and hematogenous dissemination of *S. gallolyticus* subspecies can result in a several other types of clinical presentations. *S. gallolyticus* subsp. *pasteurianus* has

been isolated from intrauterine infections and postpartum bacteremia, spontaneous bacterial peritonitis, brain abscess, septic discitis, and continuous ambulatory peritoneal dialysis-associated peritonitis.[85,309,471,1138,1156] Septic arthritis has been reported in patients with infective endocarditis, liver cirrhosis, and as the presenting sign in patients with "silent" colonic carcinoma.[256,368,745] Penicillin remains highly active against most strains of *S. gallolyticus*, although occasional isolates may show diminished susceptibility to penicillin.

Enterococcus Species

Taxonomy

The genus *Enterococcus* includes the enterococcal members previously classified with the group D streptococci.[903,904] Enterococci are microbiota of humans and animals. They are normal residents of the gastrointestinal and biliary tracts and, in lower numbers, of the vagina and male urethra. Enterococci produce smooth, gray colonies that are either non-hemolytic or alpha-hemolytic (Image Plate 13-3E). The taxonomy of *Enterococcus* species has undergone considerable change since the mid-1980s. Before the widespread use of genetic techniques for taxonomic analysis, enterococci were distinguished from streptococci and related taxa by their ability to grow at 10°C and 45°C, growth in the presence of 6.5% NaCl, growth at pH 9.6, ability to hydrolyze esculin in the presence of 40% bile, and production of pyrrolidonyl arylamidase (PYR). More than 90% of strains also possessed the Lancefield group D lipoteichoic antigen in their cell walls. Molecular studies have revealed several species that are members of the genus *Enterococcus* by genetic criteria, but that lack many of the phenotypic characteristics typical of the genus (Box 13-5). Most of these species are not often found in human clinical specimens. *Enterococci* have emerged as important agents of human disease, largely because of their resistance to antimicrobial agents to which other streptococci are generally susceptible. In vitro, enterococci have penicillin MICs that are 10- to 100-fold higher than for other streptococci, are resistant to most cephalosporins, and are nonsusceptible to the aminoglycosides. Enterococci are important agents of nosocomial infections in the United States, ranking as the second most common organisms causing bloodstream, urinary tract, and skin/soft tissue infections.[37] *E. faecalis* is the most commonly isolated species, causing about 70% of human infections. However, enterococci with acquired resistance to vancomycin (VRE, vancomycin-resistant enterococci) now account for more than 30% of enterococcal infections, and greater than 90% of VRE isolates are *E. faecium*. Only about 5% to 10% of *E. faecalis* isolates are vancomycin-resistant.[451] The emergence of VRE as nosocomial pathogens is related to the increased use of

vancomycin and broad-spectrum agents like third-generation cephalosporins. Because of their resistance to penicillins and cephalosporins of several generations, the acquisition of high-level resistance to aminoglycosides, resistance to clindamycin, and emergence of vancomycin resistance, these bacteria are often involved in serious superinfections among patients receiving broad-spectrum antimicrobial chemotherapy.

Virulence Factors

Unlike other streptococcal species discussed thus far, factors that determine virulence of the enterococci are not well understood, but ongoing research and study of these organisms has revealed several putative microbial virulence factors in enterococci.[895]About 30% of *E. faecalis* strains produce an extracellular **cytolysin/hemolysin** that acts on human, rabbit, and equine erythrocytes but not bovine or ovine erythrocytes and has demonstrable toxicity in rabbit endophthalmitis and endocarditis models.[37,102,194,516] This cytolysin/hemolysin is cidal for a number of eukaryotic cell types, including macrophages, and polymorphonuclear neutrophils. Other secreted enterococcal virulence factors include two proteases: gelatinase (GelE) and extracellular serine protease (SprE). GelE protease degrades host tissue proteins and is also involved in the activation of enterococcal autolysins and the development of biofilms.[791,1023] GelE may also be involved in the translocation of enterococci across intestinal cell barriers and the development of peritoneal infections.[1172] There is also evidence suggesting that these molecules may cause modulation of the immune response by inactivation of complement proteins.[805] **Aggregation substance proteins** are surface-bound, plasmid-encoded proteins that promote clumping of the organisms to facilitate plasmid exchange.[194] Aggregation substance proteins facilitate enterococcal adherence to cultured intestinal and renal epithelial cells and promote aggregative growth of cardiac vegetations in animal endocarditis models.[905,1110] Other studies suggest that aggregation substance may be involved in binding of *E. faecalis* to neutrophils and cultured intestinal epithelial cells and in subsequent internalization and intracellular survival of these organisms.[784,851,1080] Plasmids encoding aggregative substance proteins also carry antibiotic resistance genes. *E. faecalis* and *E. faecium* produce a unique surface protein termed called **Esp (extracellular surface protein**) and **Esp**$_{fm}$ that enable the organisms to evade antibodies by their ability to be retracted away from the cell surface. These molecules are involved in formation of biofilms and play roles in the pathogenesis of endocarditis and UTIs in animal models.[439,440,628,791,916] **Ace** and **Acm**, found in *E. faecalis* and *E. faecium*, respectively, are members of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) group, specifically bind to collagen and laminen, and play roles as virulence factors in animal endocarditis

models.[519,652,748,939]Pili are also found on the enterococcal cell surface and are involved in attachment, biofilm formation, and the pathogenesis of UTIs and endocarditis in animal models.[749,938] In *E. faecalis*, cell surface-associated polysaccharides encoded by the ***cps* (capsular polysaccharide) locus, LTAs,** and **Epa (enterococcal polysaccharide antigen**) enable these organisms to resist complement-mediated opsonization and phagocytosis, facilitate organism translocation across cellular membranes, and play pivotal roles in the pathogenesis of enterococcal urinary tract and peritoneal infections.[1016,1020,1026] Most *E. faecalis* and some *E. faecium* isolates from bacteremias produce large amounts of extracellular superoxide dismutase that may enhance enterococcal virulence in mixed-flora abscesses. Correlations have also been found between possession of various virulence factors described above and the presence of antibiotic resistance among clinical and environmental strains of *E. faecalis* and *E. faecium*.[854]

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BOX 13-5

Members of the Genus *Enterococcus*

Group/Species	Comments
Group 1	
Enterococcus avium[225]	This species is found in avian, canine, and human gastrointestinal tracts. This species may carry Lancefield group D and group Q antigens and produce H_2S. *E. avium* has been isolated from humans as a cause of bacteremia, osteomyelitis, endocarditis, meningoencephalitis, breast prosthesis infection, and brain abscesses.[8,246,277,314,528,715,722,741,816,986,1000] Bacteremia with *E. avium* is commonly of gastrointestinal or biliary origin and is often polymicrobial.[741]
Enterococcus gilvus[1058]	This 2002 species was originally isolated from bile of a patient with cholecystitis.
Enterococcus malodoratus[225]	This species was isolated from Gouda cheese and unpasteurized milk products. It is the only *Enterococcus* species that produces H_2S.
Enterococcus pallens[1058]	*E. pallens* was originally isolated from a peritoneal dialysate of a patient with peritonitis.
Enterococcus pseudoavium[211]	*E. pseudoavium* was first isolated from cases of bovine mastitis and is a rare human isolate.
Enterococcus raffinosus[211]	*E. raffinosus* is named for its ability to produce acid from raffinose. *E. raffinosus* has been isolated from blood cultures as a cause of endocarditis and has also been isolated from wounds, abscesses, decubitus ulcers, peritoneal fluid, bile, urine, sinusitis, traumatic endophthalmitis, and vertebral osteomyelitis.[178,543,686,889,1000] Multi-drug resistant and vancomycin-resistant strains of *E. raffinosus* have been a cause of nosocomial outbreaks in populations of immunocompromised hosts (e.g., hematology/oncology units).[359,888,896,898,1006] A vancomycin-resistant *E. raffinosus* was the cause of mitral valve endocarditis in an elderly male; treatment with linezolid resulted in cure.[511]
Enterococcus saccharolyticus[867]	This *S. bovis*-like organism was originally isolated from cows. Its phenotypic characteristics are similar to *Enterococcus* species (e.g., growth at 10°C and 45°C, growth in 6.5% NaCl), but it does not react with group D antisera.
E. hawaiiensis[142]	*E. hawaiiensis* is the name given to CDC "proposed new species (PNS) E3." This organism was isolated from brain tissue of an 11-month-old patient.
Enterococcus devriesei[983]	This new "*E. raffinosus*-like" species was isolated from bovine sources, from the air of a poultry slaughter by-product processing plant, and from vacuum-packaged charcoal broiled river lampreys.
Enterococcus viikkiensis[849]	This new species is based on five isolates recovered from broiler products and a broiler processing plant.
Group 2	
Enterococcus faecalis	*E. faecalis* is the most frequent isolate from human specimens and the human gastrointestinal tract; also found in the intestinal tracts of poultry, cattle, pigs, dogs, horses, sheep, and goats. It is an important cause of human infections (see text).
Enterococcus faecium	*E. faecium* is found in human clinical specimens and in the gastrointestinal tracts of various species of animals, It is an important cause of human infections and is more resistant to antimicrobial agents than *E. faecalis* (see text).
Enterococcus casseliflavus[225]	*E. casseliflavus* has been isolated from plants, soil, and the feces of chickens and was originally a subspecies of *E. faecium*. The organism is yellow-pigmented and motile. *E. casseliflavus* is an rare opportunistic agent of human infections, including bacteremia, meningitis, and endophthalmitis.[191,277,487,801,876,885,1000] Naser et al.[753] reported that *E. flavescens* (described in 1992) is a later synonym of *E. casseliflavus*, which was described in 1979 and emended in 2004. Strains of *E. casseliflavus* expressing the VanA, and VanB phenotype along with vanA-vanC genotype have been encountered in cattle.[417]
Enterococcus gallinarum[225]	The organism was originally isolated from chicken feces and is one of the motile *Enterococcus* species. This unusual *Enterococcus* has been isolated from cases of bacteremia related to the biliary and gastrointestinal tracts, hemodialysis-associated infections, native valve endocarditis, arthroplasty-associated infections, posttraumatic endophthalmitis, and meningitis.[34,54,191,239,258,456,552,788,987,997,1000] Strains of *E. gallinarum* may harbor the *vanA*, *vanB*, and *vanC* genes, resulting in high-level glycopeptide resistance.[237,641,708,757,927]
Enterococcus mundtii[217]	This species has been isolated from plants, soil, and the gastrointestinal tracts of cattle, pigs, and horses. It is named after J. O. Mundt, an American microbiologist. This species is yellow-pigmented and motile, and has been isolated from a human thigh abscess, sinus mucosa, bacteremia, and endophthalmitis.[277,452,541]

Enterococcus. haemoperoxidus[981]	*E. haemoperoxidus* is an environmental enterococcal species found in surface waters, swimming pools, and drinking water in the North Moravia region of the Czech Republic.
"E. sanguinicola"[143]	"*E. sanguinicola*" was the name given to the CDC "proposed new species" E2, which was isolated from blood cultures of a patient in Los Angeles. Subsequent hybridization and sequencing of the 16S rRNA and *rpo* genes confirmed that "*E. sanguinicola*" is the same as *E. thailandicus*, which was validly published.[921]
Enterococcus silesiacus[984]	This new species was isolated from surface water. The closest phylogenetic relatives of this species include *E. haemoperoxidus* (group 2) and *E. moraviensis* (group 5).
Enterococcus termitis[984]	*E. termitis* was isolated from the gut of a termite. This new species showed the greatest 16S rRNA sequence similarity with *E. haemoperoxidus* (group 2) and *E. moraviensis* (group 5).
Enterococcus camelliae[976]	Described in 2007, this organism was isolated from fermented tea leaves in Thailand.
Enterococcus thailandicus[143,1001]	This enterococcal species was isolated from fermented sausage in Thailand. Isolates of the proposed new species "*E. sanguinicola*" that were isolated from blood cultures are identical to *E. thailandicus*, which is validly published.[921]
Enterococcus ureasiticus[941]	This new species was isolated from water samples in Quebec.
Enterococcus quebecensis[941]	This new species was isolated from water samples in Quebec.
Enterococcus plantarum[985]	This species, described in 2011, was isolated from plants.
Group 3	
Enterococcus dispar[233]	*E. dispar* has been isolated from human stool and synovial fluid.
Enterococcus durans[225]	*E. durans* has been isolated from milk and other dairy products. This is a rare clinical isolate in cases of bacteremia, endocarditis, and ventriculoperitoneal shunt infections.[277,403,966,1000,1032,1096] Green and colleagues[397] reported a case of bacteremia in a bone marrow transplant recipient caused by a vancomycin-resistant *E. durans* strain that developed resistance to daptomycin during therapy.
Enterococcus hirae[332]	*E. hirae* was first isolated from chicken crops, chicken feces, and the gastrointestinal tracts of cattle, pigs, dogs, horses, sheep, goats, and rabbits. The type strains of this species are used in the food industry as a bioassay organism for amino acids and vitamins. In humans, *E. hirae* has been associated with bacteremia in a hemodialysis patient with end-stage renal disease, native and prosthetic valve endocarditis, bacteremic spondylodiscitis, pyelonephritis, cholangitis, and spontaneous bacterial peritonitis.[132,170,380,841,934,999,1000]
Enterococcus ratti[1011]	*E. ratti* was isolated from the intestines and feces of rats with diarrhea.
Enterococcus villorum[267,1073]	This species was first isolated from the gastrointestinal tract of dogs and pigs. *E. porcinus* is a junior synonym of *E. villorum*.
Enterococcus canintestini[752]	This species was isolated from fecal specimens from healthy dogs. 16S rRNA gene sequences showed the highest similarities with *E. dispar* (group 3), *E. asini* (group 4), and *E. asini* (group 5). This species was isolated from a case of catheter-associated bacteremia in a 72-year-old woman in Taiwan who was receiving chemotherapy for cervical cancer.[1000]
Group 4	
Enterococcus asini[279]	This enterococcal species was first isolated from the caecum of donkeys.
Enterococcus cecorum[281,283]	*E. cecorum* is found in the intestinal tracts of chickens. This species lacks the group D antigen, is PYR-negative and is unable to grow in 6.5% NaCl broth. *E. cecorum* has been isolated from humans as a cause of peritoneal dialysis-associated peritonitis, recurrent peritonitis, spontaneous bacterial peritonitis with empyema, catheter-associated bacteremia, and aortic valve endocarditis.[16,266,398,478,1000,1141]
Enterococcus sulfureus[683]	This species was isolated from plants and has not been seen in humans.
Enterococcus phoeniculicola[605]	This avian organism was isolated from the preen glands of wild red-billed Woodhoopoes. This species does not grow on BE agar or in 6.5% NaCl broth.
Enterococcus caccae[141]	This species was isolated from human stool specimens.
Enterococcus aquamarinus[982]	*E. aquamarinus* was isolated from sea water. Phenotypically, this species resembles other species in enterococcal group 4 (e.g., mannitol-, sorbitol-, arabinose-, and arginine dihydrolase-negative), but it is most closely related genotypically to *E. saccharolyticus* (group 1), *E. sulfureus* (group 4) and *E. italicus* (group 5).

Group 5	
Enterococcus columbae[282]	*E. columbae* was isolated from the intestinal tract of pigeons and it is related to *E. cecorum* and *E. avium*. This species is PYR-negative and is unable to grow in 6.5% NaCl broth.
Enterococcus canis[267]	This species was isolated from a dog with otitis externa. The organism is a resident of the canine intestine. *E. canis* grows on BE agar, grows 6.5% NaCl broth, and is PYR-positive.
Enterococcus moraviensis[981]	*E. moraviensis* is an environmental enterococcal species found in surface and drinking water in North Moravia, Czech Republic.
Enterococcus hermanniensis[578]	New species isolated from the microbial spoilage population of broiler chickens legs and also recovered from canine tonsillar tissues.
Enterococcus italicus[142,143]	This species was isolated from the blood of a patient in 1991. *E. saccharominimus* has been established to be a later synonym of *E. italicus*.[753,1074]
Newly Described *Enterococcus* Species	
Enterococcus alcedinis[360]	This species was isolated from the cloacae of common kingfishers (*Alcedo atthis*) during a study of the microbial microbiome of free-living birds.
Enterococcus lemanii[245]	This species (along with *E. eurekensis*) was isolated from swine manure storage pits.
Enterococcus eurekensis[245]	This species, and the species listed above, were isolated from swine manure storage pits.
Enterococcus lactis[727]	*E. lactis* was isolated from Italian raw milk cheeses (Bitto cheese).
Enterococcus ureilyticus[912]	This newly described yellow-pigmented, urease-positive species was isolated along with *E. rotai* from environmental sources (drinking water, plants, insects).
Enterococcus rotai[912]	This newly described yellow-pigmented, urease-positive species was isolated along with *E. ureilyticus* from environmental sources (drinking water, plants, insects).
Enterococcus rivorum[764]	This new species was isolated from "pristine" bodies of water in Finland. In this context, pristine means no human habitation, agriculture, or industry in the catchment region surrounding the flowing streams.

Clinical Spectrum of Enterococcal Infections

Enterococcus species cause complicated UTIs, bacteremia, endocarditis, intra-abdominal and pelvic infections, wound and soft tissue infections, neonatal sepsis, and, rarely, meningitis. While community-acquired enterococcal UTIs are very uncommon in the absence of anatomic predisposing factors or prior instrumentation, enterococci are the third most common agents of nosocomial UTIs.[451] While many patients with catheter-associated enterococcal UTIs are primarily colonized and not infected, catheter removal at the earliest possible time is often curative and no therapy is needed. The presence of enterococci in the urine is often asymptomatic, and, according to IDSA evidence-based guidelines, should not be treated with antibiotics except under certain conditions (i.e., pregnant women, patients undergoing urologic manipulations, patients with significant underlying disease/comorbidities).[649,762] In a study of 289 episodes of enterococcal bacteriuria, pyuria was present in 70% of 140 enterococcal UTI's (defined as significant bacteriuria with one or more additional signs/symptoms, including fever,

urgency/frequency, dysuria, suprapublic/flank pain, gross hematuria), but in only 42% of 149 episodes of asymptomatic bacteriuria (bacteriuria without other signs/symptoms).[647] Of the 339 episodes of enterococcal bacteriuria, only 7 subsequent infectious complications (6 bacteremias, 1 peritonitis) occurred within 30 days of the bacteriuria. The investigators in this study emphasized the need for adherence to established and recommended diagnostic criteria to avoid overtreatment and misuse of anti-enterococcal chemotherapy. Enterococci can, however, cause cystitis, pyelonephritis, prostatitis, perinephric abscesses, and bacteremia in older populations. Most of these are nosocomial or are associated with structural abnormalities or instrumentation of the urinary tract.

Enterococcal bacteremias can usually be traced to enterococcal infections at other sites besides the urinary tract (e.g., intravenous catheter infections, biliary tract infection, gastrointestinal/genitourinary infections) and are most often hospital-acquired. The organisms can also gain entry into the bloodstream through intra-abdominal or pelvic abscesses, wounds, decubitus ulcers, or intravenous access devices. Risk factors for the development of enterococcal bacteremia include advanced age, immunosuppression, underlying diseases and conditions (e.g., prematurity, diabetes, malignancy, congestive heart failure, renal insufficiency, deep-seated infections, prior gastrointestinal, genitourinary, or respiratory tract instrumentation, long-term hospitalization, indwelling devices, and the use of broad-spectrum antibiotics having little or no anti-enterococcal activity (e.g., cephalosporins).[434] Bacteremias caused by *E. faecium* are associated with a poorer prognosis than those due to *E. faecalis* primarily because of increased antimicrobial resistance among the former species and the inherent difficulties of adequately treating more resistant isolates.[376,696] Enterococci also cause 5% to 20% of all cases of endocarditis, and are the second or third most common cause of this infection depending on the population of patients under consideration. Enterococci, particularly *E. faecalis*, are also a common cause of prosthetic valve endocarditis.[338] Endocarditis usually occurs in older male patients with underlying valvular disease or with prosthetic valves and is generally subacute in clinical presentation, with patients having fevers, weight loss, malaise, and other vague constitutional symptoms. Endocarditis in these patients often follows procedures involving the gastrointestinal (e.g., transrectal prostatic biopsies, colonoscopy, fiberoptic sigmoidoscopy) or genitourinary (e.g., cystoscopy, prostatectomy) tracts. Complications of this infection include embolic phenomena frequently involving the CNS. In up to half of patients, enterococcal endocarditis results in acute heart failure that requires valve replacement

In intra-abdominal and pelvic infections, enterococci usually are found admixed with other indigenous aerobic and anaerobic organisms; pure spontaneous enterococcal peritonitis and enterococcal peritonitis associated with continuous ambulatory peritoneal dialysis have also been reported. Though many invasive enterococcal

infections originate in intra-abdominal sites, controversy exists regarding the organisms' role in the pathogenesis of intra-abdominal infections and peritonitis. Studies have shown that empiric anti-enterococcal antimicrobial coverage is beneficial for immunocompromised patients with nosocomially-acquired postoperative peritonitis, patients with abdominal sepsis who have received antimicrobials that select for enterococci, and patients with peritonitis and co-existing heart disease or prostheses who are at risk for enterococcal endocarditis.[426] Enterococci are often found in wound and soft tissue infections (e.g., burns, decubitus ulcers) with other facultative and anaerobic bacteria, and complications associated with such infections (e.g., enterococcal osteomyelitis) are rare. Early-onset neonatal enterococcal infections and sepsis usually result from organisms acquired from the mother during vaginal delivery and can result in bloodstream, pulmonary, wound/surgical site infections, and UTIs. Premature neonates are also at greater risk of developing serious late-onset nosocomial enterococcal infections, particularly following invasive procedures, placement of peripheral access devices or feeding tubes, and treatment with broad-spectrum antimicrobial agents. Enterococcal meningitis is a rare enterococcal infection that may be seen in both adults and children.[834] Enterococcal meningitis may develop spontaneously or as a postoperative infection. Individuals with spontaneous enterococcal meningitis usually have enterococcal infection at other sites, have severe underlying disease, and have concomitant enterococcal bacteremia more frequently than those with postoperative infections.[571] Underlying disease in patients with spontaneous meningitis include malignancy, diabetes, renal failure, and treatment with immunosuppressive agents. Patients with postsurgical enterococcal meningitis usually have an antecedent history of intracerebral hemorrhage, CNS neoplasms, head trauma, and hydrocephalus.[403] Patients with this condition often have intraventricular catheters, surgical site CSF leakage, or ventriculoperitoneal shunts in place prior to diagnosis. In these cases, fever, obtundation, and meningismus are usually present, and the CSF indices include high white blood cell counts, elevated protein, and low or normal glucose. Postsurgical enterococcal meningitis may or may not be associated with enterococcal infections at other sites.[403] In a review of enterococcal meningitis, *E. faecalis* accounted for 76% of isolates, and 15 of the 25 cases that were due to *E. faecium* were caused by vancomycin-resistant strains.[834] Although enterococcal airway and respiratory tract infections are uncommon, these organisms have been established as etiologic agents of nosocomial, aspiration-related, ventilator-associated, and community-acquired pneumonias, lung abscess, pleural empyemas, sinusitis, and bronchitis. These infections occurred in debilitated patients with various comorbidities (e.g., malignancies, postpneumonectomy esophageal–pleural fistulas, endocarditis, HIV infection, hepatic cirrhosis, myelodysplastic syndrome) and risk factors (e.g., old age, smoking, alcoholism).[897] Causative agents have included *E. faecalis*, *E. faecium*,

and *E. raffinosus*.

Antimicrobial Susceptibility of Enterococci

Resistance of enterococci to various antimicrobial agents contributes substantially to their pathogenicity.[464] Resistance genes are acquired by conjugative transfer mechanisms involving plasmids, and transposons carrying multiple antibiotic resistance genes. Enterococci display intrinsic, low-level resistance to the aminoglycosides and lincosamides, have

relatively high MICs for penicillins and cephalosporins, and are resistant to the action of sulfonamide agents in vivo. High-level aminoglycoside resistance usually results from acquisition of a transposon encoding an aminoglycoside-modifying enzyme that confers resistance to all aminoglycosides except streptomycin; high-level streptomycin resistance usually results from a ribosomal mutation involving a specific ribosomal protein or from acquisition of aminoglycoside nucleotidyltransferases such as ANT(3″)-Ia or ANT(6′)-Ia.[193] These enzymes alter the structure of the aminoglycoside molecule resulting in decreased binding of the drug to its ribosomal target. Intrinsic resistance of *E. faecium* to tobramycin, in particular, results from the presence of an AAC(6′)-modifying enzyme found all *E. faecium* isolates.[193] High-level aminoglycoside resistance has disseminated among enterococcal species other than *E. faecalis* and *E. faecium* and has been documented in strains of *E. avium*, *E. casseliflavus*, *E. gallinarum*, *E. raffinosus*, and *E. mundtii*. Elevated MICs for β-lactam agents are due to diminished affinity of cell wall PBPs for these agents. While ampicillin resistance in *E. faecalis* is uncommon, ampicillin resistance is seen in >90% of *E. faecium* isolates and is due to the presence of PBP5, which has a decreased binding affinity for penicillin and ampicillin.[352] Serious infections with enterococci are usually treated with penicillin or ampicillin in combination with an aminoglycoside. High-level resistance to aminoglycosides (i.e., streptomycin MICs higher than 2,000 μg/mL; gentamicin MICs higher than 500 μg/mL) in enterococci significantly alters the therapeutic approach to and clinical response of patients with bacteremia, endocarditis, and other serious infections, as strains with high-level resistance to aminoglycosides are not killed by the synergistic activity of theβ-lactam agent along with the aminoglycoside. Bactericidal synergism between penicillin and aminoglycosides requires that the organism not only have MICs for aminoglycosides lower than the values cited above, but also is predicated on serum penicillin/ampicillin concentrations exceeding the MIC; increasing β-lactam resistance among enterococci along with high aminoglycoside MICs further abrogates synergism. Because of the lack of reliable

penicillin–aminoglycoside synergism among high-level aminoglycoside-resistant enterococci and the emergence of methicillin-resistant *S. aureus*, vancomycin became a first-line drug effective against both staphylococci and enterococci.[799] Prior to the early 1980s, the susceptibility of enterococci to glycopeptide agents (i.e., vancomycin and teicoplanin) remained fairly predictable, but in 1986, high-level glycopeptide resistance emerged The appearance of vancomycin resistance in *E. faecium* and some *E. faecalis* heralded a major change in the enterococcal susceptibility.[620,1068]

The glycopeptides vancomycin and teicoplanin act by binding to the "D-alanyl-D-alanine" terminus of cell wall peptidoglycan intermediates and inhibiting cell wall cross-linking.[248] Acquired resistance to glycopeptides results from the synthesis of different peptidoglycan precursors that become incorporated into the cell wall and have decreased binding affinity for vancomycin, teicoplanin, or both.[37,975] Normally, vancomycin-susceptible strains have peptidoglycan side chains that terminate with the depsipeptide "D-alanyl-D-alanine," and the glycopeptide antibiotic binds to this depsipeptide. In glycopeptide-resistant strains, the "D-alanyl-D-alanine" depsipeptide is replaced by "D-alanyl-D-lactate" or by "D-alanyl-D-serine." Acquired glycopeptide resistance in *Enterococcus* species corresponds to eleven different *van* gene clusters based on DNA sequences and organization. These gene clusters encode either a D-alanyl-D-lactate (i.e., *vanA*, *vanB*, *vanD*, and *vanM*) ligase enzyme or a D-alanyl-D-serine (*vanC1*, vanC2, *vanC3*, *vanE*, *vanG*, *vanL,* and *vanN*) ligase enzyme for synthesis of peptidoglycan precursors with low affinities for glycopeptide agents. These eleven genotypes have correlated resistance phenotypes (i.e., VanA, VanB, VanC1, VanC2, VanC3, VanD, VanE, VanG, VanL, VanM and VanN), with VanA and VanB phenotypes being the most prevalent and important clinically.[111–113,341] The *vanA*, *vanB*, and *vanD* gene clusters can be differentiated on the basis of the level of expressed resistance, inducibility, and the location of the gene cluster.[378,387,1147] Genes encoding the VanA and VanB phenotypes are the most common and are carried on transposons that may be inserted into the chromosome or into plasmids. *E. faecium* and *E. faecalis* isolates that have the *vanA* genotype and VanA phenotype display vancomycin- and teicoplanin-inducible, transposon-mediated, high-level resistance to both vancomycin (MIC, 64 to 1,000 μg/mL) and teicoplanin (MIC, 16 to 512 μg/mL).[1147]Strains with the *vanB* genotype (VanB phenotype) have acquired vancomycin-inducible resistance to various concentrations of vancomycin (MIC, 4 to 1,000 μg/mL) but remain susceptible to teicoplanin (MIC, 0.5 to 1 μg/mL), although rare *vanB* strains may also be resistant to the latter antibiotic.[378,1147] Isolates expressing the VanB phenotype also include *E. faecalis* and *E. faecium* strains. Isolates that have the *vanC* genotype display intrinsic, constitutive, low-level resistance to vancomycin (MIC, 2 to 32μg/mL) and are susceptible to teicoplamin (MIC, 0.5 to 1 μg/mL). The *vanC* genotype corresponds to the intrinsic glycopeptide resistance seen

in *E. gallinarum, E. casseliflavus,* and *E. flavescens*. This *vanC* gene cluster is not transferred by conjugation to other organisms, is generally constitutively expressed, and is chromosomal in origin.[621,755] Some *E. faecium* and *E. faecalis* strains express the VanD phenotype (*vanD* genotype); these strains are intermediately and constitutively resistant to both vancomycin (MIC, 64 to 128 μg/mL) and teicoplanin (MIC, 4 to 64 μg/mL) and resistance is not transferable to other enterococci as the vanD gene cluster is chromosomal.[275,822] VanD-type vancomycin resistance is also found in some *E. avium* isolates.[274] Rare *E. faecalis* strains expressing the *vanE* genotype express inducible, low-level resistance to vancomycin (MIC, 16 μg/mL), yet remain susceptible to teicoplanin (MIC, 0.5 μg/mL).[1,111,341] The VanG phenotype is associated with low-level resistance to vancomycin (MIC, 16 μg/mL), but susceptibility to teicoplanin (MIC, 0.5 μg/mL) and was initially found in *E. faecalis* isolates from Australia and Canada.[112,703] The *vanF* genotype refers to the glycopeptide resistance gene cluster found in the former *Bacillus* species, *Paenibacillus popillae.*[812] The VanL phenotype (*vanL* genotype) is characterized by low-level vancomycin resistance (MIC of 8 μg/mL) encoded by a nontransferable, chromosomal gene cluster (*vanL*) and was detected in an *E. faecalis* isolate from VRE screening medium.[113] The *vanM* genotype was first found among glycopeptide-resistant *E. faecium* isolates that lacked the *vanA*, *vanB,* and *vanD* gene clusters.[1152] The responsible gene cluster was sequenced, found to be unique and distinct from other isolates, and was

named *vanM*. The *vanM* gene encoded a "D-alanyl-D-lactate" ligase enzyme that was related to *vanA*, vanB, and *vanD* and could be transferred by conjugation. The *vanN* operon was identified in *E. faecium* isolated from blood and showed constitutive low-level resistance to vancomycin (MIC, 16 μg/mL) and susceptibility to teicoplanin (MIC, 0.50 μg/mL).[618] The *vanN* resistance operon is located on a plasmid and is transferable. The genotypes, phenotypes, and their characteristics are summarized in Table 13-1.

Another enterococcal phenomenon is that of vancomycin dependence. This refers to strains of enterococci that grow only in the presence of vancomycin. Vancomycin-dependent enterococci (VDE) were first described in 1994, when these organisms were cultured from the urine of a patient receiving long-term therapy with vancomycin.[330,355] Since that time, other isolations of VDE have been reported.[280,396] Patients exposed to vancomycin and other agents (e.g., extended-spectrum cephalosporins) are at risk for colonization with VRE, and this exposure also places them at risk for becoming colonized with VDE as well. VRE and VDE have the ability

to use cell wall ligases to make an alternative cell wall depsipeptide ("D-alanine-D-lactate"), which replaces the normally found "D-alanine-D-alanine." If vancomycin is not present in the milieu, VRE retain their ability to make the "D-alanine-D-alanine" depsipeptide and proceed to grow normally. Because of mutations in the ligase genes that result in amino acid deletions/substitutions in the ligase enzyme, VDE strains are unable to make the original "D-alanine-D-alanine" depsipeptide.[1071] The presence of vancomycin enables these strains to use the "D-alanine-D-lactate" as a cell wall constituent, so their growth is actually dependent on vancomycin. These VDE strains are relatively uncommon. VDE strains derived from both *E. faecalis* and *E. faecium* have been described.[280,330,355,396]

Identification of *Enterococcus* species is discussed later in this chapter. Antimicrobials such as linezolid and daptomycin are commonly employed to treat patients with infections caused by VRE.[1030] Additional information on antimicrobial resistance and methods for antimicrobial susceptibility testing are discussed in detail in Chapter 17.

Genus Melissococcus *and Genus* Catellicoccus

The genus *Melissococcus* contains one species, *M. plutonius*. *M. plutonius* causes a disease of bees called "European foulbrood." Using 16S rRNA sequence data, Cai and Collins demonstrated that *M. pluton* was most closely related to genus *Enterococcus*.[126] *Catellicoccus* represents a new genus that is related to *M. plutonius,* enterococci, vagococci, and tetragenococci. The species, *C. marimammalium* was isolated from clinical specimens obtained from a porpoise and a gray seal.[608]

The "*Streptococcus* -Like" Bacteria

Abiotrophia and Granulicatella Species

"Nutritionally variant streptococci" were originally thought to be viridans streptococci that required thiol compounds (e.g., cysteine) or the active form of vitamin B_6 (i.e., pyridoxal, pyridoxamine) for growth and were also referred to as nutritionally deficient, thiol-requiring, pyridoxal-requiring, and "satelliting" streptococci. DNA relatedness studies of these organisms identified two DNA hybridization groups that corresponded to two species that were named *Streptococcus defectivus* and *Streptococcus adjacens*.[109] In 1995, Kawamura and associates in Japan performed 16S rRNA sequence studies with these species and found that they were unrelated to other species in genus *Streptococcus*. Genus *Abiotrophia* was proposed to accommodate these bacteria, and two new species—*Abiotrophia adiacens* and

Abiotrophia defectiva—were proposed.[544] In 1998 and 1999, two new members of this genus were characterized: *Abiotrophia elegans* (from a patient with endocarditis), and *Abiotrophia balaenopterae* (isolated from the minke whale, *Balaenoptera acutorostrata*).[612,869] In 2000, Kanamoto and colleagues[533] identified and characterized another new *Abiotrophia* species using genetic and phenotypic criteria that was closely related to *A. adiacens* and proposed the species name *Abiotrophia para-adiacens* for this new isolate. These new species were subsequently demonstrated to have a closer phylogenetic affinity with *A. adiacens* than with *A. defectiva,* with the latter species being phylogenetically related to non- *Abiotrophia* "*Streptococcus*-like" species, including *Globicatella sanguinis*, *Facklamia*, *Eremococcus*, and *Ignavigranum* species. The polyphyletic nature of the genus *Abiotrophia* and the close affinity between *A. adiacens*, *A. elegans*, *A. para-adiacens*, and *A. balaenopterae* resulted in reclassification of these four species in the genus *Granulicatella* as *Granulicatella adiacens*, *Granulicatella elegans, Granulicatella para-adiacens*, and *Granulicatella balaenopterae*.[230] *A. defectiva* is retained as the sole species in the genus *Abiotrophia*.

A. defectiva, *G. adiacens*, and *G. elegans* are part of the normal flora of the upper respiratory, urogenital, and gastrointestinal tracts.[713,894] *Abiotrophia* and *Granulicatella* species cause sepsis and bacteremia and are the cause of 4% to 6% of cases of infective endocarditis and bacteremic complications associated with endocarditis.[122] Endocarditis caused by *G. adiacens* is more common than that caused by *A. defectiva*, with *G. elegans* endocarditis being rare in comparison.[195] Over 17 cases of *Granulicatella* endocarditis involving mostly *G. adiacens* have been reported since 1997 and have included native valve, prosthetic valve, and pacemaker lead infections.[23,137,172,512,779,873,1078] The ability of *G. adiacens* to bind fibronectin has been postulated to be partially responsible for the higher prevalence of this organism in endocarditis than either *A. defectiva* or *G. elegans*.[915] Endocarditis caused all three species are highly associated with the development of embolic phenomena, valvular destruction, and treatment failure.[172,574,1078] *A. defectiva* and *Granulicatella* species have also been involved in other bloodstream and endovascular infections, including bacteremia without endocarditis, early-onset neonatal sepsis, infected aortic atheroma with aortic dissection, central venous catheter–related infections in compromised hosts, and Lemierre's syndrome.[6,91,827,1082] *A. defectiva*, and *G. adiacens* have been documented as causes of CNS infections, including meningitis, and brain and epidural abscesses.[163,711,1015] In almost all cases, infection was associated with prior neurosurgical procedures, including craniotomy, VP shunt placement, CT-guided myelography,

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TABLE 13-1 Genotypes and Phenotypes of Vancomycin-Resistant Enterococci

Genotype (Gene Clusters)	Phenotype	Type of Resistance	Vancomycin Resistance		Teicoplanin Resistance		Expression of Resistance	Location	Conjugative Transfer	Species With Resistance Type
			Level	MIC's (μg/mL)	Level	MICs (μg/mL)				
vanA	VanA	Acquired	High level	64 to ≥1,000	High	16–512	Inducible	Plasmid, transposon on chromosome	Yes	*E. faecium, E. faecalis, E. avium, E. casseliflavus, E. durans, E. gallinarum, E. hirae, E. mundtii, E. raffinosus*
vanB	VanB	Acquired	High–variable level	4–512	Susceptible	≤0.5	Inducible	Plasmid, chromosome, transposon, integrative conjugative element	Yes	*E. faecium, E. faecalis, E. casseliflavis, E. durans, E. gallinarum, E. hirae*
vanC1, vanC2, vanC3	VanC	Intrinsic	Low level	2–32	Susceptible	≤0.5	Constitutive	Chromosome	No	*E. casseliflavus (vanC2, vanC3), E. gallinarum (vanC1), E. flavescens (vanC3)*
vanD	VanD	Acquired	Moderate–high level	64–256	Low	4–32	Inducible/constitutive	Chromosome	No	*E. faecium, E. faecalis, E. avium, E. gallinarum, E. raffinosus*
vanE	VanE	Acquired	Low level	16	Susceptible	≤0.5	Inducible	Chromosome	No	*E. faecalis*
vanG	VanG	Acquired	Low–moderate level	≤16	Susceptible	≤0.5	Inducible	Chromosome or on integrative conjugative element	Yes	*E. faecalis*
vanL	VanL	Acquired	Low level	8	Susceptible	≤0.5	Inducible	Unknown, possibly chromosomal	No	*E. faecalis*
vanM	VanM	Acquired	High level	≥256	Susceptible to resistant	0.75 to ≥256	Inducible	Unknown	Yes	*E. faecalis*
vanN	VanN	Acquired	Low level	16	Susceptible	≤0.5	Constitutive	Plasmid	Yes	*E. faecium*

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and tumor resection. These organisms have also been isolated from significant ocular infections, including conjunctivitis, endophthalmitis following cataract surgery, infectious crystalline keratopathy, corneal ulcer following penetrating keratoplasty, and keratitis associated with extended wear hydrogel contact lenses.[548,750,815] Other infections involving *Abiotrophia* and *Granulicatella* species include pneumonia, scrotal abscess, bullous impetigo, sinusitis, septic arthritis, vertebral osteomyelitis, arthroplasty and breast implant infections, and continuous ambulatory peritoneal dialysis-associated peritonitis.[24,30,195,270,445,492] These organisms produce extracellular factors including neuraminidase and aminopeptidases that may contribute to virulence. Because of their clinical significance and exacting cultural requirements, clinical microbiologist must be familiar with the clinical settings and laboratory methods necessary for their isolation from patient specimens.

Abiotrophia and *Granulicatella* species are more resistant to antimicrobial agents than the viridans streptococci. For antimicrobial susceptibility testing, CLSI recommends broth microdilution MIC testing using cation-adjusted Mueller-Hinton broth with 2.5% to 5% lysed horse blood supplemented with 0.001% pyridoxine hydrochloride. Even with these modifications, some isolates fail to grow in this medium. Isolates in these genera display decreased susceptibility to penicillin, cefotaxime, and cefuroxime, and have variable susceptibilities to other cephalosporins

and aminoglycosides. *G. adiacens* (55% susceptible) is more susceptible to penicillin than *A. defectiva* (8% susceptible) and up to 60% and 47% of isolates may be resistant to ceftriaxone and cefipime, respectively.[137,1053,1175] Resistance to clindamycin, tetracycline, erythromycin, and ciprofloxacin but not to rifampin or vancomycin has been reported.[137,1053,1175]

Aerococcus *and* Helcococcus *Species*

Aerococci are *Streptococcus*-like organisms that are found in the environment, including air, dust, soil, vegetation, meat products, and the hospital environment. *Aerococcus viridans* causes a disease in lobsters called gaffkemia, but in humans this organism is primarily an opportunistic pathogen.[60] *A. viridans* is a rare cause of endocarditis, bacteremia, urinary tract infection, meningitis, and wound infections.[630,754,837,988] Aerococci can easily be confused with the viridans streptococci and enterococci. Aerococci tend to form tetrads when grown in broth and they are microaerophilic, with little or no growth under anaerobic conditions. Strains of *A. viridans* are usually susceptible to penicillin, macrolides, sulfonamides, and trimethoprim, although, in one case report, the blood culture isolate from an endocarditis patient was resistant to penicillin, ampicillin, cefotaxime, and gentamicin.

A second *Aerococcus* species, *Aerococcus urinae* was described in 1989 and named in 1992.[11,196,197] Inclusion of *A. urinae* in the genus *Aerococcus* is supported by 16S rRNA sequencing, and these data also support inclusion of both esculin-positive and esculin-negative biotypes within the same species.[198] When it was initially recognized, this organism was isolated in pure culture and in significant numbers from patients who were older than 50 years, and more than half of the patients had localized or systemic conditions that predisposed them to infection with opportunistic organisms, such as diabetes, urinary tract and nonurinary tract malignancies (e.g., underlying prostate disease), and urolithiasis. Subsequent studies have confirmed these findings.[917,933] Patients may be colonized in the urinary tract or may have overt symptoms; catheterized patients tend to have true infections and more debilitating underlying conditions. *A. urinae* can also cause severe, fatal bloodstream infections. DeJong and colleagues[268] described four elderly patients with various underlying diseases (e.g., benign prostatic hyperplasia, ischemic heart disease with stage IV non–small cell lung carcinoma, basal cell carcinoma, glaucoma, urolithiasis, diabetes mellitus) who developed *A. urinae* bacteremia. All of these patients also had underlying urologic conditions but all the urine cultures were negative. In recent years, *A. urinae* has emerged as a significant cause of endocarditis.[538,944,1013] Again, patients were elderly and debilitated from underlying diseases (e.g., myocardial infarction, prostate cancer, diabetes). *A. urinae* has been isolated from soft tissue infections, and

as a cause of acute pyelonephritis, spondylodiscitis, lymphadenitis, and spontaneous bacterial perionitis.[41,207,737,890] *A. urinae* may also cause peritonitis in patients on continuous ambulatory peritoneal dialsysis.[744] *A. urinae* is usually susceptible to penicillin, amoxicillin, and the fluoroquinolones, and some isolates have elevated MICs for the cephalosporins.[207,268] Susceptibility to clindamycin, erythromycin, and the tetracyclines is variable, and most isolates are resistant to the aminoglycosides and trimethoprim-sulfamethoxazole.[910,943] In a study of 66 *A. urinae* isolates, all were susceptible to penicillin, vancomycin, and ceftriaxone, but 17% of the isolates had elevated erythromycin MICs (MIC ≥ 0.5 μg/mL) and 23% had elevated levofloxacin MICs (>4 μg/mL).[917]Penicillin or vancomycin combined with an aminoglycoside (i.e., gentamicin) show bactericidal synergy, suggesting that combination therapy is the therapeutic choice for endocarditis and other serious *A. urinae* infections.

Since 1989, five new *Aerococcus* species have been described: *Aerococcus christensenii, Aerococcus urinaeequi, Aerococcus urinaehominis, Aerococcus sanguinicola*, and *Aerococcus suis*.[227,333,610,611,1089] *A. christensenii* was isolated from vaginal specimens and was originally identified as *Streptococcus acidominimus*. This organism has not been associated with any infections or pathology since its description in 1999. *A. urinaeequi* was transferred from the genus *Pediococcus* in 2005, and this species is most closely related to *A. viridans*.[333] The description of *A. sanguinicola* is based on a single isolate from a blood culture in 2001, and since then, this organism has been isolated from urine and from blood cultures.[488,917] Some patients with *A. sanguinicola* bacteremia had endocarditis caused by this organism.[488] As with *A. urinae*, patients with *A. sanguinicola* infection are usually elderly and have underlying or predisposing urologic disease, such as prostatic cancer and benign prostatic hypertrophy.[147,917] In a study of 66 *A. saguinicola* isolates, all were susceptible to penicillin, vancomycin, and ceftriaxone, but 41% of the isolates had elevated erythromycin MICs (MIC ≥ 0.5 μg/mL) and 78% had elevated levofloxacin MICs (>4 μg/mL).[917] The species *A. urinaehominis* is based on a single urine isolate and has not been associated with other pathology to date. Isolates of *A. sanguinicola* are susceptible to penicillin, amoxicillin, cefotaxime, cefuroxime,

erythromycin, vancomycin, quinupristin-dalfopristin, rifampin, linezolid, and tetracyclines, and variably susceptible to the quinolones; some strains are resistant to clindamycin, meropenem, and trimethoprim-sulfamethoxazole.[322] Information on the antimicrobial susceptibility of *A. urinaehominis* and *A. christensenii* is not available.

Other *Aerococcus*-like isolates were first described among several isolates obtained

from cultures of foot and legs wounds and from breast abscesses.[127,212] These particular strains were facultative, gram-positive, catalase-negative cocci that resembled aerococci but produced variable growth in 6.5% NaCl broth. These isolates were lipophilic in that growth on agar media and in broth was enhanced by the presence of serum or Tween 80. These organisms were shown to belong a new genus and species by genetic and phenotypic criteria and were assigned to the new genus *Helcococcus* as *Helcococcus kunzii*.[212] Studies on the ecology of this organism suggest that it is part of the bacterial skin flora of the lower extremities and that it has an association with cellulitis or wound infections along with other, more virulent organisms, such as *S. aureus*.[412] *H. kunzii* has been isolated from wounds, infected sebaceous cysts, breast abscesses, postsurgical foot abscess, and infection of a total knee prosthesis.[167,631,821,862] *H. kunzii* was isolated from infections in two patients as a cause of primary bloodstream infection and thoracic empyema, respectively, and a *Helcococcus*-like isolate was recovered from blood cultures of a patient with endocarditis.[346,1139] *H. kunzii* infection of the tissue surrounding an implantable cardiac rhythm device was successfully treated without device removal as there was no evidence of endocarditis.[706] Isolates of *H. kunzii* are susceptible to penicillin, ampicillin, ciprofloxacin, trimethoprim-sulfamethoxazole, and vancomycin, but susceptibilities to clindamycin and erythromycin are variable.[167,821,1139] In 1999, a second *Helcococcus* species, *Helcococcus ovis,* was isolated from sheep, horses, and cattle; this organism caused purulent bronchopneumonia in sheep and goats and endocarditis in cattle.[214,366,586] In 2004, a new *Helcococcus* species, *H. sueciensis,* was recovered from a wound culture, and another new species, *Helcococcus pyogenes,* was isolated from an infection of a prosthetic knee joint.[213,796,797] The latter organism most closely resembled *Pediococcus* species phenotypically, but molecular studies confirmed its association with the helcococci. This isolate was also relatively resistant to vancomycin, with an MIC of 8µg/mL.[796,797]

Leuconostoc *Species*

Leuconostoc species are gram-positive, nonmotile, non–spore-forming, heterofermentative, facultative cocci that are found in the environment (i.e., on plants, soil). These organisms have economic importance because of their use in the dairy and pickling industries and in wine making.[778] They are also found in fermented sausages, vacuum-packed meat products, cereals, and dairy products (butter, cream, fresh/raw milk, cheese). Phenotypically, these organisms are catalase-negative coccobacilli or cocci that produce gas from glucose along with formation of D(-)-lactic acid and do not hydrolyze arginine. All *Leuconostic* species are intrinsically resistant to vancomycin. Over 27 species and subspecies of *Leuconostoc* have been described but most species

are divisible into three subclusters based on 16S rRNA gene sequences: the *L. mesenteroides* subcluster, the *L. fructosum* subcluster, and the *L. fallax* subcluster (includes *L. fallax* only).[310] The four species in the *L. fructosum* subcluster were recently reclassified in the genus *Fructobacillus,* while *L. fallax* remains as the sole member of *L. fallax* subcluster. The *L. mesenteroides* subcluster includes several species, including those that have been isolated from human clinical specimens.

Leuconostoc bacteremia has been documented predominantly in patients with underlying malignancies (acute myeloid leukemia, non-Hodgkin's lymphoma, hepatocellular carcinoma) and as a complication of solid-organ (e.g., liver) and stem cell transplantation.[273,497,627,925,1022,1155] These coccal organisms also colonize indwelling intravenous catheters, resulting in catheter-related bacteremias.[349,479] Other risk factors associated with *Leuconostoc* bacteremia include prior or current treatment with vancomycin, gastrointestinal disease, and possibly collagen-vascular disease and rheumatoid arthritis.[925] *Leuconostoc* infections have been reported in extremely low-birth-weight and premature neonates with various underlying intestinal conditions, such as short bowel syndrome.[349,510,1159] In some cases, these infants were being treated with vancomycin when bacteremia developed.[510] Nosocomial infections have occurred in pediatric populations due to *Leuconostoc*- contaminated infant formula and enteral and parenteral nutrition supplements.[105] Nosocomial UTIs may also develop, again, in those patients on long-term vancomycin therapy.[133,1002] As opportunistic pathogens, *Leuconostoc* species have been isolated from brain and liver abscesses, osteomyelitis, and pulmonary infections in compromised and debilitated patients (e.g., gastroenterology patients, burn patients, AIDS patients) and as agents of spontaneous bacterial peritonitis and peritonitis in CAPD patients.[18,339,381,518,572,1069] While endocarditis due to a vancomycin-susceptible *Leuconostoc* species has been reported, details of microbial identification and antimicrobial susceptibility were not available.[962] While intrinsically resistant to vancomycin, *Leuconostoc* species are usually susceptible to azithromycin, daptomycin, tigecycline, linezolid, clindamycin, and SXT; variable susceptibilities, tolerance and full resistance to β-lactams (i.e., penicillin, ampicillin) has been described in some case reports.[105,349,497,627] *Leuconostoc* species may be misidentified as pneumococci, viridans streptococci, aerococci, or lactobacilli. Manual (e.g., ID32 Strep) and automated (i.e., Vitek2, Phoenix system, Biolog) identification systems have difficulty with identification compared with genotypic approaches (i.e., 16S rRNA sequencing), however, these systems do provide correct identifications to genus-level in most cases, which, along with susceptibility testing, is usually adequate for patient management.[105,273,627,925] Characteristics of *Leuconostoc* species that differentiate them from other *Streptococcus*-like bacteria are their resistance to vancomycin and the formation of gas

from glucose during growth in MRS broth (discussed later).

Pediococcus *and* Tetragenococcus *Species*

Pediococcus species are gram-positive cocci that, like the streptococci, produce lactic acid as the sole product of glucose fermentation. These organisms are found naturally on plants and are important in the brewing and food industries.

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Pediococci may be found in beers and ales and are also used in foods for processing and preservation. They are included as flavor enhancers in processed vegetables and soy products and are used in biotechnology as indicator strains for vitamin bioassays. As of 2013, the genus *Pediococcus* contains 14 species: *P. acidilactici*, *P. argentinicus*, *P. cellicola*, *P. claussenii*, *P. damnosus*, *P. dextrinicus*, *P. ethanolidurans*, *P. inopinatus*, *P. lolii*, *P. parvulus*, *P. pentosaceus*, *P. siamensis*, *P. solitarius,* and *P. stilesii*. *Pediococcus halophilus* represents a line of descent separate from both pediococci and aerococci so this species has been placed in genus *Tetragenococcus,* with the type species being *Tetragenococcus halophilus*.[234] The former species *P. urinaeequi* was transferred to the genus *Aerococcus* as *A. urinaeequi*.[333] The species *P. solitarius* was transferred to the genus *Pediococcus* from the genus *Enterococcus.*[311]

Pediococci have been isolated from several human clinical specimen types, including stool, urine, wounds, abscesses, and blood cultures. Patients infected with these organisms have underlying conditions, including hematologic malignancies, cardiovascular disease, chronic lung disease, gastroschisis (a birth defect in which there is a separation in the abdominal wall with a protrusion of an intestinal loop through this opening), pancreatitis, and diabetes.[59,441,893,940] Many patients with these infections had previous abdominal surgery or had nasogastric tubes or central venous catheters for total parenteral nutrition in place for prolonged periods. *P. acidilactici* endocarditis developed in a 32-year-old male following a small bowel transplant and he responded to daptomycin therapy.[501] Persistent bacteremia with pediococci in a 67-year-old female with a complicated clinical course following metastatic adenocarcinoma was also successfully treated with daptomycin.[974] Liver abscess due to *Pediococcus* species developed in a 27-year-old woman following a flare-up of Crohn's disease.[75] Blood cultures and specimens from an ultimately fatal necrotizing cellulitis following rupture of a retroperitoneal tumor grew *P. pentosaceus*.[710] Like *Leuconostoc* species, pediococci are intrinsically vancomycin-resistant, and many patients first manifest symptoms of infection while being treated with this agent. Isolates of pediococci from clinical specimens have been identified as either *P.*

acidilactici or *P. pentosaceus*.[57] Pediococci are generally susceptible to penicillin G, erythromycin, clindamycin, gentamicin, and imipenem.

Gemella *Species*

The genus *Gemella* includes seven species: *G. asaccharolytica*, *G. bergeriae, G. cuniculi, G. haemolysans*, *G. morbillorum*, *G. palaticanis,* and *G. sanguinis*.[218,219,226,475,558] *G. haemolysans*, type species of the genus, is an easily decolorized gram-positive coccus that characteristically appears as diplococci with adjacent sides flattened. Because of this, it was included with the gram-negative cocci in genus *Neisseria*. Cell wall analysis demonstrated that the organism had a thinner cell wall other gram-positive organisms, but was, nonetheless, gram-positive. Nucleic acid hybridization studies did not support any relatedness to the family *Neisseriaceae.* The negative catalase reaction initially relegated this species to family *Streptococcaceae*. But, unlike streptococci, growth is poor under anaerobic conditions. *G. morbillorum* was previously classified with the streptococci as *Streptococcus morbillorum* and was originally included among viridans streptococci. Genotypic studies of *S. morbillorum* demonstrated a closer relationship with *G. haemolysans* than to other streptococci and peptostreptococci.[558] Chromatography showed that these organisms produced acetic, formic, succinic, and pyruvic acids, plus traces of ethanol, along with lactic acid. These data supported transfer of *S. morbillorum* to genus *Gemella* as *Gemella morbillorum*. In 1998, two new *Gemella* species —*Gemella bergeriae* and *Gemella sanguinis*—were characterized and described.[218,219] Six strains of each species were recovered from blood and were distinct from the previously described *Gemella* species by biochemical and molecular characterization. Subsequently, two veterinary species—*Gemella palaticanis* and *Gemella cuniculi*—were described.[226,475] *G. palaticanis* was isolated from an oral vesicle of a Labrador retriever, and *G. cuniculi* was recovered from a submandibular abscess of a lop-eared pet rabbit. Th normal microbiome of *G. bergeriae* and *G. sanguinis* in humans is not known, and, other than recovery from blood cultures, no specific infections have been associated with them. The newest member, *G. asaccharolytica*, was isolated from an arm wound in an IV-drug user, a finger wound, and a labial abscess from a patient with a malignancy.[1064] Unlike the other *Gemella* species, *G asaccharolytica* is does not ferment any carbohydrates.

Gemella species are infrequently isolated from clinical specimens. Cultural similarity to viridans streptococci has likely resulted in misidentifications. *G. haemolysans* is part of the upper respiratory tract flora, whereas *G. morbillorum* is found in the respiratory and gastrointestinal tracts. Both organisms have been isolated as occasional causes of bacteremia and endocarditis involving both native and prosthetic heart valves in pediatric and adult patients.[20,329,482,597,989,1174] Interestingly, *Gemella* species have

also caused endocarditis following tongue piercing.[1161] As ophthalmic pathogens, *G. haemolysans* can cause infectious crystalline keratopathy and both *G. haemolysans* and *G. morbillorum* have caused postsurgical, cataract- and glaucoma-associated endophthalmitis.[530,732,747,863] Both species have also been isolated as causes of chronic ambulatory peritoneal dialysis-related peritonitis, and liver abscess.[406,672,1065] As with *S. gallolyticus* (former *S. bovis*), an association has been made between *G. haemolysans* and *G. morbillorum* bacteremia, endocarditis and presence of adenomatous polyps and possible colonic carcinoma.[443,657] Culture- and molecular-based approaches have shown that *G. morbillorum* is a consistently present and persistent pathogen in primary and secondary/persistent endodontic (i.e., root canal and periradicular abscesses) infections.[389] *G. haemolysans* has been isolated from cerebrospinal shunt fluid in a 16-year-old-boy, and recovery required antibiotic treatment and removal and replacement of the shunt.[654] *G. morbillorum*, the more commonly isolated species, is an uncommon cause of central nervous system infections, osteomyelitis and joint space infections, and pulmonary abscesses.[70,1079,1098]

The late 1990s and 2000s saw the discovery of several new *Gemella* species. The first six isolates of *G. bergeriae* were from blood cultures; three patients had clinical endocarditis.[218] Two subsequent reports of *G. bergeriae* endocarditis have appeared, including endocarditis in a 15-year-old boy with tetralogy of Fallot and pulmonary atresia.[308,656]

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G. sanguinis has been isolated from blood cultures of patients with suspected or confirmed endocarditis, with the initial isolates being from Virginia, New York, Georgia, California, and Sweden.[219] All were identified initially as *G. morbillorum*, *G. haemolysans*, or "*Gemella* species". Another case of *G. sanguinis* endocarditis was described in a 69-year-old male with poor dentition and vegetations on both mitral leaflets; valve replacement was required.[928] In this case, the organism was identified by amplification of the 16S rRNA by PCR and sequencing of the PCR product. A third case of *G. sanguinis* endocarditis was reported in 2013.[166] *G. asaccharolytica* was isolated from wound specimens (arm wound in an IV-drug user, a finger wound, and a labial abscess) from three patients.[1064] *Gemella* species are usually susceptible to a wide variety of antimicrobial agents, including penicillin, ampicillin, rifampin, and vancomycin, but low-level resistance to aminoglycosides and trimethoprim may be observed in some isolates.

Vagococcus *Species*

The genus *Vagococcus* was proposed in 1989 to accommodate motile gram-positive, catalase-negative cocci recovered from chicken feces and river water. Phenotypically, they resembled lactococci, but 16S rRNA sequencing indicated that these motile strains formed a distinct line of descent within the lactic acid-producing bacteria and were separate from the streptococci, enterococci, and *Lactococcus* species *sensu stricto*. These isolates were also phenotypically distinct. Collins and associates[210] placed these organisms into the new genus *Vagococcus* ("wandering coccus"). The motile group N *Lactococcus*-like isolates in the genus *Vagococcus* are now named *Vagococcus fluvialis* ("belonging to a river"). Strains of *V. fluvialis* have also been recovered from cutaneous lesions and tonsils of pigs, cattle, horses, and domestic cats.[839] The CDC has reported receipt of two *V. fluvialis* strains, one from a blood culture and the other from peritoneal fluid of a renal dialysis patient.[319] Additional isolates of *V. fluvialis* have been recovered from human clinical sources (i.e., blood cultures, peritoneal fluid, wounds, infected root canal lesions), animals (i.e., pigs), and the environment (i.e., well water).[21,1012]

During a genetic study of atypical lactobacilli isolated from various meat products (members of the genus *Carnobacterium*), two isolates from adult rainbow trout were described that were distinct from the poultry isolates.[1103] These two strains showed the highest degree of 16S rRNA sequence homology with strains of *V. fluvialis*, forming a genetic cluster close to the carnobacteria, streptococci, lactococci, and enterococci. These salmonid isolates were assigned to genus *Vagococcus* as *Vagococcus salmoninarum*. Subsequently, this organism was shown to cause peritoneal infections in salmon and brown trout. In 1999, a third *Vagococcus* species —*V. lutrae*—was isolated from blood, liver, lung, and spleen specimens of a common otter (*Lutra lutra*).[613] A fourth *Vagococcus* species was isolated from autopsy specimens of seals and porpoises. This organism—dubbed *V. fessus*—was recovered in pure culture from liver, lung, kidney, brain, intestinal, and placental tissues.[476] *V. carniphilus* was characterized in 2004, and another species, *Vagococcus elongatus* was described in 2007.[609,920] *V. carniphilus* was recovered from ground beef, while *V. elongatus* was isolated from swine manure. Recently, two *Vagococcus* species were isolated from a bioreactor used for treating food wastewater (*V. acidifermentans*) and from the "spoilage microbiota" of cooked shrimp in France (*V. penai*).[505,1107] Only *V. fluvialis* has been isolated from clinical specimens obtained from humans.

Alloiococcus *Species*

In 1989, Faden and Dryja reported on the recovery of a slow-growing, large, gram-positive coccus from 10 children with chronic otitis media with effusion.[324,430] The

children ranged in age from 10 months to almost 3 years, and each child had histories of two to five previous episodes of otitis media. Middle ear aspirates from these children consisted of serous, mucoid, or frankly purulent fluid; inflammatory cells were observed in all specimens. In most cases, large, gram-positive, coccal organisms could also be seen on Gram-stained smears of these fluids. A novel organism was recovered in pure culture from 11 ear aspirates and in mixed culture with nontypeable *Haemophilus influenzae*, diphtheroids, micrococci, or coagulase-negative staphylococci in five specimens. One of the children examined in the study had the same organism isolated from three separate middle ear specimens collected over an 8-month period. On culture, the organisms grew extremely slowly, leading the authors to suggest that perhaps they had been missed in the past because culture plates had not been incubated long enough. Because of the association of this slow-growing gram-positive coccus with chronic otitis media, Faden and Dryja recommended that middle ear aspirates submitted for culture should be incubated for at least 5 days to facilitate detection of these bacteria.[324] Aguirre and Collins subsequently characterized this unusual coccus and named it *Alloiococcus otitis*.[12] A revision of the name of this organism from *A. otitis* to *A. otitidis* was subsequently suggested and approved in keeping with the rules of binomial nomenclature. In addition to isolates from middle ear fluids, the CDC has received strains of *A. otitidis* that were isolated from blood cultures and sputum.[319] With adequately collected tympanocentesis specimens, Gram's stains and cultures of the material are positive if plates are incubated under microaerophilic conditions or in the presence of increased CO_2 (7.5%) for 7 days and if enrichment in BHI broth with subculture is performed.[40,272] Because of its slow growth, several workers have developed molecular amplification methods for detection of this organism is middle ear effusions.[39,44,410,542,633] These molecular methods have also demonstrated that otitis media with effusion is more often polymicrobial than acute otitis media and that carriage of *A. otitidis* in the nasopharynx of at-risk children is rare.[463,509] These highly sensitive techniques have also enabled the detection of this organism in the outer ear canal, suggesting that this site is the natural habitat and reservoir of *A. otitidis*.[301,678,1007] Isolates of *A. otitidis* are susceptible or intermediately resistant to penicillin, ampicillin, and the expanded-spectrum cephalosporins (i.e., cefixime, ceftriaxone), and are resistant to erythromycin and trimethoprim-sulfamethoxazole.[104] Some of these agents (β-lactams, erythromycin) are frequently used to treat otitis media in children. Persistence of the organism in middle ear fluids in children who have received these agents has been

demonstrated by both cultural and molecular methods.[429] Western immunoblot

techniques have demonstrated the presence of IgG_2, IgA, and IgM against *A. otitidis* in middle ear effusion specimens.[430] Typing of *A. otitidis* isolates from children by PFGE has revealed at least 13 different PFGE types.[554]

Globicatella *Species*

In 1992, Collins et al.[209] described nine unique human clinical isolates (five from blood cultures, three from urine cultures, and one from CSF). Phenotypic traits excluded these strains from the *Streptococcus*, *Lactococcus*, *Enterococcus*, or *Aerococcus* groups and genetic investigations showed a distinct line of descent for them within the "lactic acid" bacteria. These strains have been assigned to the new genus *Globicatella* (meaning a short chain composed of spherical cells). All strains were phenotypically similar and have been given the species name *Globicatella sanguinis* (formerly *G. sanguis*) Isolates of *G. sanguinis* that have been submitted to the CDC have been from blood cultures, urine cultures, wound cultures, and a CSF specimen obtained during an autopsy.[319] In 2001, Shewmaker and colleagues[922] reported on 28 strains of *G. sanguinis* that had been isolated from urine cultures and from blood cultures of patients with diagnoses of endocarditis, sepsis, and bacteremia. Since its initial description, *G. sanguinis* has been particularly associated with bacteremia and meningitis, including one report of ventriculoperitoneal shunt-associated meningitis and one describing meningitis following a head injury.[4,506,600,913] In one case, the *G. sanguinis* isolate from the CSF of a 56-year-old woman with signs and symptoms of meningitis was found colonizing the rectum and the groin area of the patient.[448] In Japan, *G. sanguinis* was isolated along with *Corynebacterium riegelii* from blood and urine cultures of an elderly male with urolithiasis.[695] Vandamme and colleagues[1075] in Belgium characterized a second *Globicatella* species that was isolated from respiratory tract and septic joint infections in calves, sheep, and swine. This isolate was named *Globicatella sulfidifaciens* due to its ability to produce H_2S.

Facklamia *Species*

In 1997, Collins and associates[215] characterized five human isolates of catalase-negative, gram-positive cocci from urine, vaginal, blood, and abscess specimens. Comparative 16S rRNA gene sequencing demonstrated that these strains were genotypically homogeneous and constituted a new phylogenetic line closely related to the genus *Globicatella*. These isolates also differed from *G. sanguinis* in their peptidoglycan murein structure. Phenotypically these isolates were quite different. *G. sanguinis* produced acid from several carbohydrates, while the new isolates were totally asaccharolytic. Some characteristics resembled those of salt-tolerant *Gemella*-like organisms (like *D. pigrum,* below). These isolates were placed in the genus

Facklamia as *Facklamia hominis*.[215] Since its description, *F. hominis* has been associated with a case of chorioamnionitis, when it was isolated from blood and placental specimens of a 34-year-old woman following spontaneous rupture of membranes and delivery at 31 weeks gestation.[436] The same organism was isolated from a gastric aspirate from the premature neonate.

Several additional *Facklamia* species have been described in the taxonomy and microbiology literature in subsequent years. Collins and associates[221,231] characterized two human blood isolates as a second asaccharolytic species, *Facklamia ignava,* and the next year the same group described *Facklamia sourekii* in their characterization of another two isolates recovered from human blood cultures. These species differed from *F. hominis* and *F. ignava* in being active fermenters. *Facklamia languida* was proposed as a new *Facklamia* species to describe three human isolates from blood and cerebrospinal fluid specimens.[607] *F. languida* is asaccharolytic, but differs phenotypically from the other species in several enzymatic reactions. *Facklamia tabacinalis* was also described in 1999 and named to describe an isolate recovered as a contaminant from snuff.[221] Its 16S rRNA gene sequence most closely resembled *F. ignava*. *Facklamia miroungae* was described in 2001 and was isolated from a nasal swab of a juvenile elephant seal (*Mirounga leonina*).[474]

Facklamia species appear to have slightly different antimicrobial susceptibility profiles. LaClaire and Facklam determined the susceptibilities of *F. hominis* (four strains), *F. ignava* (five strains), *F. languida* (six strains), *F. sourekii* (three strains), and *F. tabacinalis* (one strain), and found that one *F. hominis* and two *F. ignava* strains had penicillin MICs that tested in the intermediate range, while all other strains were penicillin-susceptible.[590] One strain of *F. ignava*, one of *F. sourekii*, and all six *F. languida* strains were resistant to cefotaxime; 5 of the 6 latter strains were also resistant to cefuroxime. While most isolates were susceptible to erythromycin, three *F. ignava* strains and two *F. languida* strains were erythromycin-resistant. Five of the six *F. languida* strains and 1 of the 5 *F. ignava* strains were resistant to clindamycin. All isolates were either intermediate or resistant to trimethoprim-sulfamethoxazole, and none of the isolates were resistant to levofloxacin or vancomycin.

Dolosigranulum, Ignavigranum, Dolosicoccus, *and* Eremococcus *Species*

Dolosigranulum and *Ignavigranum* are *Gemella*-like genera that are able grow in 6.5% NaCl. *Dolosigranulum pigrum*, the sole species in the genus, was described in 1993, and the CDC Streptococcus Laboratory has characterized several isolates from blood cultures, eye specimens, nasopharyngeal swabs, sputum, urine, gastric and sinus aspirates, and autopsy spinal cord specimens.[13] *D. pigrum* has been isolated from

blood cultures of a 64-year-old male patient with synovitis and a 76-year-old man with acute cholecystitis.[418,648] This organism was recovered from respiratory secretions of a patient with ventilator-associated pneumonia and from bronchoalveolar lavage fluid and blood cultures of a 73-year-old man with COPD and chronic respiratory insufficiency.[460,622] This organism was isolated from blood cultures of a rheumatoid arthritis patient who developed synovitis in multiple joints associated with an infected hip replacement. The organism was seen on Gram's stains of the synovial fluid but the organims was not recovered because specimens were submitted after treatment had been started.[521] LaCaire and Facklam performed susceptibility tests on 27 clinical isolates of *D. pigrum* and found that all were susceptible to penicillin, amoxicillin, cephalosporins (i.e., cefotaxime, cefuroxime),

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clindamycin, levofloxacin, meropenem, rifampin, tetracycline, and vancomycin; fifteen of the isolates were resistant to erythromycin.[589]

Ignavigranum ruoffiae is the name given to two strains of catalase-negative gram-positive cocci that were isolated from a wound and an ear abscess.[232] These salt-tolerant strains were phylogenetically related to *Globicatella* and *Facklamia* species and phenotypically resemble the asaccharolytic *Facklamia* species. Antimicrobial susceptibility data on these strains is not available.

The genus *Dolosicoccus* was created to accommodate another unique catalase-negative gram-positive coccus that was isolated from a blood culture.[228] This isolate was phylogenetically related to *G. sanguinis* and the *Facklamia* species cluster, and phenotypically resembles *F. hominis* and *F. ignava*. The sole species in the genus is *Dolosicoccus paucivorans*.

The genus *Eremococcus* was created to accommodate two phylogenetically and phenotypically unique cocci recovered from the genital tracts of thoroughbred mares.[229] Comparative 16S rRNA sequencing demonstrated that these strains were related to *A. defectiva* and *G. sanguinis* and more distantly related to *Facklamia* species and *I. ruoffiae*. The single species in the genus is *Eremococcus coleocola*.

Lactococcus *Species*

Genus *Lactococcus* contains the "lactic acid" or "milk" streptococci. About 80% of isolates carry the Lancefield group N antigen. Lactococci have important roles in the dairy industry as they are used as starter cultures for the production of various dairy products (including several types of cheese) from over 100 million metric tons of milk yearly.[335] Strains are cultivated for their different fermentative and flavor enhancement capabilities. As of 2012, 11 species and subspecies of *Lactococcus* have

been described, but those species recovered from human clinical specimens (and also the species and subspecies used most in dairy products) are *L. garvieae*, *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *cremoris*. The latter two subspecies are separated by phenotypic criteria that are strain-variable and genotypic variation (i.e., subspecies *cremoris* phenotype with a *lactis* genotype, and vice versa) has been described *L. garveiae*, *L. lactis* subsp. *lactis,* and *L. lactis* subsp. *cremoris* have mostly been associated with native- and prosthetic valve endocarditis.[348,454,637,644,879,1170] Native disease most often involves the mitral valves. Most cases of endocarditis have occurred in elderly people with valve prostheses. Commonly reported complications include the formation of bulky vegetations and the propensity to cause cerebral infarction.[637,644] In a case of *L. garviae* native valve endocarditis in a 55-year-old woman, the clinical course was complicated by cerebral and renal infarcts and a mycotic aneurysm.[1108] *L. garvieae* endocarditis also occurred in a 63-year-old patient who had previously undergone two bouts of enterococcal endocarditis.[879] Transesophageal echocardiography revealed that the heart lesion was on the native valve and spared the prosthetic aortic valve. *L. garvieae* has also been isolated from a total hip arthroplasty- and joint-site infection and from bone biopsy specimens from the lumbar spine of a patient with spondylodiscitis.[43,169] *L. lactis* subspecies *lactis* and *cremoris* are rare causes of catheter-associated bacteremia, and late-onset bacteremia and meningitis due to *L. lactis* has been seen in neonates.[385,1059] A 2-cm temporal brain abscess due to *L. lactis* subsp. *cremoris* occurred in a 19 month old Turkish child that resolved after drainage along with ceftriaxone and meropenem therapy.[1035] Bacteremia with lactococci has also lead to liver abscess with empyema and ascending cholangitis in two previously healthy individuals.[262,560] Peritoneal dialysis-associated peritonitis due to *L. lactis* subsp. *cremoris* developed in a 63-year-old male who made and ate yogurt in his home while he made his dialysis exchanges.[592]

Isolation and Identification of Streptococci and the "*Streptococcus* -Like" Bacteria

Direct Gram-Stained Smears

Gram-stained smears of clinical specimens that yield streptococci on culture will generally show gram-positive or gram-variable cocci arranged in pairs and chains. Chains of cells in both specimens and broth cultures tend to appear as chains of pairs of cells rather than as chains of individual cells. Individual cell shapes range from those that resemble diplococci to those that appear coccobacillary or coryneform. This

morphology is often observed on smears from broth cultures and from solid media as well. Viridans streptococci, in particular, tend to have cells that appear more elongated. *S. pneumoniae* will most often appear as pairs of lanceolate-shaped cells. On smears of specimens yielding mucoid, heavily encapsulated strains, the capsule may appear as a pink halo or as a nonstaining area surrounding the cells in relief against a pink background surrounding the organism. If the cellular morphology is consistent with *S. pneumoniae*, this should be reflected in the Gram's stain report as "gram-positive diplococci resembling *S. pneumoniae* (or pneumococci)."

A rapid diagnosis of group B streptococcal and pneumococcal meningitis can be made by examination of a Gram-stained smear of CSF. If sufficient CSF is submitted (i.e., more than 1 to 2 mL), the specimen should be centrifuged to obtain a pellet for examination and culture. Cytocentrifugation of CSF specimens enhances the detection of small numbers of organisms and increases the sensitivity of the Gram's stain in comparison with conventionally centrifuged or uncentrifuged specimens. Use of cytocentrifugation circumvents the need for the performance of latex or coagglutination tests for detection of group B streptococcal or pneumococcal capsular antigens (see Direct Detection section below).

Culture Media

Specimens should be inoculated onto a suitable blood-containing medium that has a peptone base. The base medium should be a peptone infusion medium without added carbohydrates, such as tryptic-soy, proteose-peptone, or Todd–Hewitt medium. Sheep blood is added to the basal medium at a 5% concentration as the indicator cells for hemolysis. Lower concentrations of blood in the media make the hemolytic reaction difficult to discern, whereas higher concentrations may obscure hemolysis entirely. Although the colonies

will generally be larger on glucose-containing basal medium such as Columbia agar after 24 hours, utilization of glucose in the medium inactivates the SLS of group A β-hemolytic streptococci and makes interpretation of hemolytic reactions less clear. Streptococci belonging to groups A, B, C, F and G are β-hemolytic (to be described in the following), whereas the majority of *Enterococcus* species and group D streptococci are α-hemolytic or nonhemolytic. *Arcanobacterium haemolyticum*, another cause of pharyngitis, also grows well on SBA, is catalase-negative, but is clearly a gram-positive rod and not a coccal organism.

Selective agar may also enhance the recovery of group A streptococci from throat cultures. The formulations that are most often used and that are commercially available employ a tryptic soy agar base containing 5% sheep blood and

sulfamethoxazole (23.75 µg/mL)-trimethoprim (1.25 µg/mL). Much of the normal oropharyngeal flora (e.g., viridans streptococci, micrococci, staphylococci, and neisseriae) will be inhibited on this medium. Use of this selective medium enhances recovery of group A and B streptococci and allows visualization of β-hemolysis without the "background" of other organism growth. Commercially available selective media include Group A Selective Strep A with 5% sheep blood (ssA; BD Diagnostic Systems, Sparks, MD), and Strep A Isolation Agar (Remel Laboratories, Lenexa KS). Most laboratories incubate media for isolation of group A β-hemolytic streptococci for 48 hours in a CO_2-enriched environment, assuming that only cultures from patients with small numbers of organisms or from those who are group A streptococcal carriers will grow under anaerobic conditions. However, even patients with small numbers of organisms may have an immune response, suggesting that they are infected and not merely carrying the organism. Small numbers of organisms on a culture plate in a patient with clinical streptococcal pharyngitis may also reflect inadequate specimen collection.

Streptococci recovered from human clinical specimens are identified on the basis of their hemolytic qualities, serologic tests for the detection of cell wall or capsular antigens, and physiologic and biochemical tests. Some of the tests performed in the laboratory for identification of these organisms provide presumptive results, whereas others provide definitive results. Before proceeding with identification tests, however, one must be sure that the gram-positive cocci under consideration are **catalase-negative** using 3% H_2O_2, placing them in the *Streptococcus* and *Streptococcus*-like bacterial groups. The catalase test is discussed in detail in Chapter 12.

Hemolysis on Blood Agar

Four types of hemolysis may be produced by streptococci on SBA (Box 13-6) (Image Plate 13-1D, 13-1E and13-1F). Observation and correct interpretation of the hemolytic properties of streptococci is very important because the performance of subsequent tests is predicated on this initial evaluation. Hemolysis is best observed by examining colonies grown under anaerobic conditions or by inspecting subsurface colonies in pour plates or streak-stab plates because, for group A streptococci, maximal activity of both the oxygen-labile (SLO) and oxygen-stable (SLS) hemolysins is observed only under anaerobic conditions. Oxygen-labile hemolysins are also produced by group C and some group G strains, so detection of hemolysis by these organisms is also enhanced by anaerobic incubation. Although routine anaerobic incubation of specimens expected to yield streptococci is not recommended, steps can be taken to maximize detection of hemolysis under aerobic or capnophilic incubation. This is the point of the "streak-and-stab" technique that is used for inoculating throat swab specimens on blood agar for diagnosis of streptococcal pharyngitis (Fig. 13-2). This technique forces some of the inoculum under the agar, thereby creating a

relatively anaerobic environment. Areas of the plate not inoculated with specimen should also be stabbed. Plates should be incubated at 35°C in air or in 5% to 7% CO_2. Although some laboratorians advocate one incubation environment over the other for throat cultures, the recovery of β-hemolytic streptococci from patients with streptococcal pharyngitis will not generally be compromised under either environmental condition.

Nonculture, Direct Detection Techniques for Group A β-Hemolytic Streptococci in Pharyngeal Specimens

Nonculture techniques for direct detection of group A streptococci on throat swab specimens have been in wide use

BOX 13-6

Hemolytic Reactions of Streptococci and *Streptococcus* -Like Bacteria

Type of Hemolysis	Description
α	Partial lysis of the erythrocytes surrounding a colony, causing a greenish-gray or brownish discoloration of the media.
β	Complete lysis of the erythrocytes, causing a clearing of blood from the medium under and surrounding the colonies.
γ	No hemolysis and, consequently, no change in the medium under and surrounding the colonies; organisms showing no hemolysis are referred to asγ-hemolytic or "nonhemolytic."
α-Prime or "wide zone α"	A small zone of intact erythrocytes immediately adjacent to the colony, with a wider zone of complete hemolysis surrounding the zone of intact erythrocytes; may be confused withβ-hemolysis.

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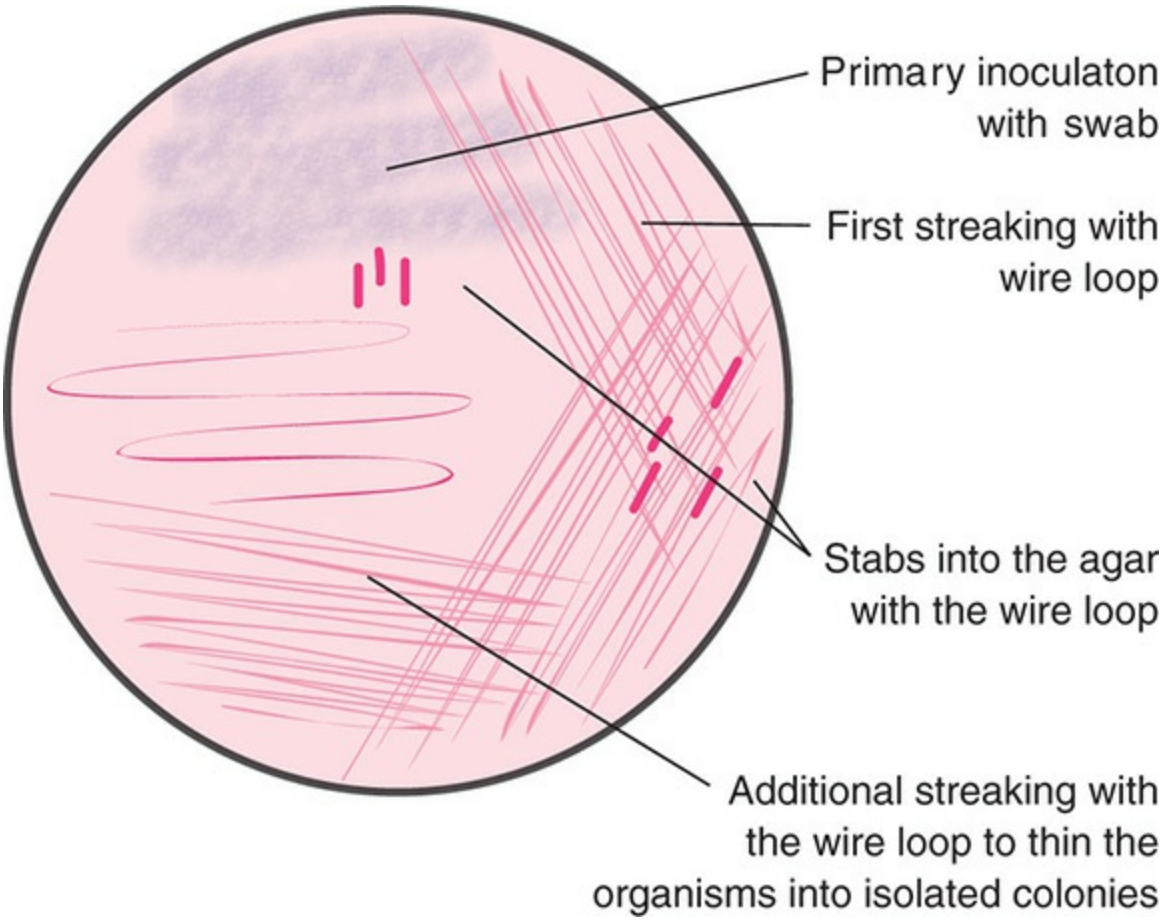

■ **FIGURE 13-2** Streak–stab technique for isolation of β-hemolytic streptococci.

in clinical laboratories since the early 1980s. In these rapid antigen detection tests (RADT's), the group A streptococcal antigen is extracted from the swab using nitrous acid or an enzymatic extraction step, followed by detection of the extracted antigen. The initially marketed kits used latex agglutination or enzyme immunoassay formats for detection, but most RADT's now use lateral flow immunoassays (e.g., Clearviw Strep Exact II cassette, Remel) or rapid immunochromatographic strip "dipstick" approaches (e.g., Clearview Strep A Exact II Dipstick, Remel; BD Chek Group A Strep, Becton-Dickinson, Sparks, MD; ImmunoCard STAT Strep A Test, Meridian Bioscience, Inc., Cincinnati, OH) for detection (Image Plate 13-1H). The sensitivities of these assays for detecting group A streptococcal antigen in throat swab specimens range from 62% up to 96%; specificities in most cases exceed 97%.[375,598,1008] While cultures and some RADTs have been deemed as moderately complex tests by CLIA, other RADT's that are easier to perform are classified as CLIA-waived and can be performed without certification.[1008] Direct DNA probe (e.g., the Gen-Probe Group A Streptococcus [GASDirect] Test, Gen-Probe, San Diego, CA) and amplified probe

(e.g., Illumagene, Meridian Bioscience, Cincinnati, OH) technologies have also been used for direct detection of group A streptococci in throat swab specimens. The Group A Streptococcus Direct Test (Gen-Probe) is a nonisotopic chemiluminescent method that uses a DNA probe to detect the complementary rRNA sequences of group A streptococci directly in an extract from a throat swab. Test sensitivities range from 88.6% to >95%, with specificities of greater than 98%.[107,108,176,442,836] The Illumigene Group A *Streptococcus* DNA amplification assay (Meridian Bioscience) uses novel loop-mediated amplification (LAMP) technology to target a highly conserved 206-base-pair region of the group A streptococcal pyrogenic exotoxin B (*speB*) gene. A 2013 multicenter clinical evaluation of the Illumigene Group A *Streptococcus* test reported a sensitivity and specificity following discrepancy analysis of 99.0% and 99.6%, respectively.[31] Other real-time PCR assays (e.g., LightCycler Strep A assay, Roche Applied Sciences, Indianapolis, IN) have also been developed that target other group A streptococcal genes (e.g., *dnaseB* gene) and have shown high sensitivity and specificity.[945,1062]

Although the specificity of RADTs is quite high, the sensitivities of the various kits for group A streptococcal antigen detection can vary widely (between 62% and 95%). As with most tests, the inoculum size on the throat swab depends on how well the specimen was collected, and swabs containing a lighter inoculum are more likely to produce negative RADT results.[206,583] While the American College of Physicians and the American Society of Internal Medicine recently proposed practice guidelines for management of adults with pharyngitis that relied on clinical criteria alone (without using rapid antigen tests or cultures) for diagnosis, this approach is considered to be controversial by other professional societies, including the Infectious Diseases Society of America, the American Heart Association, and the AAP.[88,238,379,650] A diagnostic strategy employing collection of two swabs, with a negative RADT being followed by repeat testing with the same RADT using the second swab specimen was not as sensitive as performing a culture following a negative RADT result (91.4% vs. 95.7%).[379] The American Heart Association, package inserts for many of the rapid antigen test kits, and most clinical microbiologists recommend that all specimens that yield negative rapid antigen results be confirmed by culture. Regardless of the method used or the claims of the manufacturer for a given test kit or technique, it is recommended that two throat swabs be collected. A standard throat culture should be performed with the second swab for those specimens that yield negative rapid antigen detection results with the first swab. Some laboratories have substituted the Gen-Probe Group A Strep Direct test in place of culture for confirmation of negative direct antigen results.

Nonculture, Direct Detection Techniques for Group B β-Hemolytic

Streptococci

Diagnosis of systemic group B streptococcal disease is best accomplished by culture of the organisms from appropriately collected specimens. Methods are also available for direct detection of group B streptococcal capsular antigen in cerebrospinal fluid, serum, and urine. Latex agglutination (LA) and coagglutination (COA) tests have been used in many laboratories for rapid diagnosis of systemic group B streptococcal infections, particularly meningitis in the newborn period. Currently available LA tests include the Directogen Meningitis Combo Test kit (BD Biosciences Microbiology Products, Sparks, MD), and the Wellcogen Bacterial Antigen kit (Murex Diagnostics Limited, Central Road, Temple Hill, Dartford, England) Research Triangle Park NC). The commercial COA test kit is the Phadebact CSF test (Boule Diagnostics AB, Huddinge, Sweden) Sensitivities of the LA products for detection of group B streptococcal antigens range from the mid-80% to 100%, although some studies have reported sensitivities as low as 27% to 54%. Many laboratories have discontinued offering these tests routinely. Aside from their relatively high cost, diagnostic and prognostic utility of these tests have not been borne out after years of experience. These tests may be most useful in cases of suspected meningitis where the Gram's stain is negative and/or when CSF cultures are negative after 48 hours; particularly if the patient had received antimicrobial therapy prior to specimen acquisition.

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The recommended method for optimal direct detection of group B streptococci in CSF depends on careful inspection of a Gram-stained smear of a cytocentrifuged specimen. Direct detection methods for group B streptococci in rectovaginal specimens are insensitive compared with the broth amplification methods recommended by the CDC (Box 13-3). Real-time PCR techniques targeting various group B streptococcal genes, including *cylb* (hemolysin), *cfb* (CAMP factor gene), and *ssr* (serotype antigen gene) have been described for detection of group B streptococci in rectovaginal, blood, and other clinical specimens.[288,547,1115] Rapid LA- or EIA-format assays should not be used for screening of rectovaginal swab specimens for group B β-hemolytic streptococci (see section on Clinical Significance of Group B β-hemolytic streptococci for discussion of screening methods).

Nonculture, Direct Detection Techniques for Streptococcus pneumoniae

As an adjunct to cultural diagnosis of respiratory tract and central nervous system infections by *S. pneumoniae*, methods are now available for detection of pneumococcal antigen in urine of patients with pneumococcal pneumonia and in CSF

of patients with pneumococcal meningitis. The Binax NOW *Streptococcus pneumoniae* antigen card (Alere, Inc., Waltham, MA) is a rapid immunochromatographic assay that allows a presumptive diagnosis of pneumococcal pneumonia and meningitis by detection of specific soluble capsular antigens in urine and CSF specimens, respectively.[490] The pneumococcal antigens detected by this test account for more than 90% of invasive disease occurring in the United States and worldwide. In a study conducted in the United Kingdom, the NOW assay was positive in 82% of 107 urine specimens from patients with bacteremic pneumococcal pneumonia; 3 of 106 patients with pneumonia and bacteremia due to other organisms were also NOW test-positive, yielding a specificity of 97%.[950] A subsequent study by the same clinical group found the Binax NOW was positive in 88% of 58 bacteremic pneumococcal cases while a dual-PCR assay targeting pneumolysin and autolysin genes of *S. pneumoniae* had a sensitivity of only 53.5%.[951] In a prospective, population-based study of adults with pneumonia in Spain, the test was positive in 70.4% of 27 patients with culture-documented pneumococcal pneumonia; corresponding specificity of the test in this study was 89.7%.[409] In another study, the urinary antigen was detected in 80% of 20 patients with pneumococcal bacteremia and in 52% of 54 patients with *S. pneumoniae* isolated from sputum.[736] A 3-year prospective study of the Now assay conducted in Japan found that the test detected 75.9% of 83 patients with pneumococcal pneumonia that were confirmed by routine methods (i.e., transthoracic needle aspiration and/or positive blood cultures).[496] The predictive values for positive and negative urinary antigen tests were 91.3% and 82.6%, respectively. Of note was that the diagnostic yield for a pneumococcal pneumonia diagnosis was increased by 38.9% when the antigen test was combined with other diagnostic measures. In a study of 474 cases of community-acquired pneumonia in Spain, Sorde and colleagues[955] found that the NOW test was positive in 43.8% of 171 cases due to *S. pneumoniae*, and that the test specificity was 96%. An Italian study found that a positive urinary antigen test and recovery of *S. pneumoniae* from sputum was seen in only 8.7% of 46 patients for whom both results were available.[724] Positive urine antigen tests and negative sputum cultures were seen in 17.4%, and negative urine antigen tests were seen in 28.3% of patients with sputum cultures positive for *S. pneumoniae*. The NOW urinary antigen test has also been shown to be a useful adjunctive test for diagnosis of pneumococcal empyema in children, exacerbations of pneumococcal infections in patients with COPD, and as an indicator of complications associated with pneumococcal infection (e.g., vertebral osteomyelitis, psoas muscle abscess) in compromised hosts.[32,684,692] The time to development and the intensity of positive bands on the NOW immunochromatographic strip were also found to correlate with the severity of pneumococcal pneumonia in a study of 119 children with *S. pneumoniae* pneumonia.[918] Other workers have shown

that the NOW test may be useful for detection of *S. pneumoniae* in nasal and nasopharyngeal specimens for diagnosis and management of acute otitis media as well as community-acquired pneumococcal pneumonia.[42,1102] The test was also positive with pericardial fluid obtained from a patient with purulent pneumococcal pericarditis.[746] A meta-analysis of 27 evaluations of the NOW pneumococcal urinary antigen test found the test to have a sensitivity and specificity of 74% and 97.2%, respectively.[936]

Samra and coworkers[887] evaluated the NOW test with both CSF and urine specimens from 22 patients with culture-documented pneumococcal meningitis. For CSF specimens, 21 of 22 CSF specimens were positive (95.4% sensitivity) with the rapid test; only 12 of 21 urine specimens were positive (57.1% sensitivity). Testing of CSF specimens that were culture-negative for *S. pneumoniae* or positive for other organisms were all negative, for a specificity of 100% for CSF specimens. However, 5 (18.5%) of 27 urine specimens from patients with CSF cultures positive with other bacteria, and 63 (13.4%) of 470 urine specimens from patients with negative CSF cultures were positive with the NOW test. A multicenter study on bacterial meningitis surveillance conducted in five countries throughout Africa and Asia found that 99% of 69 culture-confirmed cases of pneumococcal meningitis were detected by the NOW urinary antigen assay and 99% of 125 meningitis cases due to other bacterial agents were negative with the NOW assay.[723] Positive CSF antigen results may persist for some time after appropriate therapy and can cloud the diagnosis of recurrent disease versus a sterile aseptic meningitis syndrome that has been observed in adequately treated patients.[33]

A potential drawback of the NOW urine test is that healthy nasopharyngeal carriers of *S. pneumoniae* may also have positive tests. A study conducted in the Gambia, where the carriage rate among evaluated children was 87%, reported that 55% of urine specimens from healthy children who were pneumococcal carriers were Binax NOW test-positive.[9] Another study conducted in Ecuador found that 21.7% of children who carried nasopharyngeal *S. pneumoniae* were NOW test-positive and 4.2% of noncarriers had false-positive urine antigen tests.[419] These workers postulated that the Binax NOW urine assay may be most useful for the diagnosis of pneumococcal pneumonia and bacteremia in populations with low rates of nasopharyngeal pneumococcal carriage.

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However, in another study of 98 women in which 24 (25%) carried nasopharyngeal *S. pneumoniae*, only 3% had positive urinary antigen tests.[1056] False-positive NOW urinary antigen results have also been documented. In a retrospective cohort study

conducted at Northwestern University, 5 (9.6%) of 42 positive urinary antigen results were deemed to be falsely positive; interestingly, two of the five false-positive tests occurred in individuals who had received the 23-valent pneumococcal vaccine.[881] False-positive urinary antigen tests are, therefore, associated with nasopharyngeal pneumococcal carriage, invasive pneumococcal disease in the past, and receipt of pneumococcal vaccine.

Nonculture Techniques for the Detection of Enterococci in Blood Cultures

PNA FISH technology combines fluorescence in situ hybridization (FISH) with unique peptide nucleic acid (PNA) molecules wherein the negatively charged carbohydrate-phosphate moieties comprising the backbone of DNA and RNA are replaced with uncharged PNA probes containing the same nucleotide bases as DNA, allowing them to hybridize with complementary nucleic acid sequences. The uncharged, hydrophobic backbone of PNA probes allows the probes to penetrate the hydrophobic cell walls of bacteria and enables tighter and more specific hybridization to nucleic acid sequence targets. The PNA probes hybridize with rRNA sequences of specific enterococcal species. These PNA probes are labeled with molecules that fluoresce (i.e., fluorophores) under UV light. PNA FISH technology has found its greatest application in direct identification of pathogenic bacteria directly in positive blood cultures. The *Enterococcus faecalis*/Other Enterococci (OE) PNA FISH assay uses a mixture of a fluorescein-label *E. faecalis*-specific PNA probe and a rhodamine-labeled PNA probe specific for OE. This reagent is applied to a smear prepared from a positive blood culture. Hybridization is performed at 55°C for 30 minutes and is followed by a posthybridization wash for 30 minutes at 55°C with a stringent wash solution. The smear is mounted with a mounting medium supplied with the kit, and is then examined by fluorescence microscopy. *E. faecalis* is identified as multiple, bright green fluorescent cocci, while OE appear as multiple red fluorescent cocci. Nonenterococcal organisms do not fluoresce. A multicenter evaluation of the *E. faecalis/* OE PNA FISH was performed on 152 positive blood cultures showing gram-positive cocci in pairs and chains on Gram's stain.[728] Of these, 100% of 41 cultures positive for *E. faecalis* and 100% of 33 cultures positive for OE were correctly identified. None of the cultures growing nonenterococcal gram-positive cocci were misidentified as enterococci. In another study, the use of *Enterococcus*/OE PNA FISH resulted in earlier initiation of appropriate empirical antimicrobial therapy due to earlier recognition of *E. faecium* VRE bacteremia.[353]

Recently, the *Enterococcus* QuickFISH BC was approved for use. This test identifies *E. faecalis* and OE from blood cultures in less than 30 minutes by elimination of a wash step. This modification uses a drop from a positive blood culture

that is prepared on FISH slides with positive and negative controls already present on the slide. The slide is heat-fixed at 55°C for up to 5 minutes. Single drops of two PNA solutions—*Enterococcus* PNA blue and *Enterococcus* PNA Yellow—are mixed together on a coverslip that is placed inside a template. Mixture of the two reagents in the oval window of the template results in a uniform green color. The coverslip is then flipped over and applied to the dried/fixed blood culture drop on the specimen slide within the borders of the viewing area. The slide is incubated at 55°C for 15 to 20 minutes at the workstation. The slide is read on a fluorescence microscope by first inspecting the controls, then moving over to the specimen well in the same plane of focus. As with the longer procedure, *E. faecalis* appears as bright, apple-green fluorescent clusters of cocci, while OE appear as multiple, bright-red fluorescent cocci. This test has a turnaround time of less than 30 minutes with a hands-on time of less than 5 minutes.

Colony Morphology and Catalase Testing

After 18 to 24 hours' incubation on blood agar, colonies of group A β-hemolytic *Streptococcus* are about 0.5 mm in diameter, translucent or transparent, and have a smooth or matte surface. The zone of β-hemolysis is usually two to four times the diameter of the colony (see Image Plate 13-1D) The colonies are domed and have an entire edge. Occasional strains of group A streptococci will be mucoid due to the presence of large amounts of capsular material. Groups C and G also have a similar appearance, although colonies of some group G strains may have a golden cast on close inspection and the hemolytic zones are usually very large. Group B β-hemolytic streptococci form larger colonies on agar medium, the margin of hemolysis around the colony is comparatively smaller for group B streptococci than for the other β-hemolytic streptococci, and the hemolysis is generally "softer" and less obvious. A significant proportion of group B streptococci (up to 11%) may be nonhemolytic. Group D streptococcal colonies tend to be larger than those of group A streptococci, being 0.5 to 1.0 mm after overnight incubation. Group D isolates are α-hemolytic or nonhemolytic on SBA. The colonies are usually gray, smooth, and have an entire edge. Group F streptococci form very small, pinpoint colonies with a large zone of β-hemolysis. These extremely small colonies are called "minute colonies" and they are characteristic of the anginosus group of streptococci, *S. anginosus*, *S. constellatus*, and *S. intermedius*. Minute β-hemolytic colonies are generally pinpoint after 24 hours' incubation, yet will have a rather large, florid zone of β-hemolysis. The anginosus group organisms growing on solid media also have a distinct sweet, caramel ("butterscotch") or honeysuckle odor owing to the production of diacetyl. This characteristic may be noted with α-hemolytic, β-hemolytic, and nonhemolytic members of this group.

S. pneumoniae displays a spectrum of colony types, the appearance of which

depends on the degree of encapsulation. These colonies are generally surrounded by a large zone of intense green α-hemolysis. Colonies of heavily encapsulated strains may be several millimeters in diameter, are very mucoid, appear gray, and may resemble drops of oil on the agar surface. Colonies of the less heavily encapsulated strains are smaller. On prolonged incubation, the central portion of

the colony may collapse, giving the characteristic "checker piece" appearance. Some colonies may collapse altogether, giving the appearance of a flat nail-head on the surface of the agar.

Other species of viridans streptococci form colonies of various sizes and textures. Some may be smooth and have an entire edge, whereas others may appear rough, with the formation of scalloped edges on prolonged incubation. Colonies of *Aerococcus*, *Pediococcus*, *Gemella*, *Leuconostoc*, *Tetragenococcus*, *Vagococcus*, *Globicatella*, *Helcococcus*, *Facklamia*, *Dolosicoccus*, *Dolosigranulum*, *Ignavigranum*, and the facultative *Lactobacillus* species strongly resemble viridans streptococci or group D streptococci in their appearance, and they are either α-hemolytic or nonhemolytic.

Members of the streptococci and *Streptococcus*-like bacterial groups are catalase-negative, except for *A. otitidis*. This organism is *Streptococcus*-like in its colony morphology but is catalase-positive. Some enterococcal strains (particularly *E. faecalis* strains) produce a "pseudocatalase" that is responsible for the weak-positive catalase reaction that is seen with some strains, particularly on primary isolation. The strength of this reaction may diminish after a few serial subcultures.

Recognition and Preliminary Characterization of Streptococci and the "Streptococcus -Like" Bacteria

The determination of hemolysis and the performance of a catalase test were once the only tests that were required to preliminarily characterize streptococci. However, with the recognition of several groups of *Streptococcus*-like bacteria in human infections, it is also necessary to perform additional tests, particularly for those isolates recovered from sterile body fluids. Some of these organisms (i.e., *Leuconostoc* and *Pediococcus* species) are intrinsically resistant to vancomycin and related cyclic glycopeptide antibiotics (e.g., ristocetin, aracidin, and teicoplanin). The apparent increase in the recognition and isolation of these organisms may be partly related to the emergence of methicillin-resistant *S. aureus* and the consequent use of vancomycin as a first-line antibiotic, particularly in severely debilitated hosts. These and other "look-alike" organisms may be preliminarily differentiated from *Streptococcus* and *Enterococcus* species and from one another with the tests shown in Table 13-2.

On SBA, these organisms resemble viridans streptococci or enterococci; all of them are α-hemolytic or nonhemolytic. The characteristic streptococcal morphology—chains of cocci—is seen with the streptococci, enterococci, lactococci, *Leuconostoc/Weissella* species, *Vagococcus* species, *Abiotrophia/Granulicatella* species, *Dolosicoccus* species, *Ignavigranum* species, *Globicatella* species, and *Lactococcus* species on Gram-stained smears prepared from thioglycolate broth cultures. Gram-positive cocci arranged predominantly in pairs, tetrads, and or clusters are more characteristic of the aerococci, *Alloiococcus* species, *Gemella* species, *Pediococcus* species, and *Helcococcus* species and *Tetragenococcus* species. Some of the recently described *Streptococcus*-like bacteria (e.g., *Facklamia* species, *Tetragenococcus* species) often form pairs or short chains. Facultative lactobacilli will generally show the typical rod-shaped morphology, whereas other species will appear coccal. Observation of gram-positive cocci in short chains in blood culture media and growth of organisms on subculture to chocolate agar with no growth on a corresponding SBA subculture suggests *Abiotrophia/Granulicatella* species. On media containing blood, some isolates may produce a weak catalase reaction with 3% H_2O_2; therefore, strains showing this reaction should be subcultured onto blood-free medium and retested for catalase activity.

Viridans or nonhemolytic streptococci from sterile body fluids can be screened for susceptibility to vancomycin using the regular 30 mg disk on a SBA plate incubated at 35°C for 18 to 24 hours. Occasional isolates may require prolonged incubation (i.e., up to 72 hours) for test interpretation. Organisms that are resistant to vancomycin (i.e., *Pediococcus* species, *Leuconostoc* species) generally have MICs higher than 250 μg/mL and will grow right up to the edge of the disk. The presence of any zone around the disk indicates susceptibility to vancomycin. Production of gas from glucose, a helpful test for differentiation of *Leuconostoc* species from the other organisms, is best determined in *Lactobacillus* Mann–Rogosa–Sharpe (MRS) broth (Difco Laboratories, Detroit MI) overlaid with petrolatum (Deman–Rogosa–Sharpe, 1960). The formation of bubbles under the petrolatum seal indicates gas production and the heterofermentative nature of their metabolism. The MRS broth is incubated for up to 7 days. Other tests that are helpful for preliminary characterization of the *Streptococcus*-like organisms include pyrrolidonyl arylamidase (PYR), leucine aminopeptidase (LAP), and salt tolerance tests. The latter test is performed in heart infusion broth containing 6.5% NaCl. The presence of growth is the endpoint, as in the salt tolerance test for identification of enterococci. The PYR and LAP tests are available as rapid disk tests from several manufacturers (e.g., Remel, Carr-Scarborough) and are also on some of the kit systems (e.g., API Rapid Strep). The BactiCard *Streptococcus* test (Remel Laboratories) includes PYR, LAP, and an esculin hydrolysis test. This card, when used with vancomycin susceptibility results, can provide preliminary characterization of *Enterococcus*, *Lactococcus*, *Aerococcus*, *Gemella*, *Leuconostoc*, *Pediococcus*, and *Globicatella* species.

Occasionally, more uncommon tests may be helpful to augment traditional tests or to confirm identifications obtained with kit systems. For the growth temperature tests, a water bath set at 45°C and a 10°C refrigerator are recommended. Growth, if any, is observed after 24 to 48 hours of incubation. Motility is determined in regular semisolid motility agar medium, with or without the addition of tetrazolium. This test should be incubated for up to 48 hours at room temperature. The VP test for acetylmethyl carbinol that is used for streptococci is performed in heavily inoculated VP broth incubated overnight. After addition of α-naphthol and sodium hydroxide reagents, the tube is shaken or vortexed and incubated at room temperature for 30 minutes. Red, pink, and colorless reactions at this time correspond to positive, weakly positive, and negative reactions, respectively.

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TABLE 13-2 Phenotypic Characteristics for Presumptive Identification of Catalase-Negative, Gram-Positive Cocci

Genus	Gram Stain Morphology in Thioglyollate Broth	Hemolysis, SBA	Catalase	Growth, 10°C	Growth, 45°C	Motility	LAP	PYR	Growth, 6.5% NaCl Broth	Gas, MRS Broth	Vancomycin
Streptococcus	Chains	α, β, γ	–	–	V	–	+	–	V	–	S
Enterococcus	Pairs, chains	α, γ	–	+	+	V	+	+	+	–	S/R
Abiotrophia	Chains	α, γ	–	–	V	–	+	+	–	–	S
Granulicatella	Chains	α, γ	–	–	V	–	+	+	–	–	S
Aerococcus	Tetrads, clusters	α	–	–	–	–	V	V	+	–	S
Helcococcus	Tetrads, clusters	γ	–	–	–	–	–	+	+	–	S
Leuconostoc	Chains	α, γ	–	+	+	–	–	–	V	+	R
Weissella	Chains	α, γ	–	V	V	–	–	–	+	+	R
Pediococcus	Tetrads, clusters	α	–	–	+	–	+	–	V	–	R
Tetragenococcus	Tetrads, clusters	α	–	–	+	–	+	–	+	–	S
Gemella	Tetrads, clusters, chains	α, γ	–	–	–	–	V	+	–	–	S
Vagococcus	Chains	α, γ	–	+	–	+	+	+	+	–	S
Alloiococcus	Clusters, tetrads	α	+	–	–	–	+	+	+	–	S
Globicatella	Chains	α	–	–	–	–	–	+	+	–	S
Facklamia	Clusters, chains	α	–	–	–	–	+	+	+	–	S
Dolosigranulum	Clusters, tetrads	α	–	–	–	–	+	+	+	–	S
Ignavigranum	Clusters, chains	α	–	–	–	–	+	+	+	–	S
Dolosicoccus	Chains	α	–	–	–	–	–	+	–	–	S
Eremococcus	Chains	α	–	–	+	–	–	NA	+[w]	–	S
Catellicoccus	Pairs, short chains	γ	–	–	NA	–	+	–	NA	–	S
Lactococcus	Chains	α, γ	–	+	V	–	+	+	V	–	S

+, positive reaction; –, negative reaction; V, variable reaction; +[w], weak-positive reaction; S, susceptible; R, resistant; NA, data not available; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; MRS broth, Mann-Sharpe-Rugosa broth.

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Presumptive Identification of Streptococci and Enterococci

β-Hemolytic streptococci, pneumococci, group D streptococci, and enterococci are

definitively identified using serologic procedures (discussed later) that detect either the Lancefield group antigens (groups A, B, C, D, F, and G) or the capsular polysaccharide antigens (*S. pneumoniae*) of the organisms. Species identification of group D streptococci, *Enterococcus* species, and the viridans streptococci is accomplished primarily by biochemical, physiologic, and enzymatic tests. Laboratories use a handful of presumptive tests that correlate highly with the serologic methods, yet are less expensive to perform. Results of presumptive tests for the major streptococcal groups are summarized in Table 13-3. Detailed procedures for performance and interpretation of the more commonly used presumptive tests are described in Charts 13-1 to 13-4.

Susceptibility to Bacitracin. The bacitracin susceptibility test is used for the presumptive identification of group A β-hemolytic streptococci (Image Plate 13-2B). The test is performed on a blood agar medium with a bacitracin differential disk (e.g., TAXO A Bacitracin Disk, 0.04 units, BD Microbiology Systems, Cockeysville MD; Bacitracin Differentiation disks, 0.04 units, Remel). The procedure is described in detail in Chart 13-1. Any zone of inhibition around the disk is considered a positive test. Although this test is simple, inexpensive, and fairly accurate for presumptive identification of group A streptococci, it is not highly specific. Over 10% of group C and G streptococcal strains are also susceptible to bacitracin, as are about 5% of group B strains. Consequently, this test is often performed along with the sulfamethoxazole-trimethoprim (SXT) susceptibility test because groups C and G streptococci are usually susceptible to SXT, whereas groups A and B streptococci are resistant. Some workers have advocated the use of bacitracin disks directly on primary, nonselective blood agar for rapid detection and identification of group A streptococci in throat cultures. However, this method will identify only 50% to 60% of isolates. Placement of bacitracin disks on primary plates containing selective media is considerably more sensitive. The laboratory report should reflect the use of a presumptive method; "β-hemolytic streptococci, presumptively group A by bacitracin" or "β-hemolytic streptococci, presumptively not group A by bacitracin."

Susceptibility to Sulfamethoxazole-Trimethoprim (SXT). The SXT susceptibility test presumptively distinguishes groups A and B streptococci from other β-hemolytic streptococci (Image Plate 13-2C). When used in conjunction with the bacitracin test, the SXT susceptibility test helps screen out those non–A, non–B streptococci that may be susceptible to bacitracin because both group A and B strains are SXT-resistant, whereas groups C, F, and G are SXT-susceptible.. The test is performed the same as the bacitracin test, except that a commercial disk containing 1.25 µg trimethoprim and 23.75 µg of sulfamethoxazole is used. Any zone of inhibition indicates susceptibility to SXT (see Chart 13-1).

CAMP Test and Pigment Production. The CAMP test (named for Christie, Atkins, and Munch-Petersen) is used to presumptively identify group B streptococci (see Chart 8-1) and is performed using a β-hemolysin-producing strain of *S. aureus* (ATCC #25923 Group B streptococci secrete a protein called "CAMP factor" that interacts with the β-hemolysin produced and secreted by *S. aureus* to cause enhanced or synergistic hemolysis (Image Plate 13-2D). This appears as an arrowhead-shaped area of increased hemolysis in the area where the two streaks of growth are closest. This test is highly sensitive, and even nonhemolytic group B strains will be CAMP-positive. A small percentage of group A streptococci will also be CAMP-positive, as will *Listeria monocytogenes*; the shape of the CAMP reaction with *L. monocytogenes,* however, is rectangular rather than the arrowhead shape produced with group B streptococci. This test is frequently used in conjunction with the bacitracin and SXT tests on the same blood agar plate to presumptively identify these organisms (Table 13-3). Reports should state "presumptive β-hemolytic group B streptococci by CAMP test."

Hydrolysis of Sodium Hippurate. Group B streptococci are able to hydrolyze hippurate to its components, glycine and benzoic acid. To perform the test, broth containing sodium hippurate is inoculated with the organism and incubated overnight at 35°C. The cells are centrifuged and the supernatant is removed. Ferric chloride reagent (0.2 mL; $FeCl_3 \cdot 6H_2O$, 12 g, in 100 mL 2% aqueous HCl) is then added to the supernatant (0.8 mL), with the formation of a heavy precipitate. If the precipitate remains after 10 minutes, benzoic acid is present and the test is positive for hippurate hydrolysis. Alternatively, ninhydrin reagent may be added to the supernatant to detect free glycine. In this method, the formation of a deep blue color is positive. Hippurate-positive, β-hemolytic streptococci are reported as "presumptive group B streptococci by hippurate hydrolysis."

Bile Esculin Test. This test is used for the presumptive identification of *Enterococcus* species and group D streptococci (Image Plate 13-2F). It is generally performed on an agar slant or in a plate (Chart 13-2). Organisms that are bile esculin–positive are able to grow in the presence of 40% bile and to hydrolyze esculin. Most *Enterococcus* species and group D streptococci will blacken bile esculin medium within 24 hours; rare strains may require a 48-hour incubation before hydrolysis is apparent. Care should be taken to use bile esculin agar formulations that contain the requisite 40% bile; some manufacturers' products contain less bile than this amount, resulting in misidentification of some viridans streptococci as group D streptococci or enterococci.

Salt Tolerance Test (6.5% NaCl Broth). The salt tolerance test (6.5% NaCl broth) separates *Enterococcus* species from the group D nonenterococcal streptococci (*S. gallolyticus* subspecies) (see Chart 13-4). The organism to be identified is inoculated

into an infusion-based agar or broth containing 6.5% NaCl. After overnight incubation, the medium is observed for the presence of growth, indicating tolerance to 6.5% salt. *Enterococcus* species will be salt-tolerant. *S. gallolyticus* subspecies will not grow in 6.5% NaCl broth.

Leucine Aminopeptidase (LAP) Test. Production of leucine aminopeptidase (LAP), along with PYR, is helpful

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TABLE 13-3 Phenotypic Criteria for Presumptive Identification of Clinically Significant Streptococci and Enterococci

Organism	Hemolysis, SBA	LAP	Bacitracin	SXT	CAMP Test	HIPP	Growth, BE Agar	PYR	Growth, 6.5% NaCl Broth	Optochin	Bile Solubility
Group A *Streptococcus*	β	+	S	R	–	–	–	+	–	R	–
Group B *Streptococcus*	β, γ	+	R	R	+	+	–	–	V	R	–
Groups C, F, and G streptococci	β, γ	+	V	S	–	–	–	–	–	R	–
Group D *Enterococcus*	α, β, γ	+	R	R	–	V	+	+	+	R	–
Group D *Streptococcus*	α, γ	+	R	S	–	–	+	–	–	R	–
Viridans streptococci	α, γ	+	V	S	–	V	V	–	–	R	–
Pneumococcus	α	+	V	S	–	–	–	–	–	S	+

+, positive reaction; –, negative reaction; V, variable reaction; S, Susceptible; R, Resistant; LAP, leucine aminopeptidase; SXT; trimethoprim-sulfamethoxazole; HIPP, hippurate hydrolysis; BE agar, bile esculin agar; PYR, pyrrolidonyl arylamidase.

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for identifying streptococci, enterococci, and some of the *Streptococcus*-like organisms. This test is available as a disk spot test (Remel Laboratories; Carr-Scarborough Microbiologicals, Decatur, GA), as a part of a three-test presumptive identification system (Remel Bacti-Card Strep) or on 4-hour or overnight panels for streptococcal identification (the API Rapid Strep). Disks are moistened and heavily inoculated with colonial material from an agar plate (this may require that plates of very slow-growing isolates be incubated 2 to 3 days before sufficient inoculum is available for testing). After 10 minutes, a drop of the detection reagent is added to the disk. Development of a red color on the disk after 3 minutes indicates a positive LAP reaction; a yellow color is a negative test; and a pink color is coded as a weakly positive test. The LAP test is positive for all streptococci and enterococci, the latter also being PYR-positive.

Pyrrolidonyl Arylamidase (PYR) Test. The PYR hydrolysis test (Chart 1-6) is a presumptive test for both group A and group D enterococcal streptococci (Image Plate 13-2G). It replaces the bacitracin test and the salt tolerance test for group A streptococci and *Enterococcus* species, respectively. The enzyme that is detected is

called pyrrolidonyl arylamidase. Broth containing PYR (L-pyrrolidonyl-β-naphthylamide) is inoculated with the organism and incubated at 35°C for 4 hours. During this time, PYR is hydrolyzed. Free β-naphthylamide is then detected by addition of the diazo dye coupler, *N*, *N*-dimethylaminocinnamaldehyde. A red color develops if PYR has been hydrolyzed. This test is highly sensitive and specific for group A streptococci and most *Enterococcus* species. Several adaptations of the PYR hydrolysis test are commercially available and provide rapid results (15 minutes or less). Other organisms (e.g., most lactococci, *A. viridans, G. haemolysans*, the nutritionally variant streptococci, and some staphylococci) are also PYR-positive. This test is included along with esculin hydrolysis and leucone aminopeptidase on the Bacti-Card Strep system (Remel).

Commercial Presumptive Identification Tests. Commercially produced triplates are available for the presumptive identification of β-hemolytic streptococcal groups A and B, the group D streptococci, and *Enterococcus* species (Image Plate 13-2H). The Strep-ID II Tri-Plate (Remel) contains three compartments: an SBA quadrant for assessment of hemolysis and performance of the CAMP and SXT tests, a bile esculin agar quadrant, and a PYR medium quadrant. The Strep-ID II QUAD plate has four quadrants for the bacitracin susceptibility test, a CAMP test, growth on bile esculin agar, and growth in the presence of 6.5% NaCl, respectively. After inoculation and overnight incubation, the tests are interpreted as shown in Table 13-3.

Serologic Identification of β- Hemolytic Streptococci

The pioneering work of Rebecca Lancefield set the stage for the serologic classification of human streptococci. This classification is based on the detection of the group-specific carbohydrate antigen from the cell wall of the organism. Groupable streptococci that cause disease in humans belong to Lancefield groups A, B, C, D, F, and G. Only β-hemolytic streptococci and the α- or nonhemolytic group D organisms can be classified with this scheme. To detect the cell wall antigens of these organisms, the antigen must first be extracted from the cell wall and solubilized. This may be accomplished by acid extraction (e.g., nitrous acid), autoclave extraction (Rantz–Randall method), or by enzymatic extraction. Once extracted, the antigen may be detected by a variety of methods.

Capillary Precipitin Test. In this method used by Lancefield, the extracted antigen is layered over group-specific antisera in a capillary tube. The formation of a precipitin reaction at the extract-antiserum interface provides the group designation of the organism.

Coagglutination. In this technique, the antigen extract is reacted with *S. aureus* cells sensitized with group-specific antisera. Visible agglutination of the staphylococcal cells

coated with a specific antiserum provides the group designation of the organism. Commercial coagglutination test kits are available (Phadebact Streptococcus, MLK Diagnostics AB, Sollentuna, Sweden).

Latex Agglutination. The LA tests employ polystyrene latex beads as the carriers for the group-specific antisera that are reacted with the organism extract. Commercial kits for this method are widely used as well (Streptex, Remel; Patho-D_X Strep Grouping Kit, Diagnostic Products Corporation, Los Angeles CA; Slidex Plus Strep, bioMerieux, Inc., Hazelwood MO). LA procedures have replaced the Lancefield extraction–capillary precipitin technique as the reference method for serogrouping of β-hemolytic streptococci. The proper assessment of hemolysis is essential for test reliability, and cross-reactions with other organisms have been reported. This error may occur when organisms are tested directly from blood culture bottles without first determining the hemolytic character of the isolate. Thompson and Facklam also reported that the group B LA regent from several manufacturers cross-reacts with *S. porcinus* and *S. pseudoporcinus*.[1024] While these strains react with the group B latex reagents, the organisms form small colonies with very large, clear zones of β-hemolysis, which is very different from the soft, marginal β-hemolysis associated with group B streptococci.

Phenotypic Characteristics for Identification of Groupable Streptococci

The groupable streptococci can be identified to species on the basis of physiologic characteristics. Several of these reactions are used in the various kit systems for identifying the β-hemolytic streptococci. Biochemical identification methods may also enable detection of aberrant strains of β-hemolytic streptococci. For example, Brandt and colleagues[117] isolated three strains of β-hemolytic streptococci from blood cultures that possessed the group A antigen, but were identified as *S. dysgalactiae* subsp. *equisimilis* by phenotypic testing and 16S rDNA sequencing. These strains were acetoin-negative and PYR-negative, unlike group A streptococci. Although most clinical laboratories dealing with specimens from humans use serologic methods for

identification of these organisms, workers in veterinary microbiology may find these tests helpful because Lancefield antisera for groups other than A, B, C, F, and G are not readily available. Phenotypic characteristics for the identification of groupable streptococci are presented in Table 13-4. Molecular markers have also been identified to discriminate *S. pyogenes* from other members of the pyogenic cocci group (e.g., *S.*

dysgalactiae subsp. *equisimilis*).[705]

Identification of S. pneumoniae: Susceptibility to Optochin, the Bile Solubility Test, and AccuProbe Pneumococcus Test

The alpha-hemolytic dimpled colony morphology and Gram stain characteristics are often the first clues to recognizing pneumococcus (Image Plate 13-1C and13-2A). Susceptibility to optochin (ethyl hydrocupreine hydrochloride) is used to differentiate *S. pneumoniae* from the other viridans streptococci (see Chart 13-3). As with the bacitracin and SXT tests, the optochin susceptibility test is performed on blood agar media. Unlike the former tests, however, zones of inhibition must be measured before interpretation. A zone of 14 mm or wider around the 6-mm disk indicates susceptibility to optochin and identifies the organism as a pneumococcus (see Image Plate 13-2E). If the zone is smaller than 14 mm, an alternative identification test (e.g., serology or bile solubility) should be performed because some nonpneumococcal viridans streptococci and aerococci may show small zones of inhibition. Viridans streptococci and group D enterococci are generally optochin-resistant. The bile solubility test is another test for identification of *S. pneumoniae*. The test can be performed on a broth or saline suspension of the organism or directly on a plate. Both procedures are described in Chart 13-5. Deoxycholate, the "bile" reagent used in these procedures, activates the autolytic enzymes of the organism. Optochin-susceptible and -resistant, bile-insoluble isolates of *S. pneumoniae* and optochin-resistant, bile-soluble viridian streptococcal isolates have been reported.[36,493] Molecular assays for detection of the *ply* (pneumolysin) and the *psa* (pneumococcal surface antigen) are useful distinguishing unusual or atypical isolates of *S. pneumoniae* from other viridans streptococci that occasionally display bile solubility or susceptibility to optochin.[493]

An evaluation of the optochin test with 99 clinical isolates of *S. pneumoniae* and 101 viridans streptococci reported a sensitivity and specificity of 99% and 98%, respectively.[551] Characterization of optochin-susceptible strains of *S. mitis* revealed acquisition of genes from *S. pneumoniae* that encode subunits of the H+ ATPase, which is the target of optochin.[682] Strains of *S. pneumoniae* that are optochin-resistant have point mutations in either the a- or c-subunit of the H+ ATPase and have optochin MICs that are 4- to 30-fold higher than susceptible strains.[831] The AccuProbe DNA hybridization assay (Gen-Probe) reliably identifies typical optochin-susceptible, encapsulated pneumococci, but may not reliably identify optochin-susceptible, unencapsulated isolates.[529] The recently described species *S. pseudopneumoniae*, which is considered a member of the "mitis group" of viridans streptococci (see below), will demonstrate resistance (6 mm zone) or intermediate susceptibility (8 to 13 mm zones) to optochin when cultures are incubated in 5% CO_2 at 35°C to 37°C, but

the same isolates may test optochin-susceptible (zone of ≥14 mm) when cultures are incubated at 35°C to 37°C in ambient air.[36] While the Accuprobe hybridization test may reliably differentiate optochin-susceptible, bile-soluble *S. pneumoniae* from optochin-resistant viridans streptococci, the assay may produce equivocal or positive results with optochin susceptibility-variable, bile-solubility-negative isolates that correspond to *S. pseudopneumoniae*.[36] Wessels and colleagues[1118] studied 23 *S. pneumoniae*, 3 *S. pseudopneumoniae*, and 29 other mitis group isolates and found that optochin susceptibility testing with incubation in ambient atmosphere, bile solubility, MALDI-TOF, and sequencing of the *tuf* and *rpoB* genes resulted in false-positive, false-negative, and inconclusive results. Detection of and sequencing of the *cpsA* gene (a partial region of the capsular polysaccharide gene) and the *rpoA* (RNA polymerase α-subunit) gene, have been shown to specifically discriminate *S. pneumoniae* from other viridans streptococci.[803,804] PCR detection of the genes for pneumococcal polysaccharide antigen (*psa*) and pneumolysin (*ply*) have also proven to be helpful for the identification of atypical, nontypeable or optochin-resistant pneumococci.[493] A highly reliable molecular method for differentiation of these species involved a multiplex, real-time PCR assay targeting the *Spn9802* and *lytA* genes. The Spn9802 assay detected *S. pneumoniae* and *S. pseudopneumoniae* but not *S. mitis* or *S. oralis*, and the *lytA* PCR detected only *S. pneumoniae*, so a combination of the two PCRs could be used for rapid detection and differentiation of these organisms.[1118] MALDI-TOF MS has shown high sensitivity and specificity for identifying atypical *S. pneumoniae* isolates and for identification of *S. pseudopneumoniae*.[299]

Serologic Identification of Streptococcus pneumoniae

Definitive identification of *S. pneumoniae* involves the serologic detection of pneumococcal capsular polysaccharides using specific antisera. This is complicated because there are more than 92 different capsular serotypes, antisera for the more uncommon serotypes are not widely available, and the quality of the antisera may vary from lot to lot. Omniserum, a Scandinavian product, is capable of detecting all pneumococcal serotypes. Such antiserum pools have been used to develop commercial coagglutination (Phadebact Pneumococcus, MLK Diagnostics AB, Sollentuna, Sweden) and LA tests (Pneumoslide, BD Microbiology Systems; Slidex Pneumo, bioMerieux, Inc.) (Image Plate 13-3C) for rapid serologic identification of *S. pneumoniae*. Specific identification and assignment to an individual capsular serotype is accomplished with the Quellung test (Image Plate 13-3B). Nontypeable isolates may react if an expanded panel of high-quality antisera is used.[493] Nonencapsulated isolates of *S. pneumoniae* cannot be typed or identified by the Quellung test.

The Quellung test may employ a serum pool, as well as type-specific antisera (Pneumotest, Statens Serum Institute, Copenhagen, Denmark). A light suspension of

the organism is prepared in saline, and a loopful of this suspension is mixed with a loopful of antiserum and a loopful of methylene blue on a glass slide. A glass coverslip is applied, and the slide is incubated at room temperature for 10 minutes. The

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TABLE 13-4 Phenotypic Characteristics for Identification of the β-Hemolytic Streptococci and Other Members of the Pyogenic Streptococci

Species	Group	HEM, SBA	BAC	SXT	LAP	PYR	CAMP Test	ESC	ADH	VP	HIPP	PAL	β-GUR
S. pyogenes	A	β	S	R	+	+	–	V+	+	–	–	+	–
S. agalactiae	B	β	R	R/S	+	–	+	–	+	–	+	+	+
S. dysgalactiae subsp. *dysgalactiae*	C	β	R	S	+	–	–	V–	+	–	–	+	NA
S. dysgalactiae subsp. *equisimilis*	A, C, G, L	β	R	S	+	–	–	+	+	–	–	+	NA
S. equi subsp. *equi*	C	β	R	S	+	–	–	V	+	–	–	+	+
S. equi subsp. *ruminatorum*	C	β	NA	NA	+	–	+	–	+	–	+	+	+
S. equi subsp. *zooepidemicus*	C	β	R	S	+	–	–	V	+	–	–	+	+
S. canis	G	β	R	S	+	–	+	+	+	–	–	–	–
S. castoreus	A	β	NA	NA	+	–	NA	+	+	–	–	+	+
S. didelphis	None	β	R	S	+	–	–	–	+	–	+	+	+
S. halichoeri	B	None	NA	NA	NA	NA	NA	–	+	+	–		–
S. ictaluri	None	None	NA	NA	+	+	NA	–	NA	–	NA	NA	–
S. iniae	None	β	R/S	S	+	+	+	+	V	–	–	+	+
S. phocae	C, F, None	β	S	S	+	–	–	–	–	–	–	+	–
S. porcinus	E, P, U, V none	β	R	S	+	+	+	+	+	+	V	+	+
S. pseudoporcinus	None	β	NA	NA	+	V	NA	+	+	V	–	NA	NA
S. urinalis	None	None	R	S	+	+	–	+	+	+	–	+	–

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S. pyogenes	+	+	+	+	–	–	–	+	+	V
S. agalactiae	+	NA	+	V	–	–	+	V	V	–
S. dysgalactiae subsp. *dysgalactiae*	+	+	+	+	–	V	+	+	V	–
S. dysgalactiae subsp. *equisimilis*	+	NA	+	V	–	–	+	+	V	V
S. equi subsp. *equi*	+	NA	+	–	–	–	–	–	+	+
S. equi subsp. *ruminatorum*	+	+	–	+	–	+	+	–	NA	+
S. equi subsp. *zooepidemicus*	+	NA	+	V	–	+	–	V	+	+
S. canis	+	+	+	+	–	–	+	V	NA	–
S. castoreus	+	+	+	–	–	–	NA	–	NA	–
S. didelphis	+	NA	+	V	–	–	+	+	–	–
S. halichoeri	+	+	–	V	+	–	+	–	NA	–
S. ictaluri	+	–	–	–	–	–	–	–	NA	–
S. iniae	+	+	+	–	+	–	+	+	+	–
S. phocae	+	NA	–	–	–	–	V	–	–	V
S. porcinus	+	+	+	V	+	+	+	+	+	–
S. pseudoporcinus	+	+	+	–	+	+	+	+	+	NA
S. urinalis	+	–	+	+	–	–	+	+	NA	–

+, positive reaction; –, negative reaction; V, variable reaction; NA, data not available; S, susceptible; R, resistant; HEM, SBA, hemolysis on sheep blood agar; BAC, bacitracin, 0.04 unit disk; SXT, sulfamethoxazole-trimethoprim disk; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; ADH, arginine dihydrolase; VP, acetoin (Voges–Proskauer); HIPP, hippurate hydrolysis; PAL, alkaline phosphatase; ESC, esculin hydrolysis; β-GUR, β-glucuronidase. GLU, glucose; MAL, maltose; LAC, lactose; MNTL, mannitol; SORB, sorbitol; RIB, ribose; TRE, trehalose; SAL, salicin; GLYG, glycogen

slide is examined under the high dry objective and under oil immersion, with decreased light. Owing to a microprecipitin reaction occurring on the surface of the organism, the refractive index of the capsule changes and takes on a "swollen," more visible appearance as a halo around the blue-stained bacterial cells. Microscopic organism agglutination may also be observed, particularly with heavily encapsulated strains. Quellung results must be compared microscopically with a similar preparation made with saline instead of antisera.

Identification of the Viridans Streptococci

The viridans streptococci other than *S. pneumoniae* include several α-hemolytic and nonhemolytic species (Table 13-5, Table 13-6, and Box 13-2). Unlike the human β-hemolytic streptococci, these organisms, with the exception of members of the "*Bovis* group," lack specific Lancefield serologic group antigens, although some may carry antigens that cross-react with these antisera. Unlike pneumococci, viridian group streptococci are optochin-resistant and bile-insoluble. In cases of endocarditis and bacteremia in neutropenic patients, it may be clinically helpful to identify these organisms. Persons with preexisting valvular damage may have recurrent episodes of endocarditis or experience relapse following inadequate treatment. Knowledge of the identity and the antimicrobial susceptibility of these isolates help in differentiating treatment failures and reinfections.

Phenotypic tests were initially used to group the viridans streptococci based on their common features. In 1998, Whiley and coworkers[1124] in the Department of Oral Microbiology at London Hospital Medical College published comprehensive schemes for phenotypic identification of the viridans streptococci recognized at the time. This "conventional" approach used 24-hour carbohydrate fermentation tests and a panel of 4-methylumbelliferyl-linked substrates for the 3-hour determinations of various enzymes. The dichotomous key in Figure 13-3 incorporates the "grouping" tests from Table 13-4 and additional tests from Table 13-5 for identifying "typical" individual species within a group. The viridans streptococcal groups include the **Mitis/Sanguinis Group,** the **Mutans Group**, the **Salivarius Group**, the **Anginosus Group**, and the **Bovis Group.**[291] Organisms can be assigned to these groups based on arginine dihydrolase (ADH) production, esculin hydrolysis, acetoin production (VP), urease, and acid production from mannitol and sorbitol (Table 13-5). Box 13-2 provides details on the clinical significance of these species.

Manual and automated commercial systems do not adequately identify this difficult group of organisms, and the data bases for these systems are not updated frequently enough to provide reliable species-level identifications. Most laboratories that attempt to identify these organisms employ one or more commercial kits for this purpose.

Often, additional phenotypic tests need to be added. Commercial products use modified conventional test and chromogenic or fluorogenic substrates for detection of preformed enzymes; these approaches have become a standard part of test batteries used for streptococcal identification. Applications of molecular and chemotaxonomic methods have resulted in significant modifications in viridans streptococcal taxonomy and approaches to identification. Molecular methods proposed for identification of streptococci to the species level must have sufficient variability between different species, but little sequence variability within a species. Molecular techniques for identification of viridans streptococci and enterococci include PCR-mediated amplification/detection of 16S rRNA, 16S rDNA, and the 16S-23S rRNA intergenic spacer regions. For the most part, 16S rRNA gene sequencing does not adequately resolve species identifications among some viridans streptococci (e.g., the Mitis Group streptococci) because organisms within these groups (i.e., *S pneumoniae*, *S pseudopneumoniae*, and *S. mitis*) share >99% sequence homology among each other in the rRNA locus. Consequently, detection and sequencing of specific genes found in these organisms have been used to determine species identifications.[593,679,763] These genes include *ddl* (D-alanine:D-alanine ligase gene), *lytA* (autolysin gene), *dex* (dextranase gene),

TABLE 13-5 Phenotypic Characteristics for Differentiation of Viridans Streptococcal Groups Found in Humans

				Acid From:			
Group	**ADH**	**ESC**	**VP**	**MNTL**	**SORB**	**URE**	**Species**
Mitis group	V	V	–	–	–	–	*S. mitis, S. oralis, S. sanguinis, S. parasanguinis, S. gordonii, S. cristatus, S. peroris, S. infantis, S. australis, S. oligofermentans, S. sinensis* (VP+)
Mutans group	–	+	+	+	+	–	*S. mutans, S. sobrinus*
Salivarius group	–	V	+	–	–	V	*S. salivarius, S. vestibularis, S. infantarius, S. alactolyticus*
Anginosus group	+	+	+	–	–	–	*S. anginosus, S. constellatus, S. intermedius*
Bovis group	–	+	+	V	–	–	*S. bovis/S. equinus, S. gallolyticus* subspecies, *S. infantarius* subspecies

+, positive reaction; –, negative reaction; V, variable reaction; ADH, arginine dihydrolase; ESC, esculin hydrolysis; VP, Voges–Proskauer (acetoin); MNTL, mannitol; SORB, sorbitol; URE, urease.

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TABLE 13-6 Phenotypic Characteristics for Identification of *Viridans streptococci* Isolated From Humans

Group/Species	Hem, SBA	ESC	ADH	VP	URE	Production of: α-GAL	β-GAL	α-GLU	β-GLU	β-GUR	NAGA	α-FUC	β-FUC	α-ARAB	NAGALA	PAL
Mitis/Sanguinis Group																
S. australis	α	NA	+	–	–	–	V	NA	–	–	–	NA	NA	NA	NA	+
S. cristatus	α	–	+	–	–	–	V	–	–	–	+	+	–	–	+	–
S. danieliae	β	–	–	–	–	–	–	–	–	–	–	–	–	–	NA	V
S. gordonii	α	+	+	–	–	V	V	V	+	–	+	+	–	–	V+	+
S. infantis	α	–	–	–	–	–	+	NA	–	–	V+	V	V	NA	NA	–
S. lactarius	α	+	+	–		–	+	NA	+	–	–	NA	NA	NA	NA	+
S. massiliensis	γ	NA	+	–	–	_	_	_	_	_	_	NA	NA	NA	NA	+
S. mitis	α	–	V–	–	–	V+	V	+	V+	–	V	–	+	–	–	V+
S. oligofermentans	α	–	–	–	–	V–	–	NA	NA	NA	NA	NA	NA	NA	NA	–
S. oralis	α	–	–	–	–	V–	+	+	–	–	+	–	V	–	+	+
S. parasanguinis	α, γ	V	+	–	–	+	+	+	V–	–	+	V–	V–	V–	+	V+
S. peroris	α	_	_	_	_	_	_	NA	_	_	_	NA	–	NA	NA	V
S. pseudopneumoniae[a]	α	V	–	–	NA	_	_	NA	V	–	NA	NA	NA	NA	NA	+
S. sanguinis bio. 1	α	+	+	_	_	V+	_	_	V+	_	_	_	_	_	_	_
S. sanguinis bio. 2	α	+	+	_	_	+	V–	–	+	–	V–	–	+	_	_	_
S. sanguinis bio. 3	α	–	+	_	_	_	+	–	V–	–	+	–	V+	_	_	_
S. sinensis	α	+	+	+	_	_	_	NA	+	_	_	NA	NA	NA	NA	–
Mutans Group																
S. mutans	α, γ	+	–	+	–	–	–	+	+	–	–	_	_	_	_	_
S. sobrinus	γ, α	V	–	+	_	_	_	+	_	_	_	_	_	_	_	_
Salivarius Group																
S. salivarius	γ, α	+	–	V+	V	V–	+	V+	V+	NA	_	_	V+	+	–	+
S. vestibularis	α	V+	–	+	+	–	+	V+	–	NA	_	_	_	+	_	_

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Mitis/Sanguinis Group																
S. australis	+	+	+	+	–	–	–	NA	–	–	–	NA	–	NA	–	NA
S. cristatus	+	+	NA	V+	–	–	–	–	–	–	–	NA	+	NA	NA	–
S. danieliae	+	NA	NA	–	–	–	–	–	NA	V	–		+	+	–	NA
S. gordonii	+	+	NA	+	–	–	NA	V+	V–	V–	–	+	+	–	NA	+
S. infantis	+	+	+	+	–	–	–	V–	–	–	–	NA	–	NA	–	–
S. lactarius	+	+	+	+	–	–	–	NA	–	–	–	NA	–	NA	–	NA
S. massiliensis	+	+	–	–	–	–	–	–	–	–	–	NA	–	–	–	NA
S. mitis	+	+	NA	V+	–	–	NA	V–	V+	V+	V–	V–	V–	NA	–	–
S. oligofermentans	+	+	+	V	–	–	–	–	–	V	–	–	V	–	–	–
S. oralis	+	+	NA	+	–	–	NA	–	+	V+	V+	–	V–	NA	NA	–
S. parasanguinis	+	+	NA	+	–	V–	NA	–	V+	V+	NA	V	V+	NA	NA	V–
S. peroris	+	+	+	+	–	–	–	–	–	–	–	NA	–	NA	–	–
S. pseudopneumoniae[a]	NA	NA	NA	+	–	–	–	–	NA	–	V	NA	–	NA	–	V
S. sanguinis bio. 1	+	+	NA	+	–	V–	NA	V–	+	+	–	+	+	NA	NA	–
S. sanguinis bio. 2	+	+	NA	+	–	V+	NA	–	V+	+	–	+	+	NA	NA	+
S. sanguinis bio. 3	+	+	NA	+	_	_	NA	+	_	_	_	+	+	NA	NA	–
S. sinensis	+	+	+	+	_	_	NA	_	_	_	_	+	+	+	–	NA
Mutans Group																
S. mutans	+	+	+	+	+	+	NA	+	+	+	–	+	+	_	_	+
S. sobrinus	+	+	+	+	+	V–	NA	+	_	_	_	_	+	_	_	_
Salivarius Group																
S. salivarius	+	NA	NA	+	_	_	_	V+	V	V+	NA	+	V	NA	NA	V
S. vestibularis	+	+	NA	V+	_	_	NA	_	_	_	_	+	V	NA	NA	V+

[a]Reaction for *S. pseudopneumoniae* are those from the API 20 Strep system.
[a]Reactions shown for *S. pseudopneumoniae* are those from the API 20 Strep system.
GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; SBTL, sorbitol; ARAB, arabinose; INU, inulin; MEL, melibiose; RAFF, raffinose; RIB, ribose; SAL, salicin; TRE, trehalose; STA, starch; GLYG, glycogen; AMGD, amygdalin.
+, positive reaction; –, negative reaction; V, variable reaction; V+, variable reaction, most strains positive; V–, variable reaction, most strains negative; NA, data not available; Hem, SBA, hemolysis on sheep blood agar; ESC, esculin hydrolysis; ADH, arginine dihydrolase; VP, acetoin production; URE, urease; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GAL, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; α-FUC, α-fucosidase; β-FUC, β-fucosidase; α-ARAB, α-arabinosidase; NAGALA, *N*-acetyl-β-D-galactosaminidase; PAL, alkaline phosphatase.

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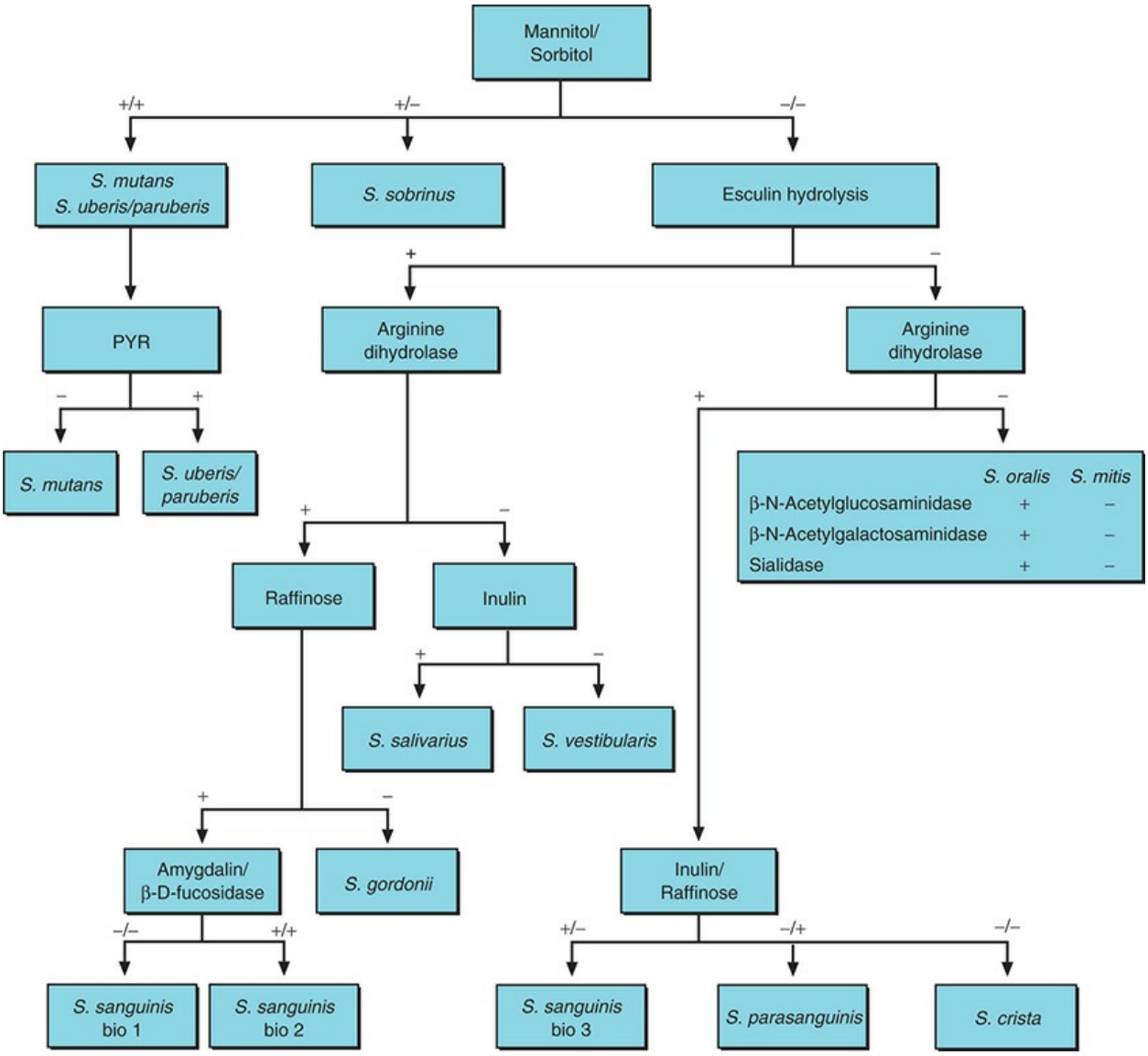

■ **FIGURE 13-3** Flow chart for identification of viridans streptococci.

groEL (genes encoding 10kDa and 60 kDa heat-shock proteins), *rnpB* (gene encoding the RNA subunit of endoribonuclease O), *sodA* (superoxide dismutase gene), *tuf* (gene encoding elongation factor Tu), *gyrA* (gene encoding quinolone-resistance-determining regions of DNA gyrase), *rpoB* (gene encoding RNase P), *parC* (topoisomerase subunit C gene), and *recN* (recombination/repair protein gene).[370,384,483,546,828,1009,1122] MALDI-TOF shows promise as a rapid and accurate method for species identification of the viridans streptococci. Identification by mass spectrometry is dependent on having an accurate database reflecting the spectral arrays found in these bacteria. The Bruker Daltronics and bioMerieux MALDI-TOF platforms contain a vast array of spectra based on species-specific PCR assays and 16S rRNA gene sequencing.

Specific applications of these methods for direct detection in clinical specimens and for identification of cultural isolates are described below and in the sections dealing with specific identification kits and methods.

Mitis/Sanguinis Group. The Mitis Group contains several closely related species, including *S. mitis, S. pneumoniae,* and *S. pseudopneumoniae*. Members of this group are more frequently isolated from blood cultures and are of some importance due to emerging resistance to penicillin and other β-lactam agents in this group. Original members of the Mitis Group did not hydrolyze esculin, did not produce ADH or aceteoin, and did not ferment mannitol or sorbitol. Some taxonomic schemes include members of the Sanguinis Group of viridans streptococci (*S. sanguinis*, *S. parasanguinis*, and *S. gordonii*) within the Mitis Group based on 16S rRNA sequencing. Isolates in the Sanguinis Group are ADH- and esculin-positive and, like members of the Mitis Group,

are negative for acetoin production and do not ferment mannitol or sorbitol. Analysis of *S. sanguinis*-like strains resulted in the descriptions of other Mitis and Sanguinis Group members (*S. gordonii* and *S. oralis*), and members of the Mitis Group genotypically (usually based on 16S rRNA sequencing) had phenotypic characteristics shared with Sanguinis Group members.[795] For example, *S. cristatus* shares features of *S. mitis*, but is ADH-positive. *S. parasanguinis* and *S. cristatus* have been isolated from the upper respiratory tract, dental plaque, urine, periodontal abscesses, and blood cultures.[422,694,882,1125] *S. peroris* and *S. infantis* are found on the teeth and in the nasopharynx.[545] *S. sinensis* is an α-hemolytic *streptococcus* that was isolated from the blood of a patient with endocarditis in 2002.[1142] Newer Mitis Group species that have been isolated from human clinical specimens (i.e., *S. australis*, *S. lactarius*, *S. massiliensis*, *S. oligofermentans*, and *S. tigurinus*) are described in Box 13-2. *S. pneumoniae* and *S. pseudopneumoniae* are discussed further below. MALDI-TOF MS has shown high sensitivity and specificity for identifying atypical *S. pneumoniae* isolates, for identification of *S. pseudopneumoniae,* and for differentiation of these organisms from other organisms in the Mitis Group.[299,1117]

Mutans Group. The Mutans Group includes oral streptococci found in humans and various animal species. (Box 13-2) Species in the Mutans Group were originally serotypes of *S. mutans* that were elevated to species status later. *S. mutans* and *S. sobrinus* are species isolated from humans. *S. hyovaginalis* is also included in the Mutans Group because of its biochemical consistency with other mutans group streptococci, but this species is found in the genital tract of swine and as part of its

normal flora. Several new Mutans Group species have been isolated from bats, rats, pigs, bears, and wild boars. *S. mutans* and *S. sobrinus* hydrolyze esculin, produce acteoin, and ferment sorbitol and mannitol, do not produce ADH, and can be differentiated by enzymatic and fermentation tests (Table 13-6).

Salivarius Group. Among species in this group, only *S. salivarius*, *S. vestibularis*, and *S. infantarius* have been recovered from human clinical specimens. *S. salivarius* and *S. vestibularis* are found in the human oral cavity, while *S. infantarius* has been isolated from humans and food products.[901,1128] *Salivarius* group organisms produce acetoin and hydrolyze esculin, but are ADH-negative and do not ferment mannitol or sorbitol. *S. infantarius* strains are also included in the Salivarius Group because some strains are bile esculin– and esculin hydrolysis–negative. *S. hyointestinalis* is a *Salivarius* group species found in the gut of swine, and *S. thermophilus* is isolated from dairy products. Strains of *S. vestibularis* and some *S. salivarius* produce urease.

Anginosus Group. The organisms that comprise the anginosus group of streptococci —*S. anginosus* subsp. *anginosus*, *S. anginosus* subsp. *whileyi*, *S. constellatus* subsp. *constellatus*, *S. constellatus* subsp. *pharyngis*, *S. constellatus* subsp. *viborgensis*, and *S. intermedius*—produce "pinpoint" colonies on blood agar and may be β-, α-, or nonhemolytic, depending on the species/subspecies. A greater proportion of *S. intermedius* strains are nonhemolytic, and *S. constellatus* strains are often β-hemolytic. For β-hemolytic strains, the size of the hemolytic zone is usually much larger than the pinpoint colony diameter. Another clue to growth of an anginosus group species is the presence of a sweet odor from the culture plate that resembles "caramel," "butterscotch," or "honeysuckle." This odor is due to production of the metabolite diacetyl.[184] Isolates may carry group A, C, F, or G antigens or may be nongroupable. *S. intermedius* rarely carries Lancefield-grouping antigens. Growth is enhanced by incubation in a CO_2-enriched environment, and some strains may require anaerobic incubation for optimal growth. Members of the Anginosus Group are positive in the esculin hydrolysis, ADH, VP, and sorbitol reactions. Individual species may be differentiated by the phenotypic tests shown in Table 13-6. These and other tests shown in Table 13-7 are found in the commercially available kit systems (e.g., API Rapid Strep, RapID STR, and the API RapidID32 kits, see discussion below).[174] These kits generally have difficulty in separating the species in this group from one another, although certain enzymatic tests on the Rapid ID32tests may prove useful for this purpose.[977] Real-time molecular tests targeting 16S rRNA genes, cpn10/60 (groES and groEL heat-shock proteins), *tuf* (elongation factor 2), *rnp* (RNase P RNA) genes, and rRNA gene intergenic spacer (ITS) regions have been developed for identification and differentiation of members of the Anginosus Group.[786,977,1117] Molecular methods (i.e., 16S rRNA gene detection and sequencing) have proved useful for direct

detection of *S. intermedius* win culture-negative clinical specimens (e.g., brain abscess aspirate).[883]

Bovis Group. As discussed previously, the Bovis Group has undergone significant taxonomic revision. Like the enterococci, the group D streptococci possess the group D antigen, hydrolyze esculin in the presence of 40% bile, and grow at 45°C (Image Plate 13-4C, 13-4D, and 13-4E). They do not grow in 6.5% NaCl broth, do not hydrolyze PYR, and do not grow at 10°C. Most isolates are LAP-positive, produce acetoin, do not produce ADH or urease, and acidify lactose but not sorbitol. *S. gallolyticus* subsp. *gallolyticus* (*S. bovis* I) strains are positive in the mannitol, raffinose, glycogen, amygdalin, and α-GAL reactions, but β-GUR-negative; most strains also ferment inulin.[64] *S. infantarius* subsp *infantarius* (S. bovis II.1) and *S. gallolyticus* subsp. *pasteurianus* (*S. bovis* II.2) are mannitol-negative, with *S. infantarius subsp infantarius* being β-GUR-negative and *S. gallolyticus* sussp. *pasteurianus* being β-GUR–positive. These phenotypic characteristics are presented in Table 13-8. *S. gallolyticus* and its subspecies are included in the data bases of automated (e.g., Vitek 2) and manual (API20 Strep; ID32 Strep) identification systems which provide reliable phenotypic identifications.[569,617,787,973] A molecular identification approach have generally used sequencing of the 16S rRNA and *sodA* (superoxide dismutase gene), with partial sequencing of the *sodA* gene being the most accurate.[350,742,787] Molecular techniques (e.g., 16S rDNA PCR) performed on resected valvular tissue have aided in the diagnosis of severe infections such as multivalve infective endocarditis caused by *S. gallolyticus* subsp. *macedonicus* and *S. gallolyticus* subsp. *gallolyticus* bacteremia.[563,674] MALDI-TOF using the Bruker Biotyper identified all of 27 *S. gallolyticus* isolates, but did not provide subspecies designations.[872] Because of the known

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TABLE 13-7 Phenotypic Characteristics for Identification of the Anginosus Group: *S. anginosus, S. constellatus,* and *S. intermedius*

Species	HEM, SBA	Lancefield Groups	Acid Produced From: GLU	LAC	AMYG	SAL	RAFF	SBTL	Production of: α-GAL	β-GAL	α-GLU	β-GLU	β-GUR	β-FUC	NAGA	NEUR	HYAL
S. anginosus subsp. *anginosus*	α, γ	A, C, F, G, none	+	+	+	V	V	–	V	V	V	+	NA	–	V	–	V
S. anginosus subsp. *whileyi*	β	C	+	+	V	V	–	–	–	–	–	+	–	–	–	–	+
S. constellatus subsp. *constellatus*	α, β, none	F, none	+	V	V	V	–	–	–	–	+	–	+	V	–	+	+
S. constellatus subsp. *pharyngis*	β	C	+	+	+	V	–	–	–	+	+	+	+	V+	+	–	+
S. constellatus subsp. *viborgensis*	β	C	+	+	V	V	–	–	–	–	–	+	+	V	–	–	+
S. intermedius	α, β, none	None	+	+	V	V	–	–	–	+	+	V	NA	–	+	+	+

+, positive reaction; –, negative reaction; V, variable reaction; SBA, sheep blood agar; GLU, glucose; LAC, lactose; AMYG, amygdalin; SAL, salicin; RAFF, raffinose; SBTL, sorbitol; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; β-FUC, β-fucosidase; NAGA, *N*-acetyl-β-D-glucosaminidase; NEUR, neuraminidase; HYAL, Hyaluronidase.

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TABLE 13-8 Phenotypic Characteristics for Identification of the Bovis Group

Species	ESC	GAL	Acid Produced From: GLU	LAC	MNTL	RAFF	TRE	INU	STA	GLYG	Production of: α-GAL	β-GAL	β-GLU	β-GUR	Comments (Former Names, Other Epithets, etc.)
S. bovis/S. equinus	+/+	–/–	+/+	–/+	–/–	–/+	V/V	–/+	–/+	–/+	–/+	–/–	+/+	–/–	*S. bovis* type strain/isolates of *S. equinus* (91%–100% sequence homology); strains represented are primarily bovine and equine in origin
S. gallolyticus subsp. *gallolyticus*	+	+	+	+	+	+	+	+	+	+	+	–	+	–	*S. bovis* I, include strains isolated from humans and several types of animals
S. gallolyticus subsp. *pasteurianus*	+	–	+	+	–	V	V	–	–	–	V	+	+	+	*S. bovis* II.2; "*S. pasteurianus*"; includes human strains
S. gallolyticus subsp. *macedonicus*	–	–	+	+	–	–	–	–	+	–	V	V+	–	–	"*S. macedonicus*"; "*S. waius*"
S. infantarius subsp. *infantarius*	V	–	+	+	–	+	–	–	+	+	+	–	V	–	*S. bovis* II.1; includes human and bovine strains
S. infantarius subsp. *coli*	V	–	+	+	–	–	V	–	+	V	+	–	+	–	"*S. lutetiensis*"; also ADH-negative, PYR-negative, NAGA-negative
S. caballi	+	–	+	–	–	+	+	+	+	+	+	–	+	–	New species from horses; does not possess the group D antigen; able to grow in presence of 40% bile; 6.5% NaCl-negative; ADH-negative, hippurate-negative, acetoin-positive
S. henryy	+	–	+	+	+	–	+	+	+	+	+	+	+	–	New species from horses; possesses the group D antigen; able to grow in presence of 40% bile; 6.5% NaCl-negative; ADH-negative, hippurate-negative, acetoin-negative
S. alactolyticus	+	–	+	–	–	–	–	–	–	–	+	–	+	–	*S. intestinalis*

+, positive reaction; –, negative reaction; V, variable reaction; V+, variable reaction, but most strains positive; ESC, esculin hydrolysis; GAL, gallate hydrolysis; GLU, glucose; LAC, lactose; MNTL, mannitol; RAFF, raffinose; TRE, trehalose; INU, inulin; STA, starch; GLYG, glycogen; α-GAL, α-galactosidase; β-GAL, β-galactosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase.

association of the former *S. bovis* II.1 with endocarditis and colonic carcinoma, the emergence of *S. gallolyticus* subsp. *pasteurianus* as a cause of bacteremia and meningitis, and the confusing changes that have occurred in the taxonomy of the Bovis Group, it, is incumbent on the laboratory to familiarize clinicians with changes in the taxonomy. Reports should include both the new name and the old name or the organisms in order to prevent the underdiagnosis of serious diseases related to this organism group.[1081]

Identification of Streptococcus suis *and Other Streptococci Isolated From Animals*

S. suis should be suspected in patients with systemic signs of sepsis and meningitis who have had contact with pigs or porcine products. The organism is a catalase-negative, nonmotile, gram-positive coccus that occurs singly, in pairs, or in short chains. *S. suis* is α-hemolytic on SBA (β-hemolytic on horse blood agar), resistant to optochin, and does not grow in 6.5% NaCl broth. Some strains will grow in the presence of 40% bile, and all strains are able to hydrolyze esculin. *S. suis* phenotypically resembles certain viridans streptococci, particularly *S. gordonii*, *S. sanguinis*, and *S. parasanguinis*. *S. suis* is in the databases of the API 20 Strep (bioMerieux) and the Vitek 2; identifications obtained with these systems usually have likelihoods in excess of 99.7%.[481,559,1028] Human isolates are usually capsular serotype 2 strains that react with Lancefield group R antisera (Statens Serum Institute, Copenhagen, Denmark) Molecular methods for identification include sequencing of 16S rRNA and housekeeping genes (e.g., *tuf* gene, *sodA* gene).[481,1028] Direct detection of *S. suis* serotype 2 in clinical specimens (e.g. CSF) and capsular typing may also be accomplished by molecular methods using sequence analysis of 16S rRNA and capsular *cps* (especially *csp2j*) genes as targets.[671,949] These genes encode part of the operon for the *S. suis* serotype 2 capsular polysaccharide. Multiplex PCR assays have also been developed for detection of other serotypes and virulence-associated genes that are pathogenic for swine.[1135]Additional characteristics that are helpful for identification of *S. suis* are presented in Table 13-9. Table 13-9 also presents the phenotypic characteristics of α- and nonhemolytic streptococci isolated from various animal species.

Detection of Vancomycin-Resistant Enterococci

Enterococcus species grow well on most bacteriological media, including 5% sheep blood CNA, and chocolate agars. On SBA, most strains are nonhemolytic or α-

hemolytic. Most strains are able to grow at 35°C to 37°C in ambient air, although incubation in a CO_2-enriched environment stimulates growth of most isolates. Specimens containing enterococci that are heavily contaminated with gram-negative bacilli may be easily isolated on media containing sodium azide (e.g., Pfizer selective enterococcus agar, bile esculin azide agar). Identification of enterococci to species level is often helpful and sometimes crucial for proper patient management and for epidemiologic and infection control purposes. Isolates of *E. faecium* tend to be more resistant to penicillin and ampicillin than *E. faecalis* isolates, and the vast majority of VRE are strains of *E. faecium*. Asymptomatic colonization of the gastrointestinal tract by VRE typically precedes infection, and colonization may persist and serve as a reservoir for colonization/infection of other patients.[725] Patients who are colonized by VRE become efficient vectors for environmental distribution and spread of VRE to healthcare workers. Patients who are colonized with VRE in the intestinal tract can remain carriers for months to years. Hospital outbreaks of VRE have been reported extensively in the United States and several other countries. The major consequences of VRE infections include increased morbidity and mortality, increased lengths of hospitalization, and increased costs related to longer hospital stays.[82,138,365,604,847,954,1112] Early detection of VRE in at-risk patients enables the timely institution of infection control practices that are known to limit the spread of these opportunistic agents. The Hospital Infection Control Practices Advisory Committee of the CDC has promulgated guidelines to prevent the spread of VRE and has recommended implementation of contact isolation precautions for patients who have gastrointestinal colonization with VRE.[154] The breakpoint for vancomycin resistance is ≥32 µg/mL, with intermediate susceptibility being 8 to 16 µg/mL. (CLSI) VREs from nosocomial outbreaks generally have vancomycin MIC's ≥32 µg/mL.

Several agar and broth media formulations have been used for detection of VRE in anatomic sites that harbor enterococci (i.e., stool specimens, rectal swabs) (Image Plate 13-3H). Broth for growth enrichment of VRE is made semi-selective by addition of vancomycin to the media; other selective agents (e.g., clindamycin, aztreonam) may also be added. Because some VRE strains may be inhibited by these combinations of antimicrobial agents, subculture from selective broth medium to both selective and nonselective agar media is suggested in order to optimize recovery of VRE. For example, VRE broth (Hardy Diagnostics, Santa Maria, CA) is a differential/selective medium for recovery of VRE from heavily contaminated specimens. The medium contains esculin and ferric citrate to detect esculin hydrolyzing organisms, bile salts to inhibit gram-positive bacteria other than enterococci, and sodium azide to inhibit gram-negative bacteria. VRE broth also contains 8 µg/mL of vancomycin for selective detection of vancomycin-resistant strains. Further testing must be performed on media such as BHI with vancomycin as per (CLSI). BEAV agar (bile esculin agar with azide and vancomycin, Remel, Lenexa, KA) is an agar medium for isolation of VRE from

contaminated specimens. As with the broth medium above, esculin, ferric citrate, bile salts, and azide are present for inhibition of other gram-positive and gram-negative bacteria, respectively. Bile tolerance and esculin hydrolysis provide presumptive identification of group D streptococci, and the presence of vancomycin in BEAV agar (8 μg/mL) selects for VRE. With 8 μg/mL of vancomycin, BEAV does not support growth of *E. casseliflavus* and *E. gallinarium* (low-level resistance). VREs must be confirmed using BHI with vancomycin according to CLSI recommendations.

Several chromogenic media formulations are available for detection of gastrointestinal VRE colonization and include Spectra VRE (Remel), BBL CHROMagar VanRE (Becton-Dickinson), VRE-BMX (bioMérieux, Marcy l'Étoile, France), chromID VRE (bioMerieux), and Brilliance VRE agar (Oxoid). These media contain chromogenic substrates having sufficient sensitivity and specificity to identify

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TABLE 13-9 Phenotypic Characteristics for Identification of α-Hemolytic and Nonhemolytic Streptococci Isolated From Animals

Species	Hem, SBA	Growth, 10°C	Growth 45°C	LAP	PYR	Growth, BE Agar	Growth, 6.5% NaCl	ESC	ADH	HIPP	URE	VP	Production of: α-GAL	β-GAL	α-GLU	β-GLU	β-GUR	NAGA	PAL
S. acidominimus	α	NA	NA	+	NA	–	–	–	–	+	–	NA	–	+	NA	+	V	–	NA
S. caballi	α	NA	NA	+	–	+	–	+	–	–	–	+	+	–	NA	+	–	–	–
S. entericus	α	–	–	+	NA	–	–	+	–	–	–	–	–	+	NA	+	–	–	–
S. gallinaceus	α	NA	NA	NA	NA	NA	NA	+	+	–	–	–	+	+	+	+	–	V	–
S. henryi	α	NA	NA	+	–	+	–	+	–	–	–	–	+	+	NA	+	–	–	–
S. hyointestinalis	α	NA	NA	+	–	–	–	+	–	–	–	+	+	–	+	+	–	–	V
S. hyovaginalis	α	–	–	+[w]	–	–	–	NA	–	+	–	+	–	+	+	V	+	+	+
S. marimammalium	None, β (3 days)	NA	NA	+	NA	NA	NA	–	V	–	V	–	–	+	NA	–	–	–	+
S. merionis	None	–	–	+	–	–	–	+	+	+	–	–	–	+	NA	+	+	NA	–
S. minor	α	NA	NA	+	–	+	NA	+	+	–	–	NA	V+[w]	–	NA	NA	–	NA	–
S. ovis	α	NA	NA	+	NA	NA	NA	+	V+	–	–	–	V	–	+	+	–	–	–
S. pluranimalium	α	–	+	+	–	+	V	+	–	+	–	–	NA	NA	NA	V	V+	NA	V+
S. plurextorum	α	–	–	+	NA	NA	NA	NA	–	–	–	–	+	+	–	–	+	–	–
S. porci	α	–	–	+	NA	NA	–	+	–	–	–	–	+	+	+	+	–	–	–
S. suis	α	–	–	+	NA	V	–	+	+	–	–	–	+	V	NA	NA	+	NA	NA
S. thoraltensis	α	–	–	+	–	+	+	+	+	V	–	+	V	–	NA	+	+	–	+[w]
S. uberis/parauberis	α, none	NA	NA	+	+	–/+[w]	+	+	+	+	–	V	NA	NA	NA	NA	+	NA	NA
Eremococcus coleocola	α	–	+	–	NA	–	+[w]	–	+	+	V	–	–	–	NA	–	–	NA	NA
Catellicoccus marimammalium	None	–	NA	+	–	NA	NA	–	+	–	V	–	–	–	–	–	–	–	NA

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S. acidominimus	+	NA	+	–	V	–	–	–	–	–	–	–	V	–	Metritis in cattle; poultry
S. caballi	+	+	+	–	–	+	+	–	–	+	+	–	+	+	Horses; associated with laminitis
S. entericus	+	+	+	+	–	–	–	–	–	+	–	–	+	+	Intestinal tract of cattle
S. gallinaceus	+	+	+	+	+	+	NA	–	–	NA	+	+	+	–	Chickens
S. henryi	+	+	+	+	+	+	+	–	–	+	–	–	+	+	Horses; associated with laminitis
S. hyointestinalis	+	+	V	V	–	+	–	–	–	+	–	–	–	–	Swine (intestinal tract)
S. hyovaginalis	+	+	+	+	+	–	–	–	+	–	–	V+	+	–	Sows (genital tract)
S. marimammalium	+	V	–	+	–	–	–	–	–	–	–	–	–	–	Gray seals, common seals
S. merionis	+	+	NA	+	–	–	+	–	–	+	+	–	+	–	Mongolian jirds
S. minor	+	+	+	+	+[w]	–	V	–	V	V	V	–	+	+	Dogs (tonsils, feces); cattle, cats (tonsils)
S. ovis	+	+	+	+	+	–	NA	–	+	NA	+	–	+	+	Sheep (clinical specimens)
S. pluranimalium	+	NA	NA	NA	V+	NA	–	–	V	–	NA	V	+	–	Cattle (mastitis, genital tract, tonsils); goats (tonsils); cats (tonsils); canaries (crop, respiratory tract)
S. pluextorum	+	+	+	+	–	–	–	–	–	–	+	–	+	–	Swine (lung, kidney tissue)
S. porci	+	+	+	+	–	+	–	–	–	+	+	–	+	+	Swine
S. suis	+	+	+	+	–	V	+	–	–	NA	V	–	NA	NA	Infections in piglets; meningitis in humans
S. thoraltensis	+	+	+	+	+	V	+	+	V	+	V	+	+	–	Sows (genital tract, intestinal tract)
S. uberis/parauberis	+	+	+	+	+	NA	V	–	+	NA	–	+	+	NA	Cattle (mastitis)
Eremococcus coleocola	+	–	–	–	–	NA	NA	–	–	NA	NA	NA	–	–	Horses
Catellicoccus marimammalium	+	–	–	–	–	–	–	–	–	–	–	–	+	–	Porpoises (*Phocoena phocoena*)

+, positive reaction; –, negative reaction; V, variable reaction; +[w], weak positive; NA, data not available; Hem, SBA, hemolysis on sheep blood agar; ESC, esculin hydrolysis; ADH, arginine dihydrolase; HIPP, hippurate hydrolysis; URE, urease; VP, acetoin production; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; PAL, alkaline phosphatase. GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; MEL, melibiose; INU, inulin; ARAB, arabinose; SORB, sorbitol; STA, starch; RAFF, raffinose; RIB, ribose; TRE, trehalose; GLYG, glycogen.

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VRE. Spectra VRE is designed to detect VRE in stool or rectal swab specimens after 24 hours' incubation. These media contain proprietary antimicrobial additives. On Spectra VRE agar, vancomycin-resistant *E. faecium* grow as pink, purple, or dark-blue colonies, while vancomycin-resistant *E. faecalis* appear as light-blue colonies. A 2010 evaluation of Spectra VRE compared with BEAV agar found that the sensitivity, specificity, and positive and negative predictive values for Spectra VRE medium were 98.2%, 99.3%, 98.2%, and 99.3% compared with 87.6%, 87.1%, 72.8%, and 94.7% for BEAV agar.[825] Similar results for Spectra VRE medium have been reported by other investigators.[514] On BBL CHROMagar VanRE, vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* appear as mauve colonies and green colonies after 24 hours' incubation, respectively. In an evaluation of this medium by Kallstrom and coworkers,[532] the sensitivity, specificity, and positive and negative predictive values for CHROMagar VanRE were 98.6%, 99.1%, 95.9%, and 99.7%, respectively. On VRE-BMX and chromID VRE agars (bioMérieux), colonies of *E. faecium* and *E. faecalis* appear as purple or green, respectively. Ledeboer and colleagues[269,623,624] compared VRE-BMX agar with BEAV agar for detection of VRE in stool specimens and found that VRE-BMX detection 94.4% of 54 vancomycin-resistant *E. faecium* and 100% of 12 vancomycin-resistant *E. faecalis* after 24 hours' incubation. Evaluations of chromID agar have reported sensitivities ranging from 80% to 100% for detection of vancomycin-resistant *E. faecium* and *E. faecalis* with

specificities exceeding 99% when direct specimen inoculation methods were used.[624] Specimen preenrichment in broth medium containing vancomycin or incubation of chromID agar for an additional 24 hours resulted in sensitivities ranging from 93% to 100%.[254,289,581,624]

Molecular approaches are also available for the detection of VRE in stool and rectal swab specimens. Widely used tests include the BD GeneOhm VanR (Becton-Dickinson) and the Gene Xpert *vanA/vanB* (Cepheid). These assays detect the *vanA* and *vanB* responsible for moderate- to high-level vancomycin resistance. The sensitivities and specificities of these assays vary depending on the prevalence of enterococci carrying the *vanA* or the *vanB* resistance determinants. In one study, the GeneXpert assay showed sensitivities for *vanA* and *vanB* detection of 73.9% and 87.5%, respectively, while corresponding sensitivities for the GeneOhm assay were 43.5% and 100%, respectively.[372] Both assays have shown low specificities ranging from 14.7% to 20.6% for detection of these resistance determinants, and lower specificities for detection of *vanB* have been reported for the BD GeneOhm assay in other studies.[961,1067] An evaluation of the GeneXpert *vanA/vanB* assay during a VanA *E. faecium* outbreak showed sensitivity, specificity, and predictive values of positive and negative test results of 61.5%, 79.2%, 61.5%, and 79.2%, respectively.[1164] The authors of this study concluded that, while a rapid (i.e., less than 1 hour) positive test enabled early recognition of some colonized patients, culture on chromogenic agar medium (in this case, chromID VRE agar) was required to detect and confirm VRE colonization in other patients. A study conducted at Johns Hopkins found that the GeneOhm VanR assay detected 96.6% of 147 VRE isolates compared with chromogenic culture media, with a specificity of 87.0%.[961] The lower specificity was due to false-positive results occurring with the *vanB* portion of the assay. While these assays primarily detect *E. faecium* and *E. faecalis* carrying vancomycin-resistance genes, positive results may be obtained with other enterococcal species (e.g., *E. raffinosus*, *E. durans*, *E. gallinarum*) carrying *vanA* or *vanB* genes.[961] Both of these molecular assays are considerably faster than culture. Other highly sensitive and specific quantitative and real-time PCR assays for detection of VRE have also been described.[1043]

Identification of Enterococcus *Species*

Identification of *Enterococcus* species is accomplished by biochemical and physiologic tests (Table 13-10). Most (about 80%) *Enterococcus* species react with antiserum against Lancefield group D by capillary precipitin or LA; the group D antigen cannot be demonstrated in strains of *E. pseudoavium*, *E. dispar*, *E. cecorum*, *E. sulfureus*, *E. columbae*, and *E. saccharolyticus*. Most species of enterococci are able to grow within 48 hours in BHI broth incubated at both 10°C and 45°C. However *E. cecorum*

and *E. columbae* fail to grow at 10°C and *E. sulfureus*, *E. malodoratus*, and *E. dispar* do not grow at 45°C. Most *Enterococcus* species hydrolyze esculin in the presence of 40% bile (BE test), growth in broth containing 6.5% NaCl, and are PYR-positive, although some species are negative in these tests as well (Table 13-10). Although *E. faecalis* and *E. faecium* constitute those species that are most commonly recovered from clinical specimens, the incidence of the other species and their roles in specific disease processes are unknown. Isolates that fit the criteria listed above are inoculated into several phenotypic test media and are identified based on these reactions. For identification of enterococci by conventional procedures, carbohydrate fermentation tests and pyruvate utilization are determined in heart infusion broth base media containing bromcresol purple and 1% filter-sterilized carbohydrates or 1% pyruvate. Deamination of arginine is determined using Moeller's decarboxylase broth, and motility is determined in semisolid motility medium. Detection of the yellow pigment produced by *E. casseliflavus*, *E. gallinarum*, and *E. mundtii* is achieved by sweeping up some of the growth on a white Dacron swab and observing the colonial material on the swab for a yellow or yellow-orange color (Image Plate 13-4A). *E. casseliflavus* and *E. gallinarum* are also motile (Image Plate 13-4B). Acid production in methyl-α-D-glucopyranoside (MGP) is a helpful differential test for differentiation of *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavaus*. The former two species do not acidify MGP broth, while the latter two species do. A handful of phenotypic tests, including motility, pigmentation, arginine dihydrolase, and acid production from MGP, arabinose and raffinose may be used for phenotypic identification of *E. faecalis*, *E. faecium*, *E. casseliflavius*, *E. gallinarum*, and *E. raffinosus*. A flowchart for identifying the clinically significant *Enterococcus* species is presented in Figure 13-4. In addition, many laboratories rely on manual (e.g., API Strep) and automated (e.g., Vitek 2, Phoenix, Microscan) identification systems for identifying enterococci. These systems and their performance for identifying enterococci are described later in this chapter. PCR-based

TABLE 13-10 Phenotypic Characteristics for Identification of *Enterococcus* and Related Species

Group/Species	Group D Antigen	Growth, BE Agar	Growth, 6.5% NaCl	Growth, 10°C	Growth 45°C	LAP	PYR	MOT	YELLOW PIGM	ADH	HIP	Acid Produced From: GLU	MNTL	SOR	ARAB	SBTL	RAFF	SUC	PYRV	MGP
Group I																				
E. avium	+	+	+	NA	+	+	+	–	–	–	V	+	+	+	+	+	–	+	+	V
E. gilvus	+	+	+	+	+	+	+	–	+	–	–	+	+	+	–	+	+	+	+	–
E. malodoratus	+	+	+	NA	–	+	+	–	–	–	V	+	+	+	–	+	+	+	+	V
E. pallens	+	+	+	+	+	+	–	–	+	–	+	+	+	+	+	+	+	+	+	–
E. pseudoavium	–	+	–	+	+	+	+	–	–	–	+	+	+	+	–	+	–	+	+	+
E. raffinosus	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	V
E. saccharolyticus	–	NA	+	NA	NA	+	–	–	–	–	–	+	+	+	–	+	+	+	–	+
E. hawaiiensis	–	–	+	+	–	+w	–	–	–	–	–	+	+	+	–	+	–	–	+	–
E. devriesei	NA	+	+	+	–	V	V	–	–	–	NA	+	+	V	V	V	+w	+	NA	–
Group II																				
E. faecalis	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	+	–	+	+	–
E. faecium	+	+	+	+	+	+	+	–	–	+	–	+	+	–	+	V	V	+	–	–
E. casseliflavus	+	+	+	NA	+	+	+	+	+	+	–	+	+	–	+	V	+	+	V	+
E. gallinarum	+	+	+	+	+	+	+	+	–	+	+	+	+	–	+	–	+	+	–	+
E. mundtii	+	+	+	+	+	+	+	–	+	+	–	+	+	–	+	V	+	+	–	–
E. haemoperoxidus	+	+	+	+	–	+	+	–	+w	+	+	+	+	–	–	–	–	+	–	+
E. silesiacus	NA	+	+w	+	–	+	+	–	–	+	–	+	–	–	–	–	–	–	NA	–
E. termitis	NA	+	+	+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	NA	+
E. camelliae	NA	NA	–	+	+	NA	NA	–	–	–	NA	+	+	–	–	–	–	+	NA	–
E. thailandicus	NA	NA	+	+	+	NA	NA	–	–	+	NA	+	+	–	–	–	–	+	NA	–
Lactococcus spp.	–							–	–	+	V	+	+	–	–	–	–	V	–	–
Group III																				
E. dispar	–	+	+	+	–	+	+	–	–	+	V	+	–	–	–	–	+	+	+	+
E. durans	+	+	+	NA	+	+	+	–	–	+	V	+	–	–	–	–	–	–	–	–
E. hirae	+	+	+	+	+	+	+	–	–	+	–	+	–	–	–	–	+	+	–	–
E. ratti	+w	+	+	+	+	+	+	–	–	+	V	+	–	–	–	–	–	–	–	–
E. villorum	+	+	+	+	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–
E. canintestini	–	+	+	–	+	V	V	–	–	+	–	+	–	–	–	–	–	–	NA	+

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Group/Species	Group D Antigen	Growth, BE Agar	Growth, 6.5% NaCl	Growth, 10°C	Growth 45°C	LAP	PYR	MOT	YELLOW PIGM	ADH	HIP	Acid Produced From: GLU	MNTL	SOR	ARAB	SBTL	RAFF	SUC	PYRV	MGP
Group IV																				
E. asini	+	+	–	V	V	+	+	–	–	–	+	+	–	–	–	–	–	–	–	V
E. cecorum	–	NA	–	–	+	+	–	–	–	–	–	+	–	–	–	+	+	+	+	–
E. sulfureus	–	+	+	+	–	+	+	–	+	–	–	+	–	–	–	–	+	+	–	+
E. phoeniculicola	NA	–	–	–	–	NA	NA	–	–	–	–	+	–	–	–	–	–	+	+	+
E. caccae	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–	+	+	+
E. aquamarinus	NA	+	+	+	+	NA	+	–	–	–	–	+	–	–	+	–	+	+	NA	–
Group V																				
E. columbae	–	+	–	–	NA	+	–	–	–	–	–	+	+	–	+	+	+	+	+	–
E. canis	NA	+	+	NA	NA	+	+	–	–	–	–	+	+	–	+	–	–	V	+	+
E. moraviensis	+	+	+	+	–	+	V	–	–	–	+	+	+	–	+	–	–	+	+	+
E. hermanniensis	–	+	V	+	–	+	+	–	–	–	–	+	+	–	–	–	–	–	NA	NA
E. italicus	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–	+	+	+
Vagococcus spp.	–	NA	+	+	–	+	+	+	–	V–	–	+	+	–	–	+	–	+	–	+
New Species																				
E. rivorum	V	+	–	+w	–	NA	NA	–	–	V	–	+	+	–	–	+	–	+	NA	+
E. ureilyticus (urease +)	+	+	+	+	–	+	+	–	+	–	+	+	–	+	–	–	–	+	NA	–
E. rotai (urease +)	+	+	+	+	–	+	+	–	+	–	+	+	–	–	–	–	–	+	NA	–
E. ureasiticus (urease +)	NA	+	–	NA	+	+	+	–	–	–	+	+	V	NA	–	–	–	+	NA	+
E. quebecensis	NA	+	–	NA	–	+	+	–	–	–	+	+	+	NA	–	–	–	+	NA	+
E. plantarum (catalase +, urease +)	+	+	+	+	+	+	+	–	+	–	+	+	–	–	–	–	–	+	NA	–
E. lactis	NA	+	+	+	+	+	+	–	–	+	–	+	+	–	V	–	–	–	NA	–
E. lemanii	–	+	+	+	+	+	+	–	–	–	–	+	+	NA	+	–	+	+	NA	+
E. eurekensis	–	+	+	+w	+	+	+	–	–	–	–	+	–	NA	–	–	+	+	NA	NA
E. alcedinis	–	+	–	–	–	+	+	–	–	–	–	+	+	–	–	–	–	+	NA	–

+, positive reaction; –, negative reaction; V, variable reaction; +w, weak positive; NA, data not available; BE agar, bile esculin agar; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; MOT, motility; PIGM, pigment; ADH, arginine dihydrolase; HIP, hippurate hydrolysis; GLU, glucose; MNTL, mannitol; SOR, sorbose; ARAB, arabinose; SBTL, sorbitol; RAFF, raffinose; SUC, sucrose; PYRV, pyruvate; MGP, methyl-α- -glucopyranoside.

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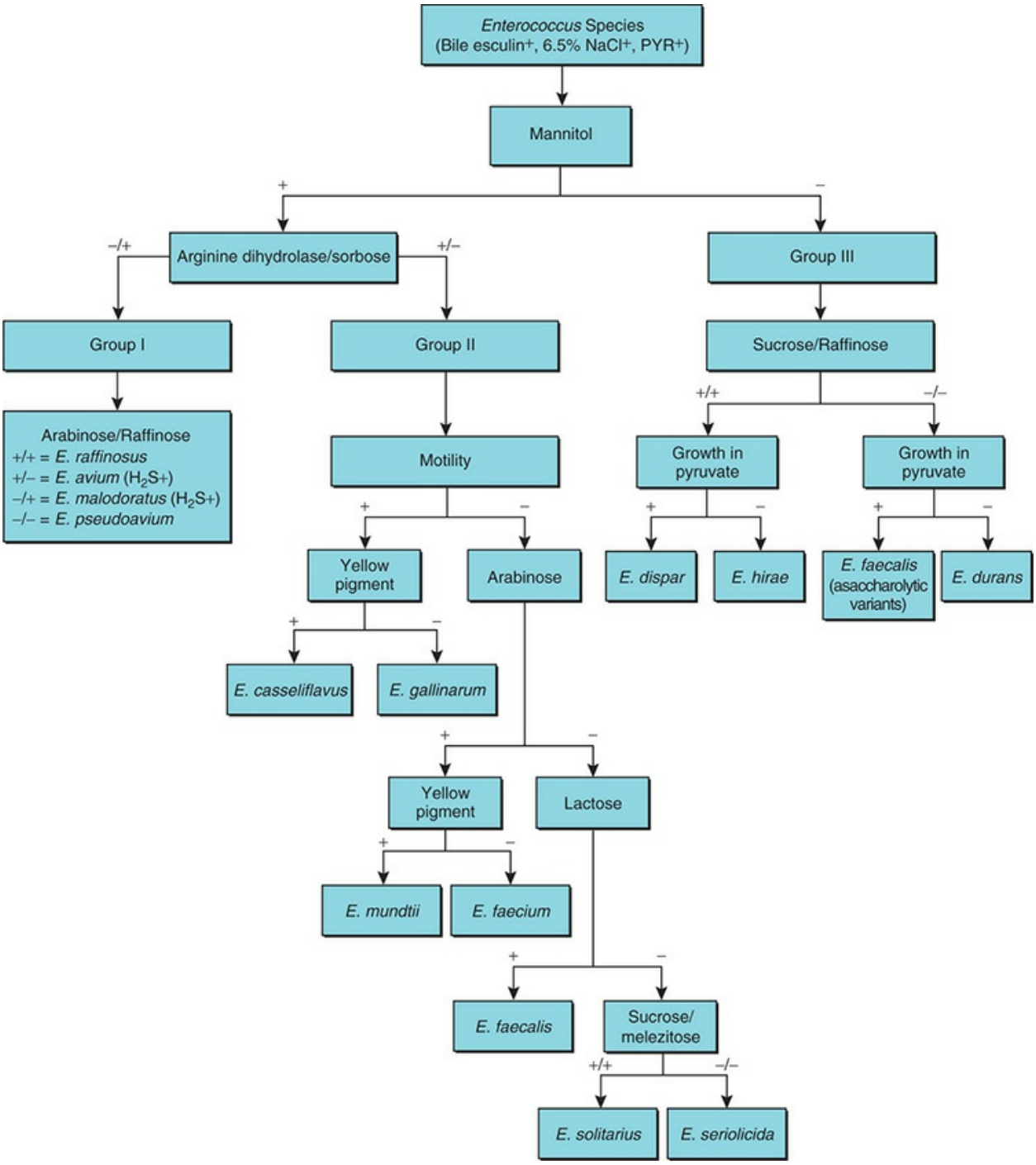

■ **FIGURE 13-4** Flow chart for identification of *Enterococcus* species (Adapted from reference 334.)

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molecular methods involving sequencing of specific genes (e.g., *tuf* gene, *rpoB* gene)

have also been useful for accurate identification of *Enterococcus* species.[638]

Identification of Abiotrophia *and* Granulicatella *Species*

A. defectiva and *Granulicatella* species should be suspected when direct Gram's stains of specimens or of positive blood cultures show streptococcal organisms that fail to grow on subsequent culture or subculture. Commercial blood culture media contain pyridoxal and will support the growth of these organisms. Subculture onto blood agar and the placement of a staphylococcal streak as is done in the satellite test for *Haemophilus* spp. will ensure growth of the nutritionally variant streptococci adjacent to the staphylococcal streak (Image Plate 13-3D). Alternatively, disks impregnated with pyridoxal may also be placed on the subculture plate, with subsequent growth of the organisms appearing as satellite colonies surrounding the disk. Commercially available chocolate agar contains pyridoxal, so these organisms will grow on chocolate agar.

When grown on pyridoxal-supplemented (10 mg pyridoxal hydrochloride per liter), cysteine-supplemented (100 mg L-cysteine/L) media, or chocolate agar, these organisms can be identified with the phenotypic tests shown in Table 13-11. Like the streptococci, these organisms are LAP-positive, but unlike the streptococci, *A. defectiva* and *Granulicatella* species are PYR-positive. Identification can be accomplished by automated (e.g., Vitek 2) or manual methods (e.g., API 20 Strep) or combinations of identification approaches.[6,23,24,270,823,873] Phenotypic testing by itself can result in incorrect identifications. For example, *G. elegans* has been misidentified as *S. acidominimus*, *G. morbillorum*, and *G. adiacens*, although the former two species grow well on unsupplemented blood agar.[6,23,137] Molecular methods for species identification include 16S rRNA, *rpoB* gene, *groES* and *groEL* heat-shock protein genes, 16S RNA, 23S RNA, and ribosomal 16S-23S intergenic spacer region sequencing performed on isolates or directly in excised valvular tissues.[84,297,484,779,1051] *Abiotrophia* and *Granulicatella* species are homofermentative (lactic acid being the end product of glucose fermentation), optochin-resistant, and vancomycin-susceptible. MALDI-TOF MS shows promise for identifying these organisms, and, in one study, *G. adiacens* was identified only to the genus-level for 33% of isolates tested.[758]

Identification of Aerococcus *and* Helcococcus *Species*

The genus *Aerococcus* contains five species: *A. viridans*, *A. urinae*, *A. sanguinicola*, *A. christensenii*, and *A. urinaehominis*. All species are α-hemolytic on SBA and grow in broth cultures as pairs, tetrads, and small clusters. Strains of *A. viridans* characteristically appear mostly as tetrads on Gram-stained smears, whereas *A. urinae* isolates occur mostly in clusters. On agar media, strains of *A. viridans* form large

colonies that resemble those of enterococci. All species except *A. christensenii* grow in 6.5% NaCl; *A. sanguinicola* and some *A. viridans* grow as black colonies on BE agar. Isolates of *A. viridans* are PYR-positive and LAP-negative, but *A. urinae* and *A. christensenii* strains are PYR-negative and LAP-positive. *A. sanguinicola* is positive for both LAP and PYR, while *A. urinaehominis* is negative in both tests. Table 13-12 provides additional phenotypic characteristics of these species.

Helcococcus kunzii, unlike most aerococci, produces variable growth in 6.5% NaCl, and growth is stimulated by

TABLE 13-11 Phenotypic Characteristics for Identification of *Abiotrophia defectiva* and *Granulicatella* Species

Characteristic	*A. defectiva*	*G. adiacens*	*G. para-adiacens*	*G. elegans*	*G. balaenopterae*
Requirement for pyridoxal	+	+	+	+	+
ADH	–	–	–	+	+
HIPP	–	–	–	+	–
Production of:					
α-GAL	+	–	–	–	–
β-GAL	+	–	–	–	–
β-GLU	–	+	–	–	–
Acid Produced From:					
GLU	+	+	+	+	+
SUC	+	+	+	+	–
TRE	+	–	–	–	+
Habitat	Humans	Humans	Humans	Humans	Cetaceans

+, positive reaction; –, negative reaction; ADH, arginine dihydrolase; HIPP, hippurate hydrolysis; α-GAL, α-galactosidase; β-GAL, β-galactosidase; β-GLU, β-glucosidase; GLU, glucose; SUC, sucrose; TRE, trehalose.

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TABLE 13-12 Phenotypic Characteristics for Identification of *Aerococcus* and *Helcococcus* Species

Characteristic	A. viridans	A. urinae	A. sanguinicola	A. christensenii	A. urinaehominis	A. urinaeequi	A. suis	H. kunzii	H. ovis	H. sueciensis
Cellular arrangement	Pairs, tetrads, clusters	Pairs, tetrads, clusters	Pairs, tetrads, clusters	Pairs, tetrads, clusters	Pairs, tetrads, clusters	Tetrads	Single cells, pairs, tetrads, small clusters	Pairs, clusters	Pairs, clusters	Pairs, short chains
Hemolysis, SBA	α	α	α	α	α	α	α	None, α	None	None
LAP	–	+	+	+	–	NA	NA	–	+	+
PYR	+	–	+	–	–	NA	NA	+	NA	NA
Growth, BE agar	V	–	+	–	–	NA	NA	–	NA	NA
Growth, 6.5% NaCl	+	+	+	–	+	NA	+	V	NA	NA
ESC	+	V	+	–	+	NA	–	+	–	–
ADH	–	–	+	–	–	–	+	NA	NA	–
HIPP	V	+	+	+	+	NA	–	–	–	–
PAL	NA	–	NA	–	+[W]	NA	+[W]	–	+	+
Production of:										
α-GAL	NA	–	–	–	–	NA	–	–	NA	–
β-GAL	+	–	–	–	–	NA	+	–	V	+
α-GLU	NA	NA	–	NA	–	NA	–	NA	NA	–
β-GLU	NA	–	–	–	V	NA	–	+	–	–
β-GUR	V	+	+	–	+	–	–	–	+	–
NAGA	NA	NA	–	–	–	NA	NA	+	–	+
Acid Produced From:										
GLU	+	+	+	+	+	+	–	+	+	+
MAL	V	–	+	–	+	+	–	+	V	+
SUC	+	+	+	–	+	+	–	–	–	–
LAC	V+	–	V–	–	–	NA	–	+	–	+
MNTL	V	+	–	–	–	V	–	–	–	–
RIB	V	V+	V	–	+	+	+ (7 days)	–	–	–
TRE	+	–	+	–	–	NA	–	+	–	+
ARAB	NA	–	–	–	–	NA	–	–	–	–
RAFF	NA	NA	–	–	–	NA	–	–	–	–
SBTL	–	+	–	–	–	NA	–	–	–	+
GLYG	+	–	–	–	–	NA	NA	V	V	–

[a]Organism was not characterized phenotypically.
+, positive reaction; –, negative reaction; V, variable reaction; V+, variable reaction, but most strains positive; V–, variable reaction, but most strains negative; +[W], weak-positive reaction; NA, data not available; SBA, sheep blood agar; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; BE agar, bile esculin agar; ESC, esculin hydrolysis; HIPP, hippurate hydrolysis; PAL, alkaline phosphatase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; RIB, ribose; TRE, trehalose; ARAB, arabinose; RAFF, raffinose; SORB, sorbitol; GLYG, glycogen.

serum or Tween 80. On the API Rapid Strep system, *H. kunzii* produces a profile that corresponds to a "doubtful" identification of *A. viridans* (API profile 4100413). Isolates of *H. kunzii* are nonhemolytic and do not grow at either 10°C or 45°C. The organism is esculin- and PYR-positive, is LAP-negative, produces acid from glucose, maltose, lactose, trehalose, and glycogen, and does not produce β-galactosidase or β-glucuronidase. These organisms are commonly penicillin-, ampicillin-, and vancomycin-susceptible. *H. kunzii* and the other human isolates, *H. sueciensis* and *H. pyogenes*, are easily differentiated from the ovine species *H. ovis* by phenotypic tests (Table 13-12).

Identification of Leuconostoc, Pediococcus, *and* Tetragenococcus *Species*

Leuconostoc species are gram-positive, catalase-negative, lactic acid-producing cocci

that are resistant to vancomycin and produce CO_2 gas from glucose. These bacteria may be differentiated from the gas-producing lactobacilli by careful scrutiny of Gram-stained smears made from thioglycolate broth. *Leuconostoc* species do not grow at 45°C and are arginine dihydrolase-negative, PYR-negative, and LAP-negative. Production of gas from glucose is best determined in Lactobacillus MRS (Mann-Rogosa-Sharpe) broth (Difco Laboratories, Detroit MI) overlaid with sterile petrolatum. The formation of bubbles under the petrolatum seal indicates gas production and confirms their heterofermentative metabolism. *Leuconostoc* species are further identified by a battery of biochemical tests. Table 13-13 shows the phenotypic characteristics of *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp.cremoris, *L. pseudomesenteroides*, *L. citreum*, *L. lactis*, and *Weissella paramesenteroides* (formerly *L. paramesenteroides*), which are the *Leuconostoc/Weissella* species that have been isolated from human clinical specimens.

Pediococcus species are also intrinsically vancomycin-resistant gram-positive cocci. However, they are generally homofermentative, similar to the streptococci and, unlike *Leuconostoc* species, they do not produce gas from glucose in MRS broth. The organisms appear as gram-positive cocci in pairs, clusters, and tetrads, and they are catalase-negative. Growth occurs over a temperature range of 25°C to 50°C. On plates, they resemble viridans streptococci in their colony morphology. They may be confused with group D streptococci or enterococci because they are bile esculin-positive, they possess the Lancefield group D antigen, and some strains grow in the presence of 6.5% NaCl. Pediococci are PYR-negative and LAP-positive. The species that have been recovered from human infections include *P. acidilactici* and *P. pentosaceus*. These organisms may be difficult to differentiate from one another, with maltose fermentation—positive for *P. pentosaceus* and negative for *P. acidilactici*—being the most reliable test for separation of the two. Both of these species are susceptible to β-lactams, clindamycin, rifampin, erythromycin, gentamicin, and imipenem and are resistant to vancomycin, teicoplanin, and the fluoroquinolones. As mentioned *P. halophilus* is phylogenetically closer to the enterococci and has been reclassified in the genus *Tetragenococcus* as *T. halophilus*. From data given in the original description of this species, *T. halophilus* characteristically grows as pairs or tetrads (hence, the name) and, occasionally, as clusters, does not grow at 10°C or at 45°C, is nonmotile, grows in 6.5% NaCl (actually will grow in up to 10% NaCl) and on BE agar, and is arginine dihydrolase-negative. Most strains produce acid from arabinose, glucose, glycerol, maltose, ribose, sucrose, and trehalose, and do not produce acid from lactose, mannose, mannitol, and sorbitol (Table 13-14). Unlike the other pediococci, *T. halophilus* is susceptible to vancomycin.

Identification of Gemella *Species*

Of the seven *Gemella* species that have been described, five—*G. haemolysans*, *G. morbillorum*, *G. bergeriae*, *G sanguinis,* and *G. asaccharolytica*—have been isolated from human clinical specimens. The two remaining species—*G. palaticanis* and *G. cuniculi*—are found in dogs and rabbits, respectively. *Gemella* species are catalase-negative, gram-positive cocci that characteristically grow as pinpoint colonies on SBA, often requiring 48 hours or more for distinct colonies to grow. Incubation in a CO_2-enriched atmosphere stimulates growth. They are α- or nonhemolytic, except for the animal species *G. cuniculi*, which is β-hemolytic. On Gram-stained smears from broth cultures, the organisms appear as single cells, pairs, or short chains. All *Gemella* species are PYR-positive, LAP-positive, nonmotile, and fail to grow at 10°C or 45°C. They do not grow on BE agar, do not hydrolyze esculin, urea, starch, or gelatin, and do not grow in 6.5% NaCl broth. *Gemella* species can be differentiated on the basis of acid formation from maltose, sucrose, lactose, mannitol, sorbitol, and trehalose. *G. haemolysans* and *G. morbillorum*, the most common clinical isolates, can also be differentiated by reduction of nitrite. Because of their thin cell walls, *G. haemolysans* and *G. morbillorum* easily decolorize on Gram's staining and may appear as gram-negative cocci in pairs, clusters, and short chains. Growth of some strains is enhanced by the presence of fermentable carbohydrates and by the addition of Tween 80 to the growth media. Because of limited numbers of clinical isolates, the performance of kit systems for identifying these bacteria cannot be assessed critically. Only *G. haemolysans* and *G. morbillorum* are included in the databases of the various kit systems at this time. Phenotypic characteristics for identification of *Gemella* species are found in Table 13-15.

Identification of Vagococcus *Species*

Of the seven species in the genus *Vagococcus*, only *V. fluvialis* has been encountered in human clinical specimens. *V. fluvialis* is *Streptococcus*-like in its colony morphology and is α- or nonhemolytic, facultative, and homofermentative in its metabolism, and is catalase-negative. On Gram's stains, the organisms appear as spherical to ovoid cells, arranged in pairs or chains. According to the original description by Collins and coauthors, *V. fluvialis* are motile cocci that produce peritrichous flagella.[210]Motility can be demonstrated with motility medium incubated at room temperature. *V. fluvialis* is PYR-positive, LAP-positive, and hydrolyzes esculin in the presence of 40% bile (Table 13-16).[1012] Most strains grow at 10°C, but growth at 45°C is variable. Most strains also grow in 6.5% NaCl and some strains produce acetoin.[319,1012]

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TABLE 13-13 Phenotypic Characteristics for Identification of *Leuconostoc* Species

Isolated From Human Clinical Specimens

Characteristic	*L. mesenteroides* subsp. *mesenteroides*	*L. mesenteroides* subsp. *dextranicum*	*L. mesenteroides* subsp. *cremoris*	*L. pseudomesenteroides*	*L. citreum*	*L. lactis*	*Weissella paramesenteroides*
Yellow pigment	–	–	–	–	+	–	–
Hemolysis, SBA	–	–	–	–	–	–	–
Growth 10°C	NA	NA	NA	+	+	NA	NA
Growth 37°C	V+	+	+	+	–	+	+
Growth 45°C	–	–	–	–	–	–	–
Esculin hydrolysis	+	V+	–	+	+	V–	V+
LAP	–	–	–	–	–	–	–
PYR	–	–	–	–	–	–	–
Acid Produced From:							
GLU	+	+	+	+	+	+	+
MAL	+	+	V–	+	+	+	+
SUC	+	+	V	V+	+	+	+
LAC	V	+	V	V	–	+	–
MNTL	+	V	–	V–	V	–	+
MANN	+	V	V	+	+	+	+
RAFF	V+	V	V–	V+	–	V+	V–
TRE	+	+	V	+	+	V	+
RIB	V+	NA	V–	+	–	–	+
SAL	+	V	–	V	+	–	V–
STA	–	–	–	+	–	–	–
MEL	+	V	V–	V+	–	V+	+
CELL	V+	V	–	V+	+	–	V
GAL	V+	V	+	V+	V–	+	+
ARAB	V+	–	–	V+	+	–	+
AMYG	V	V	–	V–	V+	–	–

+, positive reaction; –, negative reaction; V, variable reaction; V+, variable reaction but most strains positive; V–, variable reaction but most strains negative; NA, data not available; SBA, sheep blood agar; LAP, leucine amino-peptidase; PYR, pyrrolidonyl arylamidase; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; RAFF, raffinose; TRE, trehalose; RIB, ribose; SAL, salicin; STA, starch; MEL, melibiose; CELL, cellibiose; GAL, galactose; ARAB, arabinose; AMYG, amygdalin.

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TABLE 13-14 Phenotypic Characteristics for Identification of *Pediococcus* and *Tetragenococcus* Species Isolated From Human Clinical Specimens

Characteristic	*P. acidilactici*	*P. pentosaceus*	*T. halophilus*
Growth at 45°C	+	V	–
Novobiocin	S	R	NA
Acid Produced From:			
GLU	+	+	+
MAL	–	+	+
ARAB	V	+	+
STA	–	–	–
GLYC	–	–	+
MELZ	–	–	+
DEX	–	–	–
XYL	+	V	–

+, positive reaction; –, negative reaction; V, variable reaction; S, susceptible; R, resistant; NA, data not available; GLU, glucose; MAL, maltose; ARAB, arabinose; STA, starch; GLYC, glycerol; MELZ, melezitose; DEX, dextrin; XYL, xylose.

V. fluvialis produces acid from glucose, maltose, mannitol, sorbitol, ribose and trehalose, but not from arabinose, inulin, melibiose, or raffinose (Table 13-16).[1012] Strains of *V. fluvialis* have been recovered from pigs, cats, and horses; these strains were positive for the VP, alkaline phosphatase and LAP reactions; some of the strains were nonmotile.[839] Members of the genus *Vagococcus* are not included in the data bases of any of the commercial identification systems. *V. fluvialis* produce positive reactions with the AccuProbe *Enterococcus* test (Gen-Probe, Inc.). Isolates of *V. fluvialis* recovered from human clinical specimens are susceptible to ampicillin, cefotaxime, trimethoprim-sulfamethoxazole, and vancomycin, and are resistant to clindamycin and oflaxacin.[1012] Since the other *Vagococcus* species are environmental or animal isolates, their recovery from clinical specimens is unlikely.

Identification of Alloiococcus *Species,* Globicatella *Species,* Facklamia *Species*, Dolosigranulum *Species,* Ignavigranum *Species, and* Dolosicoccus *Species*

The organisms included in the genus *Alloiococcus* and the genera *Globicatella*, *Facklamia, Dolosigranulum*, and *Ignavigranum* are *Gemella*-like species that are able to grow in 6.5% NaCl (Table 13-17). *A. otitidis*, the sole member of the genus *Alloiococcus*, and has been isolated from tympanocentesis specimens obtained from the middle ear of children with chronic otitis media, from blood cultures, and from sputum.[12,633] The organism grows slowly on agar media, with visible colonies apparent only after 2 to 3 days, and does not grow in thioglycolate broth. Gram-stained smears from agar media show cocci arranged in pairs, tetrads, and sometimes clusters. Optimal growth occurs at 35°C to 37°C, and no growth occurs at either 10°C or 45°C. Colonies appear off-white, pinpoint, and nonhemolytic, although α-hemolysis and slightly yellow pigmentation may be observed after several days of incubation. Fresh clinical isolates of *A. otitidis* will grow on blood agar but may not grow on chocolate agar. Luxuriant growth in broth containing 0.5% Tween or 0.07% lecithin suggests that the organism may require lipid for optimal growth. Unlike the other organisms considered in this chapter, *A. otitidis* is catalase-positive; this reaction may be quite weak or delayed. The organism is aerobic rather than facultative and does not produce acid from any carbohydrates. *A. otitidis* grows on bile esculin agar but does not hydrolyze esculin, and grows slowly in 6.5% NaCl broth. PYR, LAP, and β-galactosidase are produced, and hippurate is hydrolyzed by some isolates. Bosley and colleagues[104] performed antimicrobial susceptibility studies on 19 *A. otitidis* strains and found that all 19 strains were resistant to trimethoprim-sulfamethoxazole, and 18 were resistant to erythromycin. The MICs for penicillin and ampicillin ranged from susceptible to intermediately resistant (MIC range for penicillin, 0.06 to 0.12 μg/mL; ampicillin MIC range, 0.12 to 0.5 μg/mL), with the same degree of relative resistance

being seen for ceftriaxone and cefixime. None of the strains they examined produced β-lactamase enzymes.[104]

G. sanguinis has been recovered from human specimens, including blood, cerebrospinal fluid, and urine. On Gram-stained smears from broth cultures, these organisms appear as gram-positive cocci in pairs and short chains. Colonies on SBA are *Streptococcus*-like and are α-hemolytic. Like the enterococci, *G. sanguinis* is bile esculin–positive, grows in 6.5% NaCl, and produces PYR; unlike both the enterococci and the streptococci, LAP is not produced. *G. sanguinis* grows at 45°C but not at 10°C.[922] These reactions also differentiate *G. sanguinis* from other viridans streptococci in that the latter species are PYR-negative, LAP-positive, and do not grow in 6.5% NaCl broth. The BactiCard Strep (Remel Laboratories), which includes rapid PYR, LAP, and esculin hydrolysis tests in a card format, can be used for these determinations.

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TABLE 13-15 Phenotypic Characteristics for Identification of *Gemella* Species

Characteristic	*G. haemolysans*	*G. morbillorum*	*G. bergeri*	*G. asaccharolytica*	*G. sanguinis*	*G. palaticanis*	*G. cuniculi*
Habitat	Humans	Humans	Humans	Humans	Humans	Dogs	Rabbits
Hemolysis, SBA	None, α	None, α	None, α	α	None, β	NA	NA
LAP	+	+	+	+	+	NA	NA
PYR	+	+	+	–	+	–	–
ESC	–	–	–	–	–	–	–
HIPP	–	–	–	+	–	–	NA
ADH	–	–	–	+	–	–	–
URE	–	–	–	–	–	–	–
PAL	+	–	–	–	+	–	+
VP (acetoin)	–	–	–	–	V	–	–
Reduction of NO_3	–	–	–	–	–	–	–
Reduction of NO_2	+	–	NA	NA	NA	NA	NA
Acid Produced From							
GLU	+	+	+	–	+	+	+
MAL	+	+	–/+^{w}	–	+	+	–
SUC	V	+	–	–	+	+	–
LAC	–	–	–	–	–	+	–
MNTL	–	V+	V	–	+	–	+
SBTL	–	V+	–	–	+	–	+
TRE	–	–	–	–	–	+	–
Production of:							
α-GAL	–	–	–	NA	–	NA	–
β-GAL	–	–	–	NA	–	NA	–
β-GLU	–	–	NA	NA	NA	NA	–
β-GUR	–	–	–	NA	–	NA	–
NAGA	NA	–	–	NA	–	NA	–

+, positive reaction; –, negative reaction; V, variable reaction; +^{w}, weak-positive reaction; –/+^{w}, negative or weak-positive reaction; V+, variable reaction, but most strains positive; V–, variable reaction, but most strains negative; NA, data not available; SBA, sheep blood agar; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; HIPP, hippurate hydrolysis; ESC, esculin hydrolysis; ADH, arginine dihydrolase; URE, urease; PAL, alkaline phosphatase; VP, Voges–Proskauer (acetoin); GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; SBTL, sorbitol; TRE, trehalose; α-GAL, α-galactosidase; β-GAL, β-galactosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase.

TABLE 13-16 Phenotypic Characteristics for Identification of *Vagococcus* Species

Characteristic	*V. fluvialis*	*V. salmoninarum*	*V. lutre*	*V. fessus*
Cellular arrangement	Single cells, pairs, short chains	Single cells, pairs, short chains	Single cells, pairs, short chains	Single cells, pairs, short chains
Hemolysis, SBA	α, γ	α, γ	NA	α
Motility	+	–	+	–
LAP	+	NA	NA	+
PYR	+	NA	NA	NA
ESC	+	+	+	NA
Growth, 6.5% NaCl	+	+	NA	NA
ADH	V	–	–	–
H_2S in KIA	–	+	NA	NA
VP (Acetoin)	V	–	–	–
Production of:				
α-GAL	–	NA	+	–
β-GAL	V	NA	V	V
β-GUR	–	NA	–	–
Acid Produced From:				
GLU	+	+	+	+
MAL	+	+	+	–
SUC	V	+	+	–
LAC	–	–	–	–
MNTL	+	–	–	–
SBTL	+	–	+	–
RIB	+	+	+	–
TRE	+	+	+	–
Habitat	Water, human clinical specimens	Salmonid fish	Otters	Seals, harbor porpoises

+, positive reaction; –, negative reaction; V, variable reaction; NA, data not available; SBA, sheep blood agar; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; ESC, esculin hydrolysis; ADH, arginine dihydrolase; KIA, Kligler's iron agar; VP, Voges–Proskauer (acetoin); α-GAL, α-galactosidase; β-GAL, β-galactosidase; β-GUR, β-glucuronidase; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; SBTL, sorbitol; RIB, ribose; TRE, trehalose.

Unlike the aerococci, *G. sanguinis* produces distinct chains in broth media instead of tetrads. This growth characteristic must be determined to differentiate *G. sanguinis* from *A. viridans*, which, like *G. sanguinis*, is LAP-negative, PYR-positive, and hippurate hydrolysis-positive. *G. sanguinis* produces acid from several carbohydrates, including glucose, maltose, sucrose, lactose, mannitol, raffinose, and trehalose; acid production from arabinose, ribose, and sorbitol is variable (Table 13-17). *G. sanguinis* is in the database of the BBL Crystal Gram-Positive ID kit, but not in the data bases of the API Rapid Strep, the RapID STR, or the ID32 identification systems.[922]Using

NCCLS breakpoints for *Streptococcus* species other than *S. pneumoniae*, Shewmaker and colleagues[922] determined the antimicrobial susceptibility of 28 strains of *G. sanguinis* and found that all strains were susceptible to amoxicillin and vancomycin, and some strains had penicillin MICs that fell in the intermediate range. Variable susceptibility was observed for other agents, including cefotaxime (52% susceptible), cefuroxime (26% susceptible), meropenem (63% susceptible), erythromycin (52% susceptible), trimethoprim–sulfamethoxazole (48% susceptible), clindamycin (70% susceptible), and tetracycline (48% susceptible). The bovine/ovine pathogen, *G. sulfidifaciens*, can easily be differentiated from *G. sanguinis*.[1075] *G. sulfidifaciens* produces H_2S and β-glucuronidase, while *G. sanguinis* does not. *G. sanguinis* also produces β-galactosidase, ferments mannitol, and hydrolyzes hippurate; *G. sulfidifaciens* is negative with these tests.

Members of the genus *Facklamia* are catalase-negative, gram-positive cocci that are related to but phylogenetically distinct from *Globicatella* species. Species isolated from human clinical specimens are *F. hominis*, *F. sourekii*, *F. ignava*,

TABLE 13-17 Phenotypic Characteristics for Identification of *Globicatella, Alloiococcus, Facklamia, Ignavigranum, Dolosigranulum,* and *Dolosicoccus* Species

Characteristic	*G. sanguinis*	*G. sulfidifaciens*	*A. otitidis*	*F. hominis*	*F. ignava*	*F. sourekii*	*F. languida*	*F. tabacinalis*	*F. mirounge*	*I. ruoffiae*	*D. pigrum*	*D. paucivorans*
Cellular arrangement	Pairs, short chains	Single cells, pairs, short chains	Pairs, tetrads, clusters	Pairs, clusters	Single cells, pairs, short chains	Single cells, pairs, short chains	Single cells, pairs, short chains	Single cells, pairs, short chains	Pairs, short chains	Single cells, pairs, clusters	Pairs, tetrads, clusters	Single cells, pairs, short chains
Hemolysis, SBA	α	α	γ	α^w	γ	α	γ	α	α	γ	α	α
Catalase	–	–	+	–	–	–	–	–	–	–	–	–
LAP	–	V	+	+	+	+	+	+	+	+	+	–
PYR	V+	–	+	V+	+	+	+	+	+	+	+	+
ESC	+	NA	–	–	–	–	–	–	–	–	+	–
Growth, 6.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	–
ADH	–	–	–	+	–	–	–	–	+	+	–	–
URE	–	–	–	V	–	–	–	–	+	+	–	–
VP (Acetoin)	–	–	NA	–	–	–	–	–	–	–	–	–
HIPP	+	–	V	+	+	+	–	–	–	–	–	–
PAL	–	–	NA	–	–	V	+	–	V	–	–	–
Production of:												
α-GAL	NA	+	–	V+	–	–	–	+	–	–	–	–
β-GAL	+	–	+	+	–	–	–	–	–	–	V	–
Acid Produced From												
GLU	+	+	–	–	$+^w$	+	+	+	+	$+^w$	+	+
MAL	+	+	–	–	–	+	–	–	–	–	+	$+^w$
SUC	+	+	–	–	–	+	–	+	–	V	–	+
LAC	V+	–	–	–	–	–	–	–	–	–	–	$+^w$
MNTL	+	–	–	–	–	+	–	–	–	V	V	$+^w$
SBTL	V+	–	–	–	–	+	–	V–	–	–	–	–
RAFF	+	+	–	–	–	–	–	–	–	–	–	+
RIB	V+	–	–	–	–	–	–	–	–	–	NA	+
TRE	+	+	–	–	–	+	+	–	+	–	NA	–
GLYG	+	+	–	–	–	–	–	–	–	–	NA	–

+, positive reaction; –, negative reaction; V, variable reaction; V+, variable reaction, but most strains positive; V–, variable reaction, but most strains negative; $+^w$, weak-positive reaction; NA, data not available; SBA, sheep blood agar; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; ESC, esculin hydrolysis; ADH, arginine dihydrolase; URE, urease; VP, Voges–Proskauer (acetoin); HIPP, hippurate hydrolysis; PAL, alkaline phosphatase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; SBTL, sorbitol; RAFF, raffinose; RIB, ribose; TRE, trehalose; GLYG, glycogen.

and *F. languida*. *F. miroungae* and *F. tabacinalis* have been isolated from elephant seals and powdered tobacco, respectively. On gram-strained smears from thioglycolate broth, *Facklamia* species appear as gram-positive cocci arranged in clusters, except for *F. languida*, which usually form pairs and short chains. On SBA, colonies are usually α-hemolytic and similar to viridans streptococci. *Facklamia* species are do not grow on bile esculin media, but do grow in 6.5% NaCl. *Facklamia* species do not grow at either 10°C or 45°C and are LAP-positive and PYR-positive (Table 13-17).

The genus *Ignavigranum*, with its single species, *I. ruoffiae*, was first described in 1999. This species grows primarily as chains in thioglycolate broth and is closely related phylogenetically to both *F. hominis* and *G. sanguinis*. *I. ruoffiae* is PYR-positive, LAP-positive, grows in 6.5% NaCl broth, and does not hydrolyze esculin. Some strains are arginine dihydrolase-positive. While most strains of *F. hominis* are also arginine dihydrolase-positive, this species is hippurate hydrolysis-positive, while *I. ruoffiae* is hippurate-negative (Table 13-17). A distinctive feature of *I. ruoffiae* is the "sauerkraut" odor produced by the organism when grown on SBA.

The genus *Dolosigranulum* contains a single species, *D. pigrum*. On SBA, this organism grows as small, grayish white, α-hemolytic colonies. On Gram's stains from broth (both thioglycolate, and, in one case report, Organon-Teknika BacT/Alert FAN blood culture medium), this organism appears in pairs, *Gemella*-like tetrads, and clusters. (Like *Facklamia* species, *D. pigrum* is PYR-positive, LAP-positive, and grows in 6.5% salt. This organism is esculin hydrolysis–positive, but this reaction may take several days to develop. No growth is observed at 10°C, 45°C, or on BE agar (Table 13-17).

The genus *Dolosicoccus*, with its single member, *Dolosicoccus paucivorans*, is a new group that is based on two isolates recovered from human blood cultures.[228] This organism is distinct from, but phylogenetically related to the *Facklamia* and *Globicatella* genera. Like, the organism discussed above, *D. paucivorans* is PYR-positive, but, unlike these others, it is LAP-negative, does not grow in 6.5% NaCl broth, and appears on broth Gram-stained smears as single cells, pairs, and short chains. Like *D. pigrum*, *D. paucivorans* does not grow on BE medium, and does not grow at 10°C or 45°C (Table 13-17).

Identification of Lactococcus *Species*

Because of the superficial resemblance to enterococci or the viridans streptococci, these organisms have been misidentified as atypical streptococci or enterococci in the clinical laboratory. The lactococci show many of the characteristics of both streptococci and enterococci; many strains are positive for the PYR, LAP, BE, and 6.5% NaCl reactions and are able to grow at 10°C. Lactococci, unlike enterococci, do

not grow at 45°C. A "tip off" to the possibility that an isolate is a possible *Lactococcus* species is the generation of a list of characteristics that do not fit either streptococci or enterococci.[319] Most clinical isolates of lactococci submitted to the CDC have been either *L. lactis* subsp. *lactis, L. lactis* subsp. *Cremoris,* or *L. garvieae.* These three species can be differentiated from common *Enterococcus* species in that these species produce acid from mannitol but do not produce acid from raffinose, sorbitol, or arabinose. *L. garvieae, L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *lactis* are included in the database of the ID32 Strep (Table 13-17). API Rapid Strep, and BBL Crystal identification kits, respectively (see the following section). Genus-specific PCR methods for separating enterococci and lactococci have also been published.[265] In most case reports, both phenotypic methods and sequencing of 16S rRNA and/or the *sodA* gene have been used for species identification.[43,169,340] Phenotypic tests for differentiation of enterococci, lactococci, and similar organisms are shown in Table 13-19.

Commercial Systems for Identification of Streptococci, Enterococci, and Selected "*Streptococcus* -Like" Bacteria

Kit systems that incorporate physiologic and enzymatic tests for streptococcal identification have been of great help in species identification of the viridans streptococci, enterococci, and the group D streptococci. Biochemical test included in the kit systems are used to generate an organism biotype number that corresponds to the identity of the organism. Although these systems include the β-hemolytic streptococci in their databases, these organisms are best handled using other methods, such as the presumptive tests described earlier or the rapid streptococcal grouping kits that are available. However, caution should also be exercised with the commercial identification kits. For example, the API Rapid Strep will misidentify *Leuconostoc* organisms as species of viridans streptococci, so vancomycin resistance and other preliminary test results must be considered along with the biochemical reactions that are observed on the strip. Commercial systems for the identification of streptococci include the following.

Vitek 2

The Vitek 2 (bioMérieux) automated identification/susceptibility testing system uses a gram-positive identification card containing colorimetric substrates for organism identification. The GP card is a 64-well card containing 43 biochemical tests and the

database includes members of both the *Streptococcaceae* (streptococci, enterococci) and the *Micrococcaceae* (staphylococci, micrococci). The card is placed in a cassette of the Vitek 2 instrument and is inoculated using a vacuum-release method. The card is automatically sealed and incubated online at 35.5°C. Wells in the card are subjected to colorimetric scanning every 15 minutes for a total incubation period of 8 hours, although some isolates provide sufficient data for identification after 180 minutes incubation. Algorithms within the machine analyze the biopatterns of interpretable and noninterpreted tests and verify whether sufficient data have accumulated to provide an accurate identification. Ligozzi and colleagues[643] found that the Vitek 2 identified 96.5% of 29 *S. agalactiae*, 96.9% of 66 *S. pneumoniae*, and 83.1% of enterococci (*E. faecalis*, *E. faecium*, *E. durans*, *E. gallinarum*). In a 2005 evaluation, the Vitek-2

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TABLE 13-18 Phenotypic Characteristics for Identification of *Lactococcus* Species

Characteristic	*L. lactis* subsp. *lactis*	*L. lactis* subsp. *cremoris*	*L. lactis* subsp. *hordniae*	*L. garveiae*	*L. plantarum*	*L. raffinolactis*	*L. xyloses*
PYR	V	–	–	+	–	–	–
HIPP	–	+	–	–	–	–	–
ADH	+	–	+	+	–	–	+
Acid Produced From:							
GLU	+	+	+	+	+	+	+
MAL	+	–	–	+	+	+	+
SUC	+	–	+	V	+	–	+
LAC	+	+	–	V	–	+	–
MNTL	+	–	–	+	+	V	+
SBTL	–	–	–	–	+	–	–
RAFF	–	–	–	–	–	+	–
TRE	+	–	+	+	+	–	+

+, positive reaction; –, negative reaction; V, variable reaction; PYR, pyrrolidonyl arylamidase; HIPP, hippurate hydrolysis; ADH, arginine dihydrolase; GLU, glucose; MAL, maltose; SUC, sucrose; LAC, lactose; MNTL, mannitol; SBTL, sorbitol; RAFF, raffinose; TRE, trehalose.

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TABLE 13-19 Differentiation of *Lactococci*, *Enterococci*, and Similar Organisms

Characteristic	*Lactococcus* Species			*Enterococcus* Species				
	L. garvieae	*L. lactis* subsp. *lactis*	*L. lactis* subsp. *cremoris*	*E. faecalis*	*E. faecium*	*Vagococcus*	*Globicatella*	*Leuconostoc*
BE agar	+	+	+	+	+	+	V	V
PYR	+	V	–	+	+	+	+	–
LAP	+	+	+	+	+	+	–	–
Growth, 10°C	+	+	+	+	+	+	V	V
Growth, 45°C	V	V	V	+	+	–	V	V
Motility	–	–	–	–	–	+	–	–
Acid Produced From:								
MNTL	+	+	–	+	+	+	+	V
SBTL	–	–	–	+	V	+	V	V
ARAB	–	–	–	–	+	–	V	V
Vancomycin	S	S	S	Usually S	Usually S	S	S	R

+, positive reaction; –, negative reaction; V, variable reaction; S, susceptible; R, resistant; BE agar, bile esculin agar; PYR, pyrrolidonyl arylamidase; LAP, leucine aminopeptidase; MNTL, mannitol; SBTL, sorbitol; ARAB, arabinose.

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GP identification card identified 92% of 153 streptococcal species (including all of 38 *S. pyogenes* and all of 39 *S. pneumoniae* isolates) and 97% of 64 *Enterococcus* species. All 13 isolates of viridans group streptococci were correctly identified. Identifications were obtained within 7 hours of incubation for over 90% of isolates tested.[363] In another evaluation by Wallet and colleagues, the colorimetric GP card correctly identified 84.8% of 132 streptococcal and enterococcal isolates. This latter study included several strains of viridans group streptococci, enterococci, and "*Streptococcus*-like" isolates (e.g., *Gemella* species, *Helcococcus* species, *Pediococcus* species).[1104] A study of the Vitek-2 by Abele-Horne et al.[7] reported that 94.2% of 121 *Enterococcus* species were correctly identified. In a comparison of enterococcal identification with the Bruker and bioMerieux MALDI-TOF MS instruments, the Vitek 2 GP card identified 131 of 132 enterococci, including 31 of 32 *E. faecalis*, 63 *E. faecium*, 16 *E. casseliflavus*, and 21 *E. gallinarum*.[328] Haanpera et al.[411] identified a collection of α-hemolytic streptococci, (102 blood culture isolates, 39 isolates from oropharyngeal cultures, and 17 invasive *S. pneumoniae* isolates) by genotypic methods and Vitek 2 and found that 75.0% of the isolates were assigned to the same streptococcal species by both methods and 46.0% reached consistent results at the species level. Ten strains remained unidentified by Vitek 2 and 4 isolates could not be assigned to any streptococcal group.[411] When compared with genotypic and molecular identification methods, the Vitek 2 system cannot adequately identify some viridans streptococci. Among 251 viridans streptococci isolated from blood cultures, 220 (87.6%) were assigned to the same species group by both methods, and only 67 of the 150 strains in the Mitis Group resulted in the same species identification by both methods. Fifteen pyogenic cocci and 54 *E. faecalis* isolates were correctly identified by both methods.[5] Teles and colleagues[1014] found that the Vitek 2 identified only 55%

of 42 viridans streptococcal isolates when compared to genotypic identification methods (i.e., *sodA* gene sequencing, restriction fragment length polymorphism of the 16S rRNA genes, and multilocus sequence analysis of seven housekeeping genes). A study by Mittman and colleagues[719] with 311 isolates of *S. pneumoniae* found that the Vitek 2 and the Phoenix system (see the following subsection) were comparable in the ability to identify *S. pneumoniae* and were preferable to routine phenotypic tests with respect to accuracy and time savings. A meta-analysis of studies evaluating the Vitek 2 found that the overall correct identification rate for the instrument was 96.1% for the streptococci and 99.70% for the enterococci.[177]

Phoenix (Becton-Dickinson Diagnostic Systems, Sparks, MD)

The BD Phoenix system is an automated identification and susceptibility test system that uses an ID panel called PMIC/ID-13 for identification of gram-positive cocci.[325] The panel contains 45 wells containing 20 enzymatic substrates, 16 carbohydrates utilization tests, and 7 assimilation carbon sources, and susceptibility to colistin and polymyxin B. A standardized suspension of the bacterial isolate is prepared in broth and poured into the panel within 30 minutes of preparation. The panel is then loaded into the instrument where it is incubated and read by the optics of the instrument. Time to providing an identification ranges from 3 to 4 hours. In a study of 179 enterococcal isolates and 15 streptococcal isolates, the Phoenix system identified 98.9% of the enterococci and 100% of the 15 streptococci. With regard to pyogenic cocci in particular, Kanemitsu et al.[534] found that the Phoenix accurately identified 85.9% of 92 *S. pneumoniae*, 95.8% of 24 *S. pyogenes*, and 90% of 10 *S. agalactiae* isolates. A second evaluation of the BD Phoenix also conducted in Japan found results similar to Kanemitsu et al. for the pyogenic species, but found that the system identified only 58% of 12 *S. mitis* group organisms and 82% of 22 *S. anginosus* group isolates.[457] Brigante and colleagues[119] compared identifications obtained by a manual identification kit (API Strep) and sequencing of 16S rRNA and three housekeeping genes with those obtained with the Phoenix system. The Phoenix system correctly identified 93.8% of 129 streptococci, 90% of 70 enterococci, 100% of 45 *S. pneumoniae*, and 49 of 50 β-hemolytic streptococci. For the viridans streptococci, the Phoenix identified 83.9%, with accurate identification of the *S. sanguinis* and *S. anginosus* groups, and less accurate identifications of *S. mitis* group isolates. Carroll and coworkers[140] found that the Phoenix identified 100% of 90 *Enterococcus* species, including *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. gallinarum*, and *E. raffinosus*. The meta-analysis of these automated systems reported that the overall accuracies of the Phoenix system for identification of streptococci and enterococci were 96.70% and 98.27%, respectively.[177]

API Rapid Strep. This strip format system (bioMerieux, Inc.) includes 20 tests,

including physiologic tests, chromogenic enzyme substrate tests, and carbohydrate utilization tests. The unique thing about this system is that the strip can be read twice. If an identification is not obtained after 4 hours' incubation using the physiologic and chromogenic substrates, the strip is reincubated and read after overnight incubation to include acid production from 10 carbohydrates. The database includes groupableβ-hemolytic streptococci, viridans streptococci, *S. gallolyticus* strains, *Enterococcus* species (*E. faecalis*, *E. faecium*, *E. gallinarum*, *E. avium*, and *E. durans*), viridans streptococci, lactococci, aerococci, *Gemella* species, and *Listeria monocytogenes* (Image Plate 13-3F and13-3G). Identification of strains that have been completely characterized by conventional biochemical tests and genetic analysis (e.g., DNA relatedness, 16S rRNA sequencing) indicate that the API Rapid Strep system is reproducible and complements existing genetic studies. The phenotypic reactions for many organisms shown in the tables of this chapter reflect the recent studies during which genetic analysis and the description of new species were performed along with API Rapid Strep characterization. In a 2011 evaluation of the ID32 Strep compared with the Vitek 2 and genotypic methods, the ID32 identified 79% of 23 clinical and 19 type strains of viridans streptococci without the need for additional tests.[1014] In a 2013 study of 27 *S. anginosus* group isolates, the API Rapid Strep correctly identified only 40.7%.[174] A 2012 study comparing API 20 Strep with genotypic methods and MALDI-TOF MS found that the manual system identified only 60.5% of viridans streptococcal isolates.[658]Percentages of correct identifications varied from as low as 36.5% for the Anginosis Group to 75% for the Salivarium and Bovis Groups of streptococci. Davies and colleagues[261] compared

the API 20 Strep system with the Bruker MS and found that only 26% 49 viridans streptococcal isolates were correctly identified to species level by API 20 Strep. At this time, the database for this system has not been updated or expanded to include newer streptococcal and enterococcal species.

Rapid ID 32 Strep. This system (bioMerieux, La Balme les Grottes, France) is a 32-test strip format identification system for the streptococci and streptococci-like bacteria. The database of this system is extensive and includes the groupable streptococci, several *Enterococcus* species, and the viridans streptococci (including newly described species such as the "mutans group" oral streptococci, *S. oralis*, *S. gordonii*, and *S. vestibularis*). A suspension of the organism is prepared in sterile water to a turbidity equivalent to a McFarland 4 standard and 55 µL of the suspension is placed into each of the 32 cupules. The strip is incubated at 35°C to 37°C for 4 hours and either read manually using a code book or with the automated reader (ATB 1520 Reader linked with an ATB 1545 computer, bioMerieux). In an evaluation of the

Rapid ID 32 Strep system with 433 strains belonging to the genera *Streptococcus*, *Enterococcus*, *Lactococcus*, *Aerococcus*, *Gemella,* *Leuconostoc*, *Erysipelothrix*, *Gardnerella*, and *Listeria*, 95.3% of strains were correctly identified.[358] Additional tests were required for identification of 25.1% of the strains. Only 16 isolates (3.7%) were not identified, and only four isolates (1.0%) were misidentified. Another evaluation by Kikuchi and coworkers[555] reported that the ID32 Strep system identified 87% of 156 viridans streptococcal strains and that most misidentification involved strains of *S. oralis, S. mitis*, and *S. gordonii*. Chang and Lo found that the Rapid ID 32 Strep identified 77.8% of 27 isolates belonging to the Anginosus Group, outperforming both the API Strep and the Vitek 2 GP card.[173] The Rapid ID 32 Strep system does not include several genera of the *Streptococcus*-like bacteria in its database and correctly produces "unacceptable identifications" when strains belonging to these species are tested.[591]

BBL Crystal Gram-Positive Identification System. The Crystal Gram-Positive identification system contains 29 dried biochemical and enzymatic substrates plus a fluorescence negative control. The inoculum is prepared by suspending colonies of the pure isolate in the fluid provided with the kit to a turbidity equivalent to a 0.5 McFarland standard. The suspension is then poured into a specific area of the base of the panel, which is then manipulated to distribute the inoculum. The panel lid, which contains the test substrates, is then snapped onto the base, with consequent hydration of the substrates with the inoculum. After incubation, tests are read for fluorescence or for changes in the colors of pH indicators using a special panel viewer. The resulting pattern of the 29 reactions is converted into a 10-digit profile number that correlates with an identification provided by the Crystal Electronic Code book. The panel can be interpreted using a 4-hour or an 18- to 24-hour incubation database. In a 1998 evaluation of this system, the Crystal Gram-Positive identification system identified all strains of groupable β-hemolytic streptococci and *S. pneumoniae*, but only identified 25 of 37 strains of viridans streptococci to the species level. The system correctly identified all of 23 *E. faecalis*, 2 of 2 *E. avium*, and all of 14 *E. faecium*.[1100] Hudson and coworkers[480] challenged the system with genotypically defined veterinary strains of enterococci and found that repeated testing of the same strains resulted in different profile numbers, some of which resulted in different identifications. In fact, 19 of the 50 strains tested were identified at different times as both *E. faecalis* and *E. faecium*. This system cannot identify or misidentifies isolates belonging to the "*Streptococcus*-like" bacterial genera.[591]

RapID STR. The RapID STR system (Remel Laboratories) employs a small cuvette containing 10 wells, four of which are bifunctional, resulting in a total of 14 biochemical tests (used in conjunction with the hemolytic reaction of the isolate). Tests include physiologic determinations (arginine dihydrolase, esculin hydrolysis),

carbohydrate utilization tests, and chromogenic enzyme substrate hydrolysis. The system is inoculated with a suspension of the organism (McFarland 1 turbidity standard) prepared in a fluid purchased with the kit, and the tests are read after 4-hour incubation at 35°C in a non-CO_2 incubator. The database of this system includes the β-hemolytic streptococci group A, B, and C/G, group D streptococci, the Anginosus Group, *Enterococcus* species (*E. faecalis*, *E. faecium*, *E. avium*, *E. cassiliflavus/mundtii*, *E. durans/hirae*, *E. gallinarum*, *E. raffinosus*, and *E. malodoratus*), viridans streptococcal species (*S. mitis*, *S. mutans*, *S. salivarius/vestibularis*, *S. sanguinis/gordonii*), *S. pneumoniae*, *Aerococcus* species, *Gemella morbillorum*, *Leuconostoc* species, *Lactococcus* species (*L. lactis*, *L. mesenteroides* group) and *Pediococcus* species (*P. acidilactici*, *P. pentosaceus*). More recently described viridans streptococci, enterococci, and *Streptococcus*-like bacteria are not included in the database. In a study by Shewmaker et al.[922] on phenotypic characterization of 28 *G. sanguinis* strains, the RapID STR misidentified 7 strains as *S. mutans* (5 strains), *E. casseliflavus* (1 strain), or *E. malodoratus* (1 strain), while the remaining strains gave inadequate profiles for identification. Another evaluation found that this system was unable to identify isolates belonging to the "*Streptococcus*-like" bacterial genera (*D. pigrum*, *I. ruoffia, Facklamia* species), indicating the need for database revision and updating.[591]

Microscan Gram-Positive Breakpoint Combo Panel. This 18-hour system provides simultaneous identification and antimicrobial susceptibility results in a microtiter format. Tritz and colleagues[1044] evaluated this system for its ability to identify enterococci and found that both *E. faecalis* and *E. faecium* were reliably identified, but less common enterococcal species were misidentified.

Matrix-Assisted Laser-Desorption/Ionization Time-of-Flight Mass Spectrometry. MALDI-TOF has been evaluated in several studies for the identification of streptococci, enterococci, and *Streptococcus*-like bacteria. The Bruker Biotyper MS instrument correctly identified 61 of 65 *S. pyogenes* isolates when compared to phenotypic methods (API 20 Strep).[1106] In a study of 76 streptococcal and 50 enterococcal isolates from the routine microbiology laboratory, MALDI-TOF identified 88% and 79% of the isolates correctly.[69] A 2012 study compared the identifications obtained with the MALDI Biotyper (Bruker Daltronics, Bruker Daltronics GmbH, Bremen, Germany), the BD Phoenix system, and the API 20 Strep for 56 α-hemolytic streptococcal isolates.[261] Using 16S rRNA gene sequence analysis as the gold standard, MALDI-TOF, BD Phoenix, and API 20 Strep identified 46%, 35%, and 26% of the isolates, respectively. Fang

and colleagues[328] in Sweden compared identifications obtained with both the Bruker and Vitek MS, the Vitek 2, and multiplex PCR and found that 100% of 132 enterococci were identified correctly by both mass spectrometry instruments, while 121 of the 132 isolates were correctly identified to species by Vitek 2. The ability of the MALDI-TOF to differentiate *S. pneumoniae* from nonpneumococcal streptococci has been studied.[1116] Early studies of *S. pneumoniae* and other *S. mitis* group streptococci (i.e., *S. mitis*, *S. oralis,* and *S. pseudopnuemoniae*) found that discrimination of these species was difficult because of very high degree of similarities in the spectra generated using MALDI-TOF. Analysis of minor mass spectral peaks using the Bruker MS instrument and defining these peaks in the spectral database resulted in discriminatory sensitivity and specificity for these species of almost 100%.[491] A study performed in Spain compared MALDI-TOF identifications with those obtained with API 20 Strep for 124 α-hemolytic streptococcal isolates obtained from blood cultures.[658] Using *sodA* gene sequencing as a reference method the API 20 Strep and MALDI-TOF provided correct species-level identifications for 60.5% and 73.4% of the isolates, respectively. The turnaround times for identifications obtained by *sodA* sequencing, API 20S, and MALDI-TOF were 12 to 24 hours, 24 to 48 hours, and 15 minutes, respectively. In a large study conducted in France, the Vitek MS MALDI-TOF instrument was evaluated for the identification of 334 *S. pneumoniae* isolates, 166 other *S. mitis* group strains, 184 non- *S. mitis* group streptococci, and 19 other α- and nonhemolytic *Streptococcus*-like bacteria.[299] Reference identification methods include optochin susceptibility, bile solubility, serotyping, and the Rapid ID32 Strep kit. Discordant results were arbitrated with genotypic methods (i.e., 16S rRNA and *recA* gene sequencing). The Vitek MS system identified 99.1% of the pneumococci, with only three bile-insoluble isolates being misidentified as *S. mitis/oralis*. Of the nonpneumococcal isolates, 90.8% were correctly identified to the species or subspecies level. Interestingly, three *S. pseudopneumoniae* isolates were identified by the Vitek system. A collaborative, multicenter US study compared the Vitek MS with nucleic acid sequence-based identifications for 1,146 isolates representing 13 genera and 42 species of gram-positive aerobic and facultative cocci, including 134 enterococci and 218 streptococci.[880] The Vitek MS identified 97% of the enterococcal isolates and 82% of the streptococci to the species level. Among the streptococci, 2%, and 5% of the 218 isolates were misidentified or not identified by the Vitek MS, respectively. In this study, only 51% of 47 *S. dysgalactiae* isolates were correctly identified to the species level. The limitation for correct identification of *S. dysgalactiae* has been noted in other studies.[908]

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CHAPTER 14

Aerobic and Facultative Gram-Positive Bacilli

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Isolation and Identification of *Lactobacillus* Species
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***Weissella* Species**

The aerobic/facultative gram-positive bacilli include a wide variety of organisms that are responsible for "classical" diseases such as listeriosis, anthrax, and diphtheria, and also for newer disease syndromes, particularly in immunocompromised hosts. The aerobic/facultative gram-positive bacilli also include several newly recognized genera that are environmental or veterinary organisms not previously associated with humans or human infections. The use of molecular techniques for characterization of many new species has required reexamination of classical pathogens as well. For example, the recognition of several new facultative species in the genus *Actinomyces* has enabled recognition of the facultative nature of classical pathogens such as *A. israelii* that were previously considered to be obligately anaerobic organisms. These new techniques have also permitted the classification of previously unnamed bacteria, such as the Centers for Disease Control and Prevention (CDC) coryneform groups, into new or existing genera, and has led to the taxonomic dissection of genera known to contain many unrelated species (e.g., the genus *Bacillus*). The classification scheme presented in the new edition of *Bergey's Manual of Systematic Bacteriology* now reflects the genotypic relationships rather than phenotypic similarities among these organisms. Box 14-1 presents the classification of the organisms to be discussed in this chapter.

Listeria Species and *Listeria monocytogenes*

Taxonomy of the Genus Listeria

The genus *Listeria* consists of gram-positive, non–spore-forming, facultatively anaerobic, regular rod-shaped bacteria (see Image Plate 14-1A). The genus is defined by a G+C%

BOX 14-1

Classification of the Aerobic/Facultative Gram-Positive Bacilli in the Domain Bacteria

Phylum	Class/Subclass	Order/Suborder	Family	Genera
Firmicutes	Mollicutes	Incertae sedis	*"Erysipelotrichaceae"*	*Erysipelothrix*
	"Bacilli"	Bacillales	*Bacillaceae*	*Bacillus, Alkalibacillus, Amphibacillus, Anoxybacillus, Cerasibacillus, Filobacillus, Geobacillus, Gracilibacillus, Halobacillus, Halolactibacillus, Lentibacillus, Marinococcus, Oceanobacillus, Paraliobacillus, Pontibacillus, Saccharococcus, Tenuibacillus, Thalassobacillus, Virgibacillus*
			"Listeriaceae"	*Listeria, Brochothrix*
			"Sporolactobacillaceae"	*Sporolactobacillus, Sinobaca, Tuberibacillus*
			"Paenibacillaceae"	*Paenibacillus, Ammoniphilus, Aneurinibacillus, Brevibacillus, Cohnella, Oxalophagus, Thermobacillus*
			"Alicyclobacillaceae"	*Alicyclobacillus, Sulfobacillus*
		"Lactobacillales"	*Lactobacillaceae*	*Lactobacillus, Paralactobacillus, Pediococcus*
			Incertae sedis	*Exiguobacterium*
Actinobacteria	Actinobacteria/ Actinobacteridae	Actinomycetales/ Actinomycineae	*Actinomycetaceae*	*Actinomyces, Actinobaculum, Arcanobacterium, Trueperella, Mobiluncus*
		Actinomycetales/ Micrococcineae	*Micrococcaceae*	*Rothia*
			Brevibacteriaceae	*Brevibacterium*
			Cellulomonadaceae	*Cellulomonas, Cellulosimicrobium, Oerskovia*
			Dermabacteriaceae	*Dermabacter, Helcobacillus*
			Intrasporangiaceae	*Sanguibacter*
			Jonesiaceae	*Jonesia*
			Microbacteriaceae	*Microbacterium (Aureobacterium), Leifsonia*
		Actinomycetales/ Corynebacterineae	*Corynebacteriaceae*	*Corynebacterium*
		Bifidobacteriales	*Bifidobacteriaceae*	*Gardnerella*
			"Unknown affiliation"	*Turicella*

DNA content of 36% to 38%, a typical gram-positive cell wall with a peptidoglycan murein layer containing *meso*-DAP attached to the cell membrane by lipoteichoic acid, and membrane-associated polyribitol teichoic acids. Genetic studies indicate that *Listeria* organisms exhibit the closest phylogenetic relationship to the genus *Brochothrix* (an environmental organism) and are more distantly related to organisms in genus *Bacillus,* related spore-forming genera, and the genus *Lactobacillus*, rather than to organisms in the genera *Streptococcus*, *Enterococcus*, and *Lactococcus.* The relationship between *Listeria* and *Brochothrix* species is also supported by chemotaxonomic data. Although some taxonomists have favored the inclusion of *Listeria* species in the family *Lactobacillaceae*, Collins and other workers believe that

Listeria species merit inclusion in a new family. In the current edition of *Bergey's Manual of Systematic Bacteriology*, *Listeria* and *Brochothrix* species are included in the proposed family "*Listeriaceae*" in the order *Bacillales*, class "*Bacilli*" in the phylum *Firmicutes*.[695] The genus *Erysipelothrix* is included in the phylum *Firmicutes* along with *Listeria* species, but is currently classified as species incertae sedis (Box 14-1). Table 14-1 presents the major characteristics that differentiate *Listeria*, *Erysipelothrix, Lactobacillus*, and *Brochothrix* from one another.

Genus *Listeria* includes *L. monocytogenes*, *L. ivanovii* subsp. *ivanovii*, *L. ivanovii* subsp. *londoniensis*, *L. seeligeri*, *L. innocua*, *L. grayi*, *L. welshimeri*, *L. marthii*, *L. rocourtiae,* *L. fleischmannii,* and *L. weihenstephanensis*.[99,122,433,633,648,996] *L. rocourtiae* was initially isolated from precut lettuce in Salzburg, Austria, in 2002.[648] *L. marthii* was originally isolated from river water, soil, puddles, and run-off environmental samples collected in the Finger Lakes National Forest and the Connecticut Hill Wildlife Management Area in New York State.[433] The newest species in the genus, *L. fleischmannii* and *L. weihenstephanensis*, were isolated from Swiss cheese and from a water plant (*Lemna trisulca*) in a freshwater pond in Bavaria, Germany, respectively.[99,633] *L. monocytogenes* can be divided into 13 serotypes based on somatic (S) and flagellar (H) antigens. Among the 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b/4bX, 4c, 4d, 4e, and 7), most diseases are due to types 4b, 1/2a, and 1/2b. *L. monocytogenes* and *L. ivanovii* are closely related pathogenic species, while *L. innocua*, *L. welshimeri*, *L. seeligeri*, *and L. marthii* are regarded as nonpathogens. *L. grayi* is distantly related to the other *Listeria* species, and in 1974, a proposal of a new genus, *Murraya*, to accommodate *L. grayi* was made but never approved.[1063] *L. monocytogenes* can infect a wide range of hosts, while *L. ivanovii* is largely a pathogen of sheep.

Virulence of L. monocytogenes

L. monocytogenes is a facultative intracellular pathogen that is able to adhere to, invade, and survive within mammalian cells, including macrophages and several human tissue culture cell lines.[782] This organism employs several cellular proteins that belong to a family known as the **internalins.**[108] These proteins are members of the LRR (leucine-rich repeat) protein superfamily. The two major proteins that *L. monocytogenes* employs are internalin A and internalin B. The receptor for internalin A on human epithelial cells is **E-cadherin,** while the receptor for internalin B is the globular portion of the complement component C1q. These interactions result in induction of organism phagocytosis by cells that are not usually phagocytic cells.[741] Cadherins are 110-kDa transmembrane glycoproteins that are structurally related and

tissue specific. Experiments using several different cell lines have established that the internalin A/E-cadherin interaction promotes both specific binding and entry of *L. monocytogenes* into epithelial cells.[741] Recent work has established that other cellular proteins, such as clathrins (which function in the endocytosis of macromolecules) and septins (GTP-binding proteins involved in cell division), are also involved in the internalization of *L. monocytogenes*.[1151] Once sequestered within the phagosomes, the organisms produce **listeriolysin O** and several phospholipases that enable the organism to escape from the phagosome before lysosomal fusion, thereby avoiding intracellular killing. Listeriolysin O is encoded by the *hly* gene and is a pore-forming hemolysin that is similar to streptolysin O of group A streptococci.[987] Listeriolysin O acts by binding to membrane cholesterol and then inserting itself into the target membrane, resulting in the formation of transmembrane pores. These pores inhibit lysosome fusion by altering pH and intracellular calcium levels.[1005] Once within the cytosol, another listerial surface protein, **ActA**, promotes polymerization of actin to form actin "tails," which enable the organism to be actively motile and spread directly from cell to cell.[424] At this point, other mechanisms that promote intracellular survival also become

TABLE 14-1 Phenotypic Characteristics for Differentiation of *Listeria, Brochothrix, Erysipelothrix,* and *Lactobacillus*

Genus	Growth at 35°C–37°C	Catalase	H_2S in KIA	Motility	Acid From Glucose	Peptidoglycan Di-Amino Acid
Listeria	+	+	–	+[a]	+	meso-DAP
Brochothrix	–	+	–	–	+	meso-DAP
Erysipelothrix	+	–	+	–[b]	+	L-lysine
Lactobacillus	+	–	–	–	+	L-lysine or meso-DAP or L-ornithine

[a] All strains motile at 20°C–25°C; poorly motile or nonmotile at 35°C–37°C.
[b] Most species are nonmotile, although a few motile species have been described.
+, positive reaction; –, negative reaction; KIA, Kligler's iron agar.

involved. These include the production of peptidoglycan-modifying enzymes by the organism that deacetylate D-glucosamine residues in the bacterial cell wall, rendering them resistant to lysis by intracellular lysozyme, and ActA-mediated inhibition of the normal autophagic enzymes involved in normal turnover of cellular constituents.[126,783,1236] These mechanisms enable the organism to move directly from cell to cell without exposure to soluble immune factors such as antibodies and complement.

Epidemiology of L. monocytogenes

Although all *Listeria* species may be isolated from the environment and from a wide variety of animals, both as pathogens and as commensals, only *L. monocytogenes* is a well-recognized animal and human pathogen. This organism can be isolated from soil, water, sewage, and vegetation and as a part of the fecal flora of a wide variety of animals, including rodents, rabbits, sheep, and ruminants.[1074] Infections in these animals include sepsis, rhombencephalitis, prematurity, and abortion. Many foods are contaminated with *L. monocytogenes,* and the organism can be recovered from 15% to 70% of raw milk samples, cheese, raw vegetables, and meats, including processed chicken and beef products from delicatessen counters. *L. monocytogenes* is able to grow in biofilms on the surface of various foods, and refrigeration actually augments further organism growth because of the ability of the organisms to grow at 4°C. Because of their ubiquity in foods, humans probably have contact with these organisms on a daily basis, and as a result, some individuals become fecal carriers of *L. monocytogenes.* The organism is a transient constituent of the human intestinal flora and may be excreted in the feces by 3.5% to 5% of healthy humans.[438,1074] Human listeriosis occurs mostly on the spring and summer months, and sporadic cases (outside of food-borne outbreaks) occur at annualized rates of less than 1 case per 100,000 population. In the year 2000, listeriosis was made a nationally reportable illness. With the development and application of food industry regulations formulated to minimize risks of food-borne disease, incidence rates in the United States declined from 7.4 per million population in the mid-1980s to 4.4 cases per million by 1993.[207,1093]

Clinical Significance of L. monocytogenes

L. monocytogenes is associated with a spectrum of clinical syndromes.[24,736,788,789,879] The most common result of organism acquisition is a transient asymptomatic gastrointestinal carrier state that usually results from ingestion of contaminated food. During this time, the organism may be excreted in the feces. Acute symptomatic infection with this organism often occurs during pregnancy. *Listeria* infection is 18 times more common in pregnant than nonpregnant females, and 16% to 27% of all *L. monocytogenes* infections occur in pregnant women.[541,543] Pregnancy-associated infection usually occurs during the third trimester, although cases have occurred at earlier gestational ages.[631] About two-thirds of patients usually develop mild, influenza-like symptoms of fever, malaise, sore throat, myalgias, lower abdominal pain, and back pain.[788] Occasionally, a vaginal discharge, diarrhea, and urinary tract symptoms are noted. Peripheral leukocytosis may be present, and blood cultures collected during the acute phase of this transient illness may be positive. During

maternal infection, occult bacteremia and transplacental transmission of the organism may occur, leading to intrauterine infection of the fetus. Infection of the fetus in utero may induce labor, resulting in spontaneous abortion in 10% to 20% of cases. Over 50% of infants infected in utero are delivered preterm, and intrauterine fetal death may occur in about 11%.[736] Symptoms in the mother usually subside following the delivery of the infected infant and the placenta. Fetal survival is partly determined by the length of gestation, with spontaneous abortion occurring when the infection is acquired early in pregnancy, and neonatal infection resulting when the infection is acquired later.[788]

Neonates become infected either by inhalation and ingestion of infected amniotic fluid in utero, by transplacental infection via the maternal circulation, or by ascending infection from the vagina. The hematogenous route is probably the most common avenue of neonatal infection. Infection of the neonate occurs in about 8.6 cases of every 100,000 live births.[186] In more than half of the cases, the mother is likely to have had the antecedent flu-like syndrome, and in 44% to 89% of cases, *L. monocytogenes* may be isolated from blood or cervicovaginal cultures.[631,677,736] Like that of group B streptococci, there are two forms of the disease in infected neonates that are categorized as either "early onset" or "late onset." The symptoms of early-onset disease generally occur with 36 to 48 hours after birth. Newborns who are infected in utero generally present with acute sepsis with positive blood cultures in 80% to 90%, respiratory distress with pneumonia in about 38%, and meningitis in about 24% of cases.[788,789] Pustular skin lesions and inflammatory granulomas containing *L. monocytogenes* may be found in the brain, liver, kidneys, lung, and spleen ("granulomatosis infantisepticum"). Placental tissues from these infections usually show evidence of acute chorioamnionitis and the presence of microabscesses.[1112] The mortality rate of early-onset listeriosis may be as high as 20% to 60%, but fatal neonatal infections are more common among immunocompromised children.[726,736,788] Listeriosis in infants may also occur as "late-onset" infections. These infections become clinically apparent between 5 days and 2 or more weeks after delivery, and infants with this condition are usually born at term. Late-onset listeriosis usually presents as neonatal meningitis, with signs and symptoms of fever and irritability.[879] Gram's stains of cerebrospinal fluid (CSF) will show polymorphonuclear leukocytes along with high protein and low glucose indices; the gram-positive rods of *L. monocytogenes* will usually be seen in over 50% of cases. Neonatal blood cultures are positive in 17% to over 90% of cases of late-onset disease. Postpartum transmission or nosocomial acquisition is assumed to occur in infants with late-onset disease. Usually, the mother has had an uncomplicated pregnancy, with no signs of infection (e.g., fever) or sepsis (e.g., positive blood cultures) being present.

Nonpregnant adults may also become infected with *L. monocytogenes*, and those

individuals with compromised immune systems are at greater risk for severe, life-threatening listeriosis. Adults with acute listerial sepsis usually are greater than 60 years old and have underlying malignancies or are otherwise immunocompromised (e.g., lymphoma,

organ transplantation, diabetes, chronic liver/renal disease, collagen-vascular disease, alcoholism, HIV infection, and AIDS).[407,849] Persons who are receiving treatment with immunosuppressive agents such as tumor necrosis factor inhibitors and corticosteroids are also at increased risk.[467] Immunocompromised patients usually present with acute sepsis, subacute meningitis, meningoencephalitis, or, rarely, rhombencephalitis.[736] Positive blood cultures and clinical findings similar to sepsis associated with gram-negative bacteremia are usually observed. In some patients, the organisms cross the blood–brain barrier, resulting in infection of the meninges, the ventricles, and the brain.[88,788] Meningitis is usually seen in neonates, the elderly, and patients who are immunosuppressed. Oftentimes, central nervous system (CNS) infection is subacute, develops over several days, and is characterized by low-grade fever, headaches, and nuchal rigidity. The Gram's stain of the CSF is positive in less than 40% of patients. CSF cell counts are generally lower, and in 60% of cases, CSF glucose levels are normal. The protein levels in the CSF are usually elevated, with higher levels correlating with a poorer prognosis.[879] Meningoencephalitis caused by *L. monocytogenes* is associated with several unique features. Nuchal rigidity is less common, being found in 80% to 85% of adults. Motor disorders, such as ataxia, tremors, cranial nerve palsies, and seizure activity, are more common with listerial infection than with other etiologic agents of meningoencephalitis.[736,788,999] The development of ventriculitis may necessitate insertion of extraventricular drains to treat acute hydrocephalus and brain edema.[88] Fluctuating mental status is another clinical feature in these patients. In some cases, CNS listerial infection may progress to include brainstem involvement (i.e., rhombencephalitis), and imaging studies may show microabscesses in the diencephalon and cerebellum.[999] Rhombencephalitis usually occurs in previously healthy individuals without predisposing or underlying conditions, and presents with a prodrome of nonspecific CNS symptoms (e.g., nausea, vomiting, and fever) for 5 to 10 days, followed by development of brain stem involvement as evidenced by asymmetric cranial nerve palsies, sensory deficits, respiratory depression, impaired consciousness, and seizure activity.[811,1131] Brain abscesses, both solitary and multiple, may occur as complications of listerial meningoencephalitis and rhombencephalitis.[260,1161] Abscesses are usually found in subcortical regions of the brain parenchyma (i.e., the thalamus, pons, and medulla), while spinal cord abscesses

are rare.[788] Vertebral osteomyelitis due to *L. monocytogenes* may develop as a sequelae of spinal abscesses.[590] Diagnosis of meningoencephalitis and rhombencephalitis is established by culture of CSF and blood specimens. Listerial meningoencephalitis is rare in nonimmunocompromised patients, but has been reported.[652]

Hematogenous dissemination of *L. monocytogenes* may result in a variety of focal infection, including cutaneous infections, abscesses, arthritis, peritonitis, intra-abdominal, liver and splenic abscesses, endophthalmitis, prosthetic joint/vascular graft infections, osteomyelitis, pericarditis, myocarditis, and endocarditis.[983] Infection at sites other than the CNS and the bloodstream is usually seen in patients with underlying acute or chronic diseases and conditions (i.e., malignancies, systemic lupus erythematosus [SLE], rheumatoid arthritis, organ transplantation, ulcerative colitis, diabetes, renal disease, alcoholic cirrhosis, and HIV infection) or in individuals being treated with immunosuppressive or immunomodulating therapies (i.e., corticosteroids, cytotoxic chemotherapy, tumor necrosis factor [TNF]-α inhibitors). Cutaneous infections have been observed in previously healthy persons (i.e., veterinarians, animal handlers, laboratory workers) with histories of occupational contact with infected animals or animal tissues (i.e., placental tissues, amniotic fluids).[738] Cutaneous lesions may also be manifestations of early-onset disease in neonates.[1037] A positive culture of *L. monocytogenes* from a biopsy of a thigh lesion provided the diagnosis in a bone marrow transplant patient with cerebritis, since all other cultures were negative.[630] Cutaneous lesions may be papular or pustular, and disseminated infections have occurred following primary cutaneous inoculation.[151,1037] Intra-abdominal infections with *L. monocytogenes* are rare and include solitary and multiple liver abscesses, diffuse or granulomatous hepatitis, splenic rupture, and spontaneous bacterial peritonitis.[682,691,989,1017,1077] Patients with intra-abdominal complications usually have diabetes, renal disease, liver cirrhosis/hepatitis, or underlying carcinoma. *L. monocytogenes* is also capable of causing infections in continuous ambulatory peritoneal dialysis (CAPD) patients and in those with ventriculoperitoneal shunts.[107,1120,1199] Several reports have appeared describing *L. monocytogenes* as a cause of septic arthritis in patients receiving TNF-α inhibitors (e.g., methotrexate, etanercept, infliximab) for treatment of rheumatoid arthritis.[545,587,791] During bacteremia,*Listeria* organisms may seed implanted prostheses, such as knee joints and hip arthroplasties, causing subacute infection and eventual failure of the implanted device. Most patients with these infections are elderly, and the severity of infection usually requires replacement of the failed hardware.[194,235,603,731] In one report, 18 patients with infected hip arthroplasties who underwent replacement were cured, but 5 of 13 patients for whom the prostheses was not removed had protracted infection

despite chemotherapy.[194] Septic arthritis has been described in native joints and an unusual case of *L. monocytogenes* septic arthritis from Taiwan occurred following acupuncture, with inadequately sterilized needles being the suspected vehicle for transmission.[744,1107] *L. monocytogenes* is an uncommon cause of eye infections in humans, although ocular infections have been described in sheep, cattle, and horses. Case reports have been published documenting *L. monocytogenes* as a rare cause of keratitis, uveitis, hypopyon, and endogenous endophthalmitis in humans.[529,740,1096]

L. monocytogenes endocarditis results from bacteremia and usually involves damaged native aortic/mitral valves or prosthetic valves. A survey of all reported cases of *L. monocytogenes* endocarditis found that 60% of patients had some type of valvular compromise, and 23% had some type of cardiac prosthesis in place. The remainder of cases have occurred in patients with rheumatic valvular disease, mitral valve prolapse, and hypertrophic myocardiopathy.[39] Patients with endocarditis involving native valves have a lower mortality rate (about 31%) than those with prosthetic valve infection (about 41% mortality).[1052] Septic embolization to distant sites is relatively uncommon with *L. monocytogenes* endocarditis.[266,876,1068] Endocarditis involving prosthetic valves is associated with large vegetations, perivalvular leaks, dehiscence of the prosthesis, fistula formation, and

pericarditis.[334,570,876] Listerial pericarditis with pericardial effusion has also been reported without the presence of valvular lesions lesion or cardiac involvement.[277] Prosthetic valve endocarditis with *L. monocytogenes* may be complicated by paravalvular and aortic root abscesses.[707] Listerial infection of pacemaker leads is extremely rare, and, in the single case report available, pacemaker infection and vegetations were documented and the patient died due to hemodynamic instability, shock, requirement for chest tube placement due to a pleural effusion, and the development of renal failure requiring dialysis.[1068] Endocarditis with *L. monocytogenes* has also been reported in a patient with psoriatic arthritis who was being treated with infliximab.[578] Pleural fluid infections with *L. monocytogenes* have been documented in patients with malignancies, where pleural infection occurred by hematogenous seeding of malignant pleural effusions rather than secondary to a parapneumonic process.

L. monocytogenes is present as a contaminant in many kinds of foodstuffs, including raw milk, raw vegetables, fish, poultry, and both fresh and processed meats. The organism can be recovered from 15% to 70% of raw vegetables, raw milk, meats, and cheese.[320] In 1983, a widespread outbreak of *L. monocytogenes* was associated

with contaminated coleslaw, and since then, numerous outbreaks involving soft cheese, processed ready-to-eat delicatessen meats (pork, turkey), pates, butter, milk, and fish products have been documented.[1074] As a corollary to the recognition of food-borne outbreaks of listeriosis, a syndrome of febrile gastroenteritis caused by *L. monocytogenes* has also been described.[966] This syndrome includes noninvasive, nonbloody diarrhea, nausea, and vomiting accompanied by fever, fatigue, chills, and myalgias.[249,927,1074] Retrospective serologic analysis of patients with febrile gastroenteritis has been able to demonstrate immune responses to listeriolysin, a putative virulence factor of *L. monocytogenes*.[249] This acute illness usually occurs 6 hours to a few days after ingestion of foods with high listerial burdens and lasts 2 to 7 days. Individuals with this syndrome have fever, watery diarrhea, nausea, headache, and joint pain. Febrile gastroenteritis due to *L. monocytogenes* is usually self-limited, but invasive disease may occur in immunocompromised patients and those with other underlying bacterial or viral gastrointestinal infections.

With the recognition and documentation of food-borne outbreaks due to *L. monocytogenes*, the CDC has promulgated guidelines for the prevention of food-borne listeriosis.[179] Guidelines that apply to all individuals include thoroughly cooking raw food from animal sources, thoroughly washing raw vegetables before eating, keeping uncooked meats separate from vegetables, cooked foods, and "ready-to-eat foods," and avoidance of the consumption of raw (unpasteurized) milk or foods made from raw milk. Hands, knives, and cutting boards should be thoroughly washed after handling uncooked foods. For persons at high risk for listeriosis (i.e., immunocompromised persons, pregnant women, elderly individuals), soft cheeses (e.g., Mexican-style, feta, Brie, Camembert, and blue-veined cheese) should be avoided, although there is no need to avoid hard cheeses (e.g., Swiss cheese, Colby cheese), cream cheese, cottage cheese, or yogurt. Leftover foods or "ready-to-eat" foods (e.g., hot dogs) should be re-heated until steaming hot before eating. "Delicatessen counter" food (e.g., cold cuts, salami) should be avoided or thoroughly heated before eating. During the mid-1990s, the food industry introduced an initiative called Hazard Analysis at Critical Control Points (HACCP) to improve the surveillance and control of *L. monocytogenes* and other food-borne pathogens in the food processing plant environment, and the US Food and Drug Administration (FDA) has mandated a "zero tolerance" approach to the control of listeriosis, with increases in both the numbers of FDA inspections of food processing centers and recalls of foods suspected of harboring pathogens.

Isolation of L. monocytogenes *From Clinical Specimens*

L. monocytogenes may be isolated from the blood, cerebrospinal fluid, genital tract specimens, amniotic fluid, and biopsy specimens derived from maternal/fetal tissues.

Direct inoculation of both agar and broth media with overnight incubation will usually yield the organism. On Gram's stains of clinical specimens and from blood cultures, the organism may appear as regular, gram-positive bacilli or as short, plump coccobacilli. If polymorphonuclear or mononuclear cells are present, organisms are found both intra- and extracellularly. Because of variations in Gram's stain morphology that are observed with this organism, *L. monocytogenes* morphotypes may be mistaken for diphtheroids or for certain streptococci, particularly *Streptococcus pneumoniae*, enterococci, and some viridans streptococci.

Methods for the recovery of *L. monocytogenes* and other *Listeria* species from foods and environmental samples differ significantly from those used for isolation from human clinical specimens. Additional information regarding methods used for the detection and recovery of *L. monocytogenes* from food and environmental samples may be found in the indicated references.[25,26,165]

Identification of Listeria *Species*

L. monocytogenes grows well on sheep blood agar (SBA), producing gray-white colonies that closely resemble those of group B β-hemolytic streptococci. After 18 to 24 hours' incubation, colonies may show a narrow zone of β-hemolysis that does not extend very far beyond the edge of the colony (see Image Plates 14-1B and 14-1C). Before this time, β-hemolysis may only be noted directly under the colony after removal of the colonial growth from the agar surface with a swab. On longer incubation, or in areas of the plate that have been stab-inoculated, the β-hemolysis of the organism becomes more obvious. Among the other species, *L. ivanovii* subspecies produce wide zones or even multiple zones of β-hemolysis on sheep blood- or horse blood-containing media, while *L. seeligeri* produces hemolytic zones that are narrower than those of *L. monocytogenes*. Because of the resemblance of the colonies of *L. monocytogenes* to those of enterococci and group B β-hemolytic streptococci, short cuts in laboratory identification are to be discouraged. Gram stains and catalase tests should always be performed.

L. monocytogenes is a catalase-positive, facultative, gram-positive bacillus (Table 14-2). The organism is motile, showing "tumbling motility" particularly in hanging drop preparations prepared from overnight broth cultures

TABLE 14-2 Phenotypic Characteristics for Identification of *Listeria* Species

Test	*L. monocytogenes*	*L. ivanovii* subsp. *ivanovii*	*L. ivanovii* subsp. *londoniensis*	*L. innocua*	*L. grayi*	*L. seeligeri*	*L. welshimeri*	*L. rocourtiae*	*L. marthii*
Hemolysis, SBA	β	β[a]	β	None	None	β	None	None	None
Hippurate hydrolysis	+	+	+	+	–	NA	NA	NA	NA
CAMP test with *S. aureus*	+	–	–	–	–	+	–	–	NA
CAMP test with *R. equi*	V	+	+	–	–	–	–	–	NA
Acid Production From:									
Mannitol	–	–	–	–	+	–	–	+	–
D-Arabitol	+	+	+	+	+	+	+	–	NA
α-methyl-D-mannoside	+	–	–	+	+	–	+		NA
L-Rhamnose	+	–	–	V	–	–	V	+	–
D-Xylose	–	+	+	–	–	+	+	+	–
Ribose	–	+	–	–	+	–	–	+	–
N-acetyl-β-D-mannosamine	NA	V	+	NA	NA	NA	NA		NA
Soluble starch	–	–	–	–	+	NA	NA		NA

[a] Wide zones or multiple zones of β-hemolysis.
+, positive reaction; –, negative reaction; V, variable reaction; NA, data not available.

incubated at 25°C. This greater motility following room temperature incubation is also apparent in semisolid motility medium, where the organism displays a characteristic "umbrella" of motility near the surface of semisolid motility medium containing 0.2% to 0.4% agar after incubation at 25°C (Image Plate 14-1E). The organism also grows in the presence of 40% bile and hydrolyzes esculin; therefore, bile esculin agar slants used for presumptive identification of group D streptococci (i.e., *Streptococcus gallolyticus*) and enterococci may be used to demonstrate esculin hydrolysis (Image Plate 14-1D). The organism is fermentative, producing acid from glucose, and produces acetoin, resulting in a positive Voges–Proskauer reaction. All strains of *L. monocytogenes* ferment α-methyl-D-mannoside and do not ferment D-xylose. The β-hemolytic reaction, positive catalase test, lack of H_2S production in TSI agar, room temperature motility, and esculin hydrolysis differentiate *L. monocytogenes* from *Erysipelothrix rhusiopathiae*. Motility differentiates this organism from all *Corynebacterium* species. *Leifsonia aquatica*, the former "*Corynebacterium aquaticum,*" is also a motile, catalase-positive, gram-positive bacillus, but this organism is nonhemolytic, grows as yellow-pigmented colonies, and is Voges–Proskauer-negative (see section on *Leifsonia* species). While the biochemical characteristics of *L. monocytogenes* are usually uniform and unambiguous, aberrant strains lacking catalase activity have been isolated from patients with typical listerial sepsis and meningitis.[146,306] Phenotypic characteristics for identification of *Listeria* species are presented in Table 14-2.

The CAMP test has also been used to identify *L. monocytogenes*.[23] With *Listeria* species, the CAMP test is performed with a β-lysin-producing *S. aureus* strain and

with a strain of *Rhodococcus equi*. Synergistic hemolysis is seen between *S. aureus* and putative *L. monocytogenes* and *L. seeligeri* strains, whereas enhanced hemolysis is seen between *R. equi* and putative *L. ivanovii* strains. Other *Listeria* species (i.e., *L. innocua*, *L. welshimeri*, and *L. grayi*) are CAMP test-negative with both *S. aureus* and *R. equi*. With *L. monocytogenes*, a positive CAMP test with *S. aureus* is indicated by enhanced hemolysis in the region between the two nonintersecting streaks of growth at right angle to one another, while that seen between *R. equi* and *L. ivanovii* appears as an area of shovel-shaped enhanced hemolysis. The CAMP test for *Listeria* species may be difficult to interpret, as some investigators have reported synergistic hemolytic reactions between *L. monocytogenes* and *R. equi*.[23] Test performance is somewhat dependent on the basal media used to perform the test and the experience of the user in test interpretation.[737]

Kit systems are also available for identifying *Listeria* species. The API Coryne (bioMérieux, Inc., Marcy l'Étoile, France) and the RapID CB-Plus systems (Remel, Inc., Lenexa, KA) contain *Listeria* species in their data bases and have been shown to reliably identify these organisms to at least the genus level. In an evaluation of this system for identification of *Listeria* species, the API Coryne identified all 72 *L. monocytogenes* strains that were tested as *L. monocytogenes/innocua*, with definitive species identification being dependent on the presence of β-hemolysis and a positive CAMP reaction (Image Plate 14-1F).[584] Similar results have been reported by other investigators for both the API Coryne and the RapID CB-Plus.[379,384,528] The API *Listeria* (bioMérieux, Inc.) and the Micro-ID *Listeria* (Organon-Teknika, Durham, NC) are identification systems designed specifically for identification of *Listeria* species. The API *Listeria* is a 10-test, chromogenic enzyme substrate system that has been shown to reliably identify several species in the genus, including *L. monocytogenes*, within 24 hours.[737,853] This identification panel includes a test for glycyl arylamidase (DIM test) that differentiates *L. monocytogenes* (DIM test-negative) from *L. innocua* (DIM test-positive) without the need for assessing hemolytic activity on SBA. The other tests on the API *Listeria* include esculin hydrolysis, detection of α-mannosidase, acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate, and D-tagatose. Stand-alone tests for detection of alanyl arylamidase using either DL-alanine-β-naphthylamide or DL-alanine-*p*-nitroanilide can also be used to identify *Listeria* species.[210] *L. monocytogenes* does not hydrolyze these compounds due to absence of specific arylamidases, while all other species, including *L. innocua*, are alanyl or glycyl arylamidase-positive. This test is particularly helpful in identifying *L. monocytogenes* strains that are poorly hemolytic or nonhemolytic. Some strains of *L. ivanovii* may be alanyl- or glycyl-arylamidase-negative as well.[737] The 15-test Micro-ID *Listeria* also identifies these organisms correctly, but, like the API Coryne, requires an assessment

of hemolysis for differentiation of *L. monocytogenes* and *L. innocua*.[68] Tests on the Micro-ID *Listeria* include MR/VP, nitrate reduction, phenylalanine deaminase, H_2S production, lysine and ornithine decarboxylase, malonate utilization, urea and esculin hydrolysis, β-galactosidase, and fermentation of xylose, rhamnose, mannitol, and sorbitol.[101] In an evaluation of the Micro-ID *Listeria*, this identification method agreed with conventional biochemical identification for 98% of *L. monocytogenes*, 77.1% of *L. seeligeri*, 90% of *L. ivanovii*, 96% of *L. grayi*, 73.9% of *L. welshimeri*, and 100% of *L. innocua* strains tested.[492] In a comparative evaluation with 207 *Listeria* species isolates, the API *Listeria* showed 95.2% agreement and the Micro-ID showed 93.2% agreement with conventional biochemical testing.[101] Certain substrates on the API 50CH (bioMérieux, Inc.) carbohydrate assimilation panel have also used for identification of *Listeria* species.[585]

Molecular approaches to the identification of *Listeria* species have also been developed. A highly sensitive and specific chemiluminescent DNA probe assay—the AccuPROBE *Listeria monocytogenes* Culture Identification kit (Gen-Probe, San Diego, CA)—and a spectrophotometric DNA probe assay—the GeneTrak DLP *Listeria* (GeneTrak Systems, Framingham, MA)—are also available for rapid identification of *L. monocytogenes* from colonies grown on primary isolation media.[804,818] The AccuPROBE *Listeria* hybridization assay has also been evaluated using broth media seeded with *L. monocytogenes* and showed variable results. Three nonselective broth media (BHI, tryptic soy, and Todd-Hewitt broth) gave positive AccuPROBE results, but media selective for *L. monocytogenes* gave negative reactions, even with organism loads as high as 10^8 to 10^9 cfu/ml.[804] Other workers have also noted false-negative results with AccuPROBE *Listeria* when testing is performed from certain *L. monocytogenes* enrichment broths.[842] The GeneTrak DLP (Direct Labeled Probe) *Listeria* was developed and is used primarily

for the detection of *L. monocytogenes* in foods and environmental samples following broth enrichment. The assay uses *L. monocytogenes*-specific probes directly labeled with horseradish peroxidase and a colorimetric detection system. The test takes 2 hours after a 40- to 48-hour broth enrichment step. In a side-by-side evaluation of the AccuPROBE and the Gene-Trak assays for identification of *L. monocytogenes* from plated media, all 86 *L. monocytogenes* were identified correctly, while 121 isolates of other *Listeria* species were negative with both hybridization assays.[101]

Kits are also available for the rapid detection of *L. monocytogenes* in foods and environmental samples. These tests are performed on aliquots of enrichment broth inoculated with food or environmental materials and incubated for 24 to 48 hours. The

Reveal *Listeria* (Neogen, Lansing, MI) is a lateral flow assay that is able to detect 10^6 cfu/mL of *Listeria* organisms after overnight incubation. An evaluation of the Reveal assay relative to reference methods found that the test has a sensitivity of 85.9% after 27 hours of enrichment and 97.1% sensitivity after 30 hours of enrichment.[26] The Reveal assay out-performed reference culture methods for detection of *Listeria* species on surfaces such as stainless steel, plastic, and cast iron, and was equivalent to reference culture procedures for ceramic tile and sealed concrete surfaces. NeoGen also manufactures GeneSequence, which uses molecular technology in a microtiter format for detection of *Listeria* and other pathogens present in foods. In this assay, an aliquot of the enrichment culture is placed into a test tube, lysis reagent is added, and a portion of the lysed sample is transferred to a microwell and the probe reagents are added. Probe reagents consist of an oligonucleotide capture probe specific to rRNA sequences of the target organism and labeled at the 3′ end with polydeoxyadenylic acid (poly dA) and an oligonucleotide detector probe, also specific to rRNA sequences of the target organism, that is labeled at the 5′ end with horseradish peroxidase (HRP). The tray is then incubated for 1 hour. If target rRNA is present in the sample, both probes hybridize to their complementary sequences on the target molecule and the resulting complex is captured onto the solid phase that is coated with polydeoxythymidylic acid (poly dT) (complementary to the poly dA portion of the capture probe). Unbound probe is then washed away, and a substrate of HRP is added. After another incubation, stop solution is added and results are determined spectrophotometrically. The sensitivity of this assay compared with reference USDA–FSIS (US Department of Agriculture–Food Safety and Inspection Service) procedures exceeds 90% with greater than 99% specificity.[25]

Antimicrobial Susceptibility and Treatment of Listeria *Infections*

Except for those women who deliver infants with early-onset disease, infection with *L. monocytogenes* is usually fatal if left untreated. *L. monocytogenes* is generally susceptible to penicillin, ampicillin, the aminoglycosides, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, and imipenem.[721] Cephalosporins (first, second, and third generations) and the fluoroquinolones are not active against *L. monocytogenes*.[317,506] Ampicillin is considered the therapy of choice, but the standard of practice includes adding gentamicin to the ampicillin regimen for bacteremia, endocarditis, meningitis, and immunocompromised patients.[506,692] For those patients with penicillin allergy, trimethoprim-sulfamethoxazole with or without rifampin is believed to be the best alternative agent for treating *L. monocytogenes* infections.[747] Amoxicillin with trimethoprim-sulfamethoxazole was also found to be as effective as ampicillin with gentamicin in one retrospective study.[747] Patients should be treated for at least 3 weeks or longer, depending upon their immune status and clinical response.

Patients with advanced HIV disease may require lifelong therapy to prevent relapses. Interestingly, the use of trimethoprim-sulfamethoxazole as a prophylactic agent for *Pneumocystis jirovecii* infection in HIV-positive patients may be partially responsible for protecting these individuals from *Listeria* infections, although widely promulgated dietary guidelines may also have some impact on disease incidence in this population. Cephalosporins, erythromycin, and tetracyclines have no utility in the therapy of *L. monocytogenes* infections, and high rates of resistance to clindamycin have been reported in a large cancer center.[968] Vancomycin is an appropriate therapeutic agent for primary *L. monocytogenes* bacteremia, but it does not cross the blood–brain barrier sufficiently well to be useful in treating meningitis. Naturally occurring, high-level resistance to vancomycin among the listeriae has never been reported, but the *vanA* genes that code for vancomycin resistance in *Enterococcus faecium* (VRE) have been transferred to *L. monocytogenes* and four other *Listeria* species in the research laboratory.[106]

The Clinical and Laboratory Standards Institute (CLSI) has published a standard for microbroth dilution antimicrobial susceptibility testing of *L. monocytogenes*.[214] The medium used for testing is cation-adjusted Müeller–Hinton broth with lysed horse blood (2.5% to 5% v/v). The inoculum is a direct colony suspension equivalent to a 0.5 McFarland turbidity standard. Incubation is at 35°C in ambient air for 20 to 24 hours. Breakpoints are only available for penicillin, ampicillin, and trimethoprim-sulfamethoxazole. For penicillin and ampicillin, only a susceptible category is defined since resistance of *L. monocytogenes* to these agents has not been described, but breakpoints defining susceptible, intermediate, and resistant categories are available for trimethoprim-sulfamethoxazole. For penicillin and ampicillin, *L. monocytogenes* isolates will have minimum inhibitory concentrations (MICs) of ≤2 μg/mL. Isolates that are susceptible to trimethoprim-sulfamethoxazole have MICs of ≤0.5/9.5 μg/mL, while isolates that are resistant will have MICs of ≥4/76 μg/mL. Strains with MICs of 1/19 to 2/38 μg/mL are considered intermediate in susceptibility to trimethoprim-sulfamethoxazole.

Pathogenicity of Other Listeria *Species*

In both mouse and tissue culture models, only *L. monocytogenes* and *L. ivanovii* demonstrate pathogenic properties, while the other species in the genus are considered to be nonpathogenic. Gouin et al.[423] demonstrated that the gene cluster associated with virulence of *L. monocytogenes* is also found in *L. ivanovii* and *L. seeligeri*. While both *L. monocytogenes* and *L. ivanovii* are able to invade mammalian cells in tissue culture and to spread from cell to cell, the latter species lacks the cytotoxin associated with *L. monocytogenes*, suggesting

that lack of cytotoxin may account for the lower virulence of *L. ivanovii*.[571] In 1994, Cummins and colleagues[245] isolated *L. ivanovii* from blood cultures of a patient with AIDS complicated by non-Hodgkin's lymphoma. Lessing and coworkers[669] also recovered L. *ivanovii* from a blood culture of an alcoholic 26-year-old IV-drug user. In 2010, *L. ivanovii* was isolated from the blood of a 55-year-old man with a history of kidney transplantation and hepatitis C. The same organism was isolated from the patient's diarrheal stool specimen.[450] In 2003, *L. innocua* was reported for the first time as a cause of fatal bacteremia in a nonimmunocompromised 62-year-old patient with cholangitis and septic shock.[863] The organism was identified by conventional methods and confirmed by the API *Listeria* and sequencing of the 16S rDNA. *L. grayi* has also been isolated from human clinical specimens in two cases. In the first, *L. grayi* was isolated from two sets of blood cultures from a 20-year-old female with relapsed T-cell leukemia who was being conditioned for receipt of an allogeneic peripheral blood stem cell transplant.[970] The isolate was resistant to vancomycin (MIC, >32 µg/mL) and susceptible to ampicillin (MIC, 0.5 µg/mL). In the second case, *L. grayi* was isolated from one of two sets of blood cultures from a 57-year-old male heart transplant recipient.[899]

Erysipelothrix Species

Taxonomy of the Genus Erysipelothrix

The genus *Erysipelothrix* includes three species of catalase-negative, gram-positive bacilli and is classified in the Phylum Firmicutes in the Class Mollicutes (Box 14-1). Within this class, orders I through IV include the mycoplasmas and related organisms, while order V (incertae sedis) includes the genus *Erysipelothrix* and the genus *Holdemania* within the proposed family *Erysipelotrichaceae*.[1155] *Erysipelothrix* strains can be subdivided into serotypes, the most recently described being serotype 26. These are also some strains, termed "N strains", which do not produce any precipitating antibodies against homologous or heterologous heat-stable extracts in rabbits. DNA–DNA hybridization studies performed with these various serotypes indicated that the genus *Erysipelothrix* contained two species: *E. rhusiopathiae* (composed of serotypes 1, 2, 4 to 6, 8, 9, 11, 12, 15 to 17, 19, 21, and type N) and *E. tonsillarum* (composed of serotypes 3, 7, 10, 14, 20, 22, and 23).[1079,1080] Strains representing the first group of serotypes exhibited more than 73% hybridization with the type strain of *E. rhusiopathiae* but less than 24% hybridization with the type strain

of *E. tonsillarum*.[1080] Conversely, strains belonging to the second group of serotypes exhibited more than 66% hybridization with the *E. tonsillarum* type strain and less than 27% with the *E. rhusiopathiae* type strain.[1080] Isolates of *E. tonsillarum* are phenotypically identical to *E. rhusiopathiae*, except that the former species is able to ferment sucrose, and *E. tonsillarum* strains are not pathogenic for swine.[1081] (Table 14-3). Studies of the 16S rRNA genes of *E. rhusiopathiae* and *E. tonsillarum* have shown that the nucleotides sequences are 99.8% concordant, with only a three-nucleotide difference between the two sequences.[599] Two serotypes (13 and 18) are distinct from the others by DNA hybridization and may represent separate, unnamed genomic species. *E. tonsillarum* has been isolated from porcine tonsillar tissue and from the blood of dogs with endocarditis; it has not been isolated from human infections.[1079,1080,1082] Strains belonging to the same serotype are genetically diverse as demonstrated by ribotyping, randomly amplified polymorphic DNA (RAPD) analysis, amplified fragment-length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), and nucleotide sequencing of a hypervariable region of the *spaA* gene.[17,241,242,792,815,816,826] PFGE appears to be more sensitive and discriminatory than RAPD or ribotyping and is considered the method of choice for epidemiological studies.[826] In 2004, a third *Erysipelothrix* species was isolated as a contaminant from a "sterile"-filtered vegetable broth medium prepared for pharmaceutical manufacturing. This new species was named *Erysipelothrix inopinata*.[1155]

Clinical Significance of E. rhusiopathiae

E. rhusiopathiae is widely distributed in nature, where it is found in various species of animals, mainly swine. It is also found in fish, birds, shellfish, and other wild and domestic mammals.[345,1081] The organism is also commonly found in decomposing organic matter and may be isolated from the soil. In swine, the organism causes the economically important disease known as **erysipelas**, but it may cause significant illness in other species of wild and domesticated animals. Swine erysipelas results from the ingestion of contaminated food and water. The organisms multiply in the tonsils and lymphoid tissues in the gut and gain access to the bloodstream. Swine erysipelas may have an acute, subacute, or chronic presentation. Acute infection presents with generalized septicemia within 24 hours of infection. Cutaneous lesions (e.g., erythema, vesicular lesions, necrosis) may also be present. Subacute infection presents as cutaneous lesions that develop over a few days. Swelling of tissues results in pink-to-red skin lesions that overlap and involve most of the skin surface. Lesions may go on to heal and desquamate, or may develop into septicemic lesions that eventually cause death. A live attenuated *E. rhusiopathiae* vaccine for swine immunization (Suvaxyn

E-Oral, Fort Dodge Animal Health, New Zealand) is available and appears to be safe and to afford protection against disease.[799] *E. rhusiopathiae* is also pathogenic for sheep, lambs, many species of wild and domestic birds, and, rarely, for cattle. In lambs, sheep, and cattle, this organism causes polyarthritis, but rarely causes death. Infections in birds occur most frequently in turkeys, but may occur in chickens, ducks, geese, hens, pigeons, parrots, and quail. In birds, *E. rhusiopathiae* causes hemorrhagic lesions in muscle tissue, with subsequent development of fatal diarrheal illness. Humans generally acquire the organism through contact with the tissues of infected animals or animal products and most infections occur via skin scratches or puncture wounds. Infection is usually, but not always, limited to individuals in certain occupations, such as butchers, veterinarians, abattoir workers, farmers, and fishermen.[1179]

In humans, *E. rhusiopathiae* may cause a mild, localized cutaneous infection, a generalized cutaneous infection, or a septicemic infection that is often associated with endocarditis.[944] **Erysipeloid**, the most common form of infection,

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TABLE 14-3 Phenotypic Characteristics for Identification of *Erysipelothrix* Species

Test	*E. rhusiopathiae*	*E. tonsillarum*	*E inopinata*
HEM SBA	None, α	None, α	None, α
CAT	–	–	–
H_2S KIA	+	–	–
NO_3	+	+	NA
VP	+	+	–
PAL	–	+	–
β-GLU	–	+	+
β-GUR	–	–	–
Acid produced from:			
GLU	+	+	+
MAL	–	–	–
SUC	–	+	–
LAC	+	–	–
MNTL	–	–	–
SBTL	–	–	–
TRE	–	–	+
Hydrolysis of:			
STA	–	–	–
GEL	–	–	–
CAS	–	–	–
Serotypes included[a]	1a, 1b, 2a, 2b, 4–6, 8, 9, 11, 12, 15–17, 19, 21, type N	3, 7, 10, 14, 20, 22, 23	

[a] Serotypes 13 and 18 are distinct from the others by DNA hybridization and may represent another genomic species.
HEM SBA, Hemolysis on SBA; CAT, Catalase; H_2S KIA, Production of hydrogen sulfide in Kligler's iron agar; NO_3, Reduction of nitrate to nitrite; VP, Voges–Proskauer (production of acetoin); PAL, Alkaline phosphatase; β-GLU, β-Glucosidase; β-GUR, β-Glucuronidase; GLU, Glucose; MAL, Maltose; SUC, Sucrose; LAC, Lactose; TRE, Trehalose; MNTL, Mannitol; SBTL, Sorbitol; STA, Starch; GEL, Gelatin; CAS, Casein.

develops following cutaneous inoculation of the organism, and is usually found on the fingers or hands.[1154] Lesions appear 2 to 7 days after organism acquisition and are characterized by an elevated, violaceous, erythematous, advancing border and a faded central area. Localized throbbing pain, itching, swelling, and purplish erythema typically develop and may spread to the wrist and forearm. Lymphadenopathy, fever, and joint pain may be present. Erysipeloid usually resolves in a few weeks without systemic therapy. Erysipeloid may progress to a more generalized cutaneous infection with the appearance of lesions that are remote from the initial inoculation site. Fever, joint pain, frank arthritis, and the development of bullous lesions along with regional lymphadenitis or lymphangitis may also be a part of this generalized cutaneous infection. As the organism is located in the perivascular regions of the dermis, biopsy specimens of the advancing margin of the lesion are required to isolate the organism from localized infections.

Systemic infection occurs infrequently and is characterized by sepsis and

bacteremia.[10,471,1179] In these patients, *E. rhusiopathiae* endocarditis (particularly left-sided) is common, causes extensive damage to healthy heart valves and cardiac tissues, and has a high mortality rate.[1133] Both native valve endocarditis and prosthetic valve endocarditis (including both Starr–Edwards prosthetic valves and porcine xenograft valves) caused by *E. rhusiopathiae* have been reported.[481] Native valve endocarditis with *E. rhusiopathiae* usually requires valve replacement along with antimicrobial chemotherapy for treatment and cure. Individuals with systemic infections frequently have no prior history of primary cutaneous infection.[1133] Patients with systemic involvement are usually debilitated by immunosuppressive therapy (e.g.,

corticosteroid administration) or underlying conditions that result in immunocompromised states, such as cancer, diabetes, alcoholism, or systemic lupus erythematosus.[1007]Multisystem disease evidenced by shock and renal failure has also been reported, particularly among those with endocarditis.[408] Complications and unusual manifestations of *E. rhusiopathiae* bacteremia and endocarditis include prolonged hypertension, paravalvular and myocardial abscesses, septic arthritis, endogenous endophthalmitis, necrotizing fasciitis, and cerebral infarcts.[47,307,502,611,954,1019,1070] Recently, *E. rhusiopathiae* was isolated from infections of total hip and total knee arthroplasties that required total revision of both implants.[1114,1117] One patient worked in a tanning factory, where he worked with hides of pigs, cows, and deer and suffered frequents cuts and abrasions on his hands.[1114] The second patient had no direct exposure to wild animals, but she had a dog that was used for hunting and frequently carried wild animals in its mouth.[1117] In 2010, *E. rhusipathiae* was reported as the cause of an abdominal abscess in a patient with a recent history of a surgical duodenocephalopancreatectomy for distal bile duct adenocarcinoma.[326] This organism is rarely isolated from the CNS, but two reports, one in 2007 and the other in 2011, document meningitis in two patients, neither of whom had any overt animal exposures.[558,595] In one case, the patient presented with chronic headache that had lasted for 2 months, and subsequently developed hydrocephalus requiring placement of a ventriculoperitoneal shunt. *E rhusiopathiae* was isolated from the CSF.[595] In the second case, onset of meningeal signs and symptoms were acute.[558] Although the CSF failed to grow the organism, the cellularity of the CSF and magnetic resonance imaging (MRI) scans showed chronic ischemia in the cerebral white matter, with no indications of infarcts or hemorrhage. Echocardiography revealed severe mitral regurgitation compatible with infective endocarditis, and *E. rhusiopathiae* was isolated from blood cultures. In 2012, *E.*

rhusiopathiae pneumonia was diagnosed in an immunocompetent male stockyard worker who spent most of his time feeding cattle.[746] *E. rhusiopathiae* has also been documented as a rare cause of peritonitis in patients on CAPD.[160,472,1012]

Isolation and Identification of E. rhusiopathiae

E. rhusiopathiae may be isolated from biopsy specimens of erysipeloid lesions and from blood cultures. The specimen of choice for isolation of the organism from skin lesions is an aspirate obtained by injection and aspiration of sterile saline from the leading edge of the cellulitis or a punch biopsy from the same area. The organism grows well in commercially available blood culture media. On SBA, *E. rhusiopathiae* forms small (0.1 to 0.5 mm in diameter), circular, convex, smooth colonies. A weak α-hemolytic reaction may be noted in the media, particularly in areas where the growth is more confluent (Image Plate 14-1H). On Gram-stained smears, the organisms appear as slender rods; chains of nonbranching bacilli may also be observed and the individual bacterial cells may be straight or somewhat curved (Image Plate 14-1G). *E. rhusiopathiae* is catalase-negative and nonmotile (Table 14-3). The most useful property for identifying this organism is the production of H_2S in the butt of a TSI agar or Kligler's iron agar (KIA) slant (see Image Plate 14-2A). Failure to look for H_2S production by suspect isolates may result in the misidentification of these organisms as *Lactobacillus* species.[297] A properly streaked and stabbed KIA slant will show an acid slant and acid butt, due to acid production from glucose and lactose; H_2S may be noted throughout the butt or only along the streak line in the butt. This reaction helps to differentiate *E. rhusiopathiae* from other catalase-negative bacilli such as *Arcanobacterium haemolyticum*; this latter species is also β-hemolytic on SBA. The negative catalase reaction, lack of hemolysis or weak α-hemolysis on SBA, and production of H_2S in KIA or TSI agar also help to differentiate *E. rhusiopathiae* from *L. monocytogenes*. Stab inoculation of a tube of gelatin agar and incubation at 22°C results in the production of "bottle brush" or "pipe cleaner" growth, where the organisms grow out in straight lines perpendicular to the stab inoculation, giving the appearance of "bristles".

E. rhusiopathiae is weakly fermentative, producing acid from glucose, galactose, fructose, lactose, maltose, and *N*-acetyl-glucosamine. No acid is produced from glycerol, arabinose, xylose, adonitol, inositol, mannitol, sorbitol, amygdalin, malibiose, trehalose, cellobiose, inulin, melezitose, raffinose, and glycogen. Acetoin and indole are not produced and nitrate is not reduced to nitrite. *E. rhusiopathiae* is included in the data bases of both the API Coryne and the RapID CB-Plus identification systems. Isolates of *E. tonsillarum* are phenotypically identical to *E. rhusiopathiae*, except that the former species also produces acid from sucrose. Biochemical characteristics for identification of *Erysipelothrix* species are shown in Table 14-3. Rapid molecular

methods, including real-time polymerase chain reaction (PCR) approaches, have been developed for detection of *E. rhusiopathiae* and differentiation of *E. rhusiopathiae* and *E. tonsillarum* in animal specimens.[837,1221]

Antimicrobial Susceptibility of E. rhusiopathiae

E. rhusiopathiae strains are generally susceptible to penicillin, cephalosporins, imipenem, piperacillin, and ciprofloxacin.[343,345] Of considerable therapeutic importance is the fact that *E. rhusiopathiae* strains are intrinsically resistant to vancomycin, teicoplanin, and other glycopeptide drugs.[343] MICs for vancomycin are generally >64 μg/mL.[343] Penicillin, amoxicillin clavulanate, imipenem, and the fluoroquinolones have been found to be the most active agents against this organism.[746] In one study of 60 isolates from animal and human infections, the MIC50s and MIC90s were both 0.03 μg/mL for penicillin, both 0.06 μg/mL for ciprofloxacin, and 0.06 μg/mL and 0.125 μg/mL, respectively, for ceftriaxone.[343] Some isolates may have elevated MICs or may be resistant to clindamycin, erythromycin, tetracycline, trimethoprim–sulfamethoxazole, the fluoroquinolones, and gentamicin. In an antimicrobial susceptibility study of 149 *E. rhusiopathiae* strains isolated from pig tissues in Japan, 37.6% were resistant to erythromycin, 2.7% were resistant to lincomycin, 12.1% were resistant to ofloxacin, and 12.8% were resistant to enrofloxacin (a fluoroquinolone used in veterinary medicine).[206] Daptomycin and linezolid also have excellent activity against this organism.[826,871] Even with

appropriate antimicrobial therapy, the mortality associated with disseminated disease may be as high as 35% to 40%. This high mortality rate is largely attributable to complications resulting from endocarditis. Cases of human cutaneous infection may be effectively treated with oral penicillin, but severe disease requires penicillin G, 12 to 20 million units/day for 10 to 14 days.

In 2010, CLSI published an approved standard for microbroth dilution antimicrobial susceptibility testing of *E. rhusiopathiae*.[214] The interpretive criteria are based on a synthesis of previously/currently published standards for susceptibility testing of staphylococci and streptococci. The medium used is cation-supplemented Müeller–Hinton broth with lysed horse blood (2.5% to 5% v/v), with inoculum coming from a direct colony suspension equivalent to a 0.5 McFarland turbidity standard. Tests are incubated at 35°C in ambient air for 20 to 24 hours. Only susceptible breakpoints are available for the penicillins, cephalosporins, carbapenems, and fluoroquinolones, while susceptible and resistant breakpoints are published for clindamycin and erythromycin.

Vancomycin does not need to be tested because of intrinsic resistance. In the document, CLSI points out the importance of correct and rapid identification of the organism because of its association with complicated endocarditis and its resistance to vancomycin, which is often used as a frontline drug for empiric antimicrobial therapy.

Bacillus Species and Related Genera

Taxonomy and the Taxonomic Dissection of Genus Bacillus

The genus *Bacillus* is comprised of a large group of aerobic and facultative, catalase-positive, gram-positive rods that are characterized by the ability to form spores under aerobic conditions (see Image Plate 14-2B). Because the description of genus *Bacillus* placed particular emphasis on endospore formation as a taxonomic criterion, an extremely diverse collection of species was assigned to this genus over several decades. Within this criterion, endospore shape (i.e., spherical, oval, ellipsoidal, cylindrical), spore location within the cell (i.e., central, subterminal, terminal), and spore-induced changes in cell shape (i.e., cells swollen or not swollen by the intracellular spore) were also used as taxonomic descriptors. Until recently, members of genus *Bacillus* included aerobic and facultative organisms that were also acidophiles, alcaliphiles, psychrophiles, mesophiles, and thermophiles, reflecting growth and metabolism over wide temperature and pH ranges. Some species are able to metabolize a wide assortment of carbon sources, including methanol, cellulose, and chitin. Significant variation also is found in the amino acids comprising the cross-linkages within the cell wall peptidoglycan. At the genetic level, diversity within the genus is exemplified by G+C contents, which range from 33 mol% to 69 mol%. Such genetic heterogeneity is incompatible with inclusion in a single genus as defined by current bacterial taxonomic methods. Not surprisingly, 16S rRNA sequencing studies demonstrated that extant "genus *Bacillus*" was phylogenetically heterogeneous, with at least eight highly divergent phyletic lines being represented. Aerobic and facultative spore-forming bacilli are now included in three families: family *Bacillaceae*, family *Paenibacillaceae*, and family "*Alicyclobacillaceae*," all of which are in the order Bacillales, class "Bacilli" in the phylum Firmicutes.[695] The family *Bacillaceae* includes genus *Bacillus* and at least 18 other genera, most of which are environmental species. The family *Paenibacillaceae* includes the genus *Paenibacillus* and at least 6 other genera. Members of genus *Paenibacillus* are facultatively anaerobic spore-forming rods that produce swollen sporangia and have G+C contents ranging from 45 mol% to 54 mol%.[51,1008]Currently there are over 60 species in the genus *Paenibacillus* and some of these have been isolated from human clinical specimens. The family "*Alicyclobacillaceae"* includes species that are acidophilic thermophiles that contain unique cycloheptane/cyclohexane cell membrane fatty acids. The family

"*Sporolactobacillaceae"* is composed of three genera of anaerobic, motile, endospore-forming lactic acid-producing bacilli (*Sporolactobacillus*, *Sinobaca,* and *Tuberibacillus*) and is also classified in phylum Firmicutes.

The genus *Bacillus sensu stricto* is composed of aerobic gram-positive rods that produced endospores under aerobic conditions. At the present time, the genus contains more than 269 species. With rare exceptions, all species in the genus are mesophilic and aerobic or facultative. The primary pathogen in this group is *Bacillus anthracis*, the causative agent of anthrax. These organisms are catalase-positive, and, with the exception of *B. anthracis* and *B. mycoides*, many other species are motile via peritrichous flagella (H antigens). Most non-*anthracis Bacillus* species are ubiquitous in the environment (dust, soil, water, and materials of both plant and animal origin), and some species are recognized pathogens in animals and insects. *Bacillus* species are used in antibiotic and vitamin manufacturing and production, in bioassays, and as indicator organisms for monitoring the efficacy of disinfectants and sterilization procedures (e.g., autoclaving, heat, and radiation sterilization). Various species within this group, particularly *B. cereus*, are being increasingly recognized for their potential to cause significant human infections, particularly among immunocompromised hosts. *B. anthracis* belongs to the "*B. cereus* group," which includes eight very closely related species: *B. anthracis*, *B. cereus, B. thuringiensis, B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. gaemokensis*, and *B. maliponensis*.[563,564,647,793] The *B. cereus* group can be divided into three distinct clades.[279] *B. anthracis* is restricted to clade 1 along with virulent emetic toxin-producing *B. cereus* strains.[1056] Other pathogenic *B. cereus* strains are placed in clade 2 along with most *B. thuringiensis,* and clade 3 includes *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis. B. anthracis, B. cereus, B. thuringiensis*, and *B. mycoides* share a high degree of genetic similarity by DNA–DNA hybridization and comparisons of rRNA and rDNA at the 16S, 23S, and the 16S-to-23S spacer region.[48–50] Some researchers have even suggested that these organisms should be considered as a single species based on gene sequence analysis and multilocus enzyme electrophoresis studies.[647] *B. cereus* is a mesophilic organism that is ubiquitous in the environment as a soil saprophyte in food of plant and animal origin. *B. cereus* is also a cause of food poisoning and, in this role, is a serious problem for the commercial

food industry. This organism can also cause localized and systemic opportunistic infections in immunocompromised hosts, intravenous drug users, and neonates, as well as keratitis (see section on *B. cereus* below). Genotypic analyses of *B. cereus* and *B. thuringiensis* strains from the environment, animals, and humans have

demonstrated a great deal of diversity within and between the species.[161] *B. thuringiensis* is a pathogen of insects and is a rare cause of opportunistic infections and food poisoning in animals and humans. During sporulation, this organism forms parasporal crystals composed of insecticidal proteins called Cry proteins. Cry proteins have molecular weights of 40 to 140 kDa and are toxic to several orders of insects, including members of the Coleoptera (beetles), Diptera (mosquitoes), and Lepidoptera (butterfly and moth larvae). The genes for these toxins are located on large transmissible plasmids. Several other extracellular products of *B. thuringiensis* such as Vip proteins and β-exotoxin are also potent insecticides. All of these insecticides are purified, manufactured, tested for safety and investigated for environmental impacts before they can be licensed and marketed. Products of *B. thuringiensis* account for about 90% of the world's microbial pest control agents market. *B. mycoides* is a nonmotile species that forms rhizoidal colonies on agar. For many years, *B. mycoides* was considered a variant of *B. cereus*, but they are genetically distinct taxa.[794] DNA studies and chemotaxonomic analysis of whole-cell fatty acids delineated a new species within "*B. mycoides,*" resulting in the species, *B. pseudomycoides*.[793] *B. weihenstephanensis* is closely related to *B. cereus* and includes primarily psychrotolerant strains that grow at 4°C to 7°C but not at 43°C.[647] *B. gaemokensis* and *B. manliponensis* are new members of the *B. cereus* group that were isolated from tidal flat sediment of Gaemok Harbor and Manlipo Beach, both of which are in the coastal region of the Yellow Sea in the Republic of Korea.[563,564]

Bacillus anthracis

Epidemiology of Anthrax. Anthrax, the classical disease caused by *B. anthracis*, is primarily a disease of wild and domestic herbivorous mammals, including cattle, sheep, goats, bison, antelope, and deer. The organisms exist as a spore and as a vegetative, actively replicating, large gram-positive bacillus. The spores are dormant forms that are found in the soil, particularly in warmer, wetter climates.[294] Animals ingest the spores while grazing on contaminated vegetation. Illness in animals usually appears within 3 to 7 days after exposure to the spores. Animals usually develop fulminant systemic disease with edema, hemorrhage, hemorrhagic discharges from body orifices, and necrosis.[531]In infected animals, the bacteria are shed in blood and other body fluids, and the organisms subsequently sporulate and contaminate the soil and water, where they can remain viable for decades. Because of this ability to persist, animals suspected of having anthrax should be incinerated after death to prevent dissemination of spores. Outbreaks of anthrax in North America usually occur in the hot, dry summer months. Disruption of the soil by winds or construction in regions where animals have died may result in further dissemination of spores and to additional cases of disease. Other animals feeding on the carcasses of dead animals

may help to disseminate both vegetative bacilli and spores, and biting insects can act as mechanical vectors by spreading spores to vegetation after feeding on infected carcasses.[1124] Anthrax is most common in South America, Central America, sub-Saharan Africa, Central and Southwestern Asia, and Southern and Eastern Europe.[530,1127] In the United States, this disease has been essentially eradicated because of the widespread use of effective human and animal vaccines since the late 1930s, improvements in hygienic procedures used in factories processing animal products, and increased use of synthetic fibers as alternatives to animal hides, fur, and hair. However, because the spores of *B. anthracis* are highly resistant to adverse environmental conditions and because it is exceedingly difficult to be sure that the organism has been truly eradicated from former endemic regions, a high index of suspicion should be maintained if a clinically compatible illness suddenly appears. Historically, anthrax is usually defined in terms of its mode of acquisition. **Industrial anthrax** refers to cutaneous inoculation or inhalation of anthrax spores by those individuals employed in the cleaning and industrial processing of contaminated leather hides, wool, hair, or bone from various animals.[63] **Nonindustrial anthrax** refers to the acquisition of the organisms from close contact with infected animals or with their carcasses and tissues.[63,182] At-risk individuals in this category would include slaughterhouse workers, veterinarians, butchers, and ranchers, with cutaneous anthrax being the most common clinical presentation.

B. anthracis has been exploited and developed as an agent of biologic warfare by several countries, including Japan, the former Soviet Union, the United Kingdom, Iraq, and the United States.[926] In 1992, the Soviets admitted their ongoing efforts in the weaponization of anthrax over the previous few decades, and divulged that, in 1979, an accidental release of anthrax spores from a Soviet military laboratory in Sverdlovsk resulted in over 77 cases of inhalational anthrax, with more than 66 human fatalities.[183,184,748] The potential for bioterrorism associated with *B. anthracis* was brought into sharp focus in the United States during the last 4 months of 2001, when spores of *B. anthracis* were delivered in mailed letters and packages via the US Postal Service to locales within the continental United States.[183,184] As a consequence of these exposures, 22 people developed anthrax, including 11 cases of cutaneous disease and 11 cases of inhalational anthrax. Five people with pulmonary disease died. Since these incidents occurred soon after the September 11 attack on the World Trade Center, the Pentagon, and United Airlines Flight 93, several laws were passed by Congress placing limitations on access to and possession of select agents—a CDC- and USDA-sanctioned listing of pathogens believed to be the most dangerous. Additional funding for research on these agents was also made available. These incidents also resulted in the passage of anti-terrorism legislation in the United States and the development the Bioterrorism Preparedness Initiative. Through this initiative, the

CDC, along with the Association of Public Health Laboratories (APHL), various federal agencies (i.e., the Federal Bureau of Investigation [FBI], and the Department of Defense [DOD]), and the various states' public health laboratories, established the Laboratory Response Network (LRN).[177,178] In the LRN, clinical microbiology laboratories throughout the country

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are integrated into a four-tier network. Sentinel laboratories include clinical microbiology laboratories in hospitals; advanced-level sentinel laboratories must have class II biologic safety cabinets and establish and maintain compliance with biosafety level-2 laboratory practices. Through the LRN, technologists in laboratories perform basic tests to rule out potential biothreat agents and to refer possible agents to the next level—reference laboratories.[31,1168] These laboratories are usually the state public health laboratories, where confirmatory testing of referred isolates can be done in a timely manner. Referrals from this level are made to national laboratories that have biosafety level 4 facilities: the CDC and the US Army Medical Research Institute for Infectious Diseases (USAMRID). Most hospital-based clinical laboratories with microbiology capabilities are sentinel members of the LRN. Sentinel laboratory tests for detection of possible *B. anthracis* are found in Table 14-4 and will be addressed in the "Isolation and Identification" section.

Virulence Factors of *B. anthracis.* *B. anthracis* is highly monomorphic, shows little genetic variation, and produces toxins that are composed of three distinct proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF).[614,866]Virulent strains also are encapsulated. *B. anthracis* harbors two large plasmids called pX01 (182 kb) and pX02 (95 kb).[765] The structural genes for PA, EF, LF—*pagA*, *cya*, and *lef*, respectively—are found on the pX01 plasmid. These genes reside noncontiguously in a 30-kb area on the pXO1 plasmid within a 44.8 kb "pathogenicity island".[817] Another gene in this region, *atxA*, encodes the trans-activating transcriptional regulator AtxA and the repressor PagR, which regulate expression of the toxin genes.[614] pX02 contains a five-gene operon consisting of *capB*, *cap C*, *capA*, *capD*, and *capE;* these genes function in the synthesis of the poly-γ-D-glutamic acid capsule, which enables vegetative cells of *B. anthracis* to evade phagocytosis and killing by macrophages. The five capsular genes and the genes for capsular transcriptional regulators AcpA (*acpA*) and AcpB (*acpB*) are located on a 35-kb "pathogenicity island" on pX02. Expression of the transcriptional regulators for the capsule are also under the control of AtxA from pX01.[860] PA is synthesized and secreted by the organism and binds to TEM8 (tumor endothelial marker 8) and CMG 2 (capillary morphogenesis protein 2)

sites on target cells, where it undergoes proteolytic cleavage.[134] A 20-kDa amino-terminal fragment is released, and the remaining 63-kDa PA fragment polymerizes with other similar fragments to form a ring-shaped heptamer, which forms a pore through the cell membrane.[321,1194] Each 63-kDa PA monomer in the heptamer acts as an "adhesion subunit" and binds one molecule of either EF or LF, and the PA-EF and PA-LF complexes enter the target cell by endocytosis. Acidification of the phagosome allows these complexes to be released from the phagosome and the complexes dissociate to free LF and EF. LF is a zinc metalloprotease and EF is a calmodulin-dependent adenylate cyclase. EF increases intracellular cAMP levels, which leads to inhibition of phagocytosis, and deregulation of cytokine production.[1092] In macrophages, LF inhibits macromolecular synthesis, promotes apoptosis, and hydrolyzes protein kinases involved in intracellular signal transduction. Interruption of signaling pathways results in the inactivation of transcriptional factors in the cell nucleus and induction of proinflammatory cytokines.[764] During infection, LF and EF are able to suppress the cellular immune response by all of these mechanisms. Toxin gene expression and toxin production are specifically enhanced at the genetic level by CO_2 and growth at 35°C to 37°C.[613] Organism growth under these conditions results in fourfold to sixfold enhancements in the transcription of toxin genes and in toxin synthesis.[765]

Interestingly, several *B. cereus* group strains carry plasmids that are related genotypically to pX01 and pX02, but these plasmids do not carry the anthrax toxin genes, capsular genes, the pathogenicity islands, or the regulatory genes found on the *B. anthracis* plasmids.[614,850,851] In addition, certain important phenotypic properties of *B. anthracis* (i.e., nonmotility, β-hemolysis, sensitivity to γ-bacteriophage, failure to produce phospholipase C) result from a unique single base mutation in a chromosomal gene called *plcR*, which encodes a global transcriptional regulatory protein called PlcR.[11,1033] This gene is functional in *B. cereus* and *B. thuringiensis*, but the single-point mutation in the *B. anthracis plcR* gene renders the gene product nonfunctional. In *B. cereus*, PlcR interacts with a second molecule in a quorum-sensing system that controls expression of genes encoding phospholipases, proteases, hemolysins, and collagenases.[412] In most cases, *B. anthracis* can be easily distinguished from *B. cereus* and *B. thuringiensis*, but there are strains of *B. cereus* that contain the toxin and capsular virulence genes and that also have the *plcR* mutation and, that may express a phenotype like *B. anthracis*. In fact, three recent clinical isolates of *B. cereus* were isolated from metal workers in Texas and Louisiana with pneumonias similar to inhalational anthrax.[507,508] These isolates contained the entire *B. anthracis* pX01 plasmid with the full complement of virulence and regulatory genes. In addition, two *B. cereus* group strains recovered from 10 great apes in the Ivory Coast and Cameroon contained both pX01 and pX02 plasmids, with all the required regulatory

genes and capsular biosynthesis genes.[602,658,659,1164] Further genotypic analysis, phenotypic studies, and MLST-based phylogenetic taxonomic studies established that these isolates were indeed, **not** *B. anthracis* in the classic sense. The nine chimpanzees and a single gorilla died from anthrax-like illnesses. Much work is now being done to further characterize these and other isolates among the "nonanthrax *B. cereus*" group organisms.

Clinical Presentations of Anthrax. Human anthrax may be divided into four clinical forms, depending upon the mode of organism acquisition. These forms include cutaneous anthrax, oropharyngeal/gastrointestinal anthrax, inhalational anthrax, and injectional anthrax.[1075] **Cutaneous anthrax** accounts for 95% to 99% of cases worldwide and most cases occur in Africa, Asia, and eastern Europe, where livestock and animal worker vaccinations are not readily available. Infection results from direct mucosal contact with infected animals, animal products (e.g., hair or animal hides), soil containing the organism, or exposure to spores.[288] Spores enter through small cuts or abrasions and germinate subcutaneously within local macrophages and in the regional lymph nodes.[1212] Lesions of cutaneous anthrax usually occur on exposed areas of the skin (e.g., hands, arms, neck, wrist,

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TABLE 14-4 Phenotypic Characteristics for Identification of Members of the "*Bacillus cereus* Group"

Characteristic	*B. anthracis*	*B. cereus*	*B. thuringiensis*	*B. mycoides*	*B. pseudomycoides*	*B. weihens-tephanensis*	*B. gaemokensis*	*B. manliponensis*
CAT	+	+	+	+	+	+	+	+
OX	–	V	–	–	–	–	–	–
MOT	–	+	+	–	–	NA	+	+
CHAINS	+	+	+	+	+	NA	–	+
SPORE shape	Oval	Oval	Oval	Oval	Oval	NA	Oval	Oval
SPORE LOC	ST	ST, C	ST	ST	ST, C	NA	T	T
NO_3 RED	–	+	+	–	+	+	+	+
GROWTH TEMP	NA	10–50	10–43	7–30	15–40	5–43	15–40	15–40
ANAER	+	+	+	+	+	–	+	+
ADH	–	+	+	–	+	–	+	–
VP	–	+	+	+	+	+	+	–
CIT	+	+	+	–	–	–	–	–
LEC	+	+	+	+	+	NA	NA	NA
Acid produced from:								
GLU	–	+	+	+	+	+	+	+
MAL	–	+	+	+	+	+	+	+
FRU	–	+	+	+	+	+	+	–
SUC	–	+	+	+	–	–	+	–
RIB	–	+w	+	–	+	–	+	+
TRE	–	+	+	+	+	+	+	–
MNE	–	–	+	–	–	–	+	–
SAL	–	+	+	–	–	+	+	–
CELL	–	+	–	–	–	–	–	–
STA	–	+	+	–	+	–	+	–
GLYG	+	+	+	–	+	–	+	–
TUR	–	–	+	–	–	–	+	–
NAG	–	+	+	–	+	+	–	+
Hydrolysis of:								
GEL	+	+	+	+	+	+	+	+
STA	+	+	+	+	–	+	+	–
CAS	+	+	+	+	+	+	–	–

CAT, Catalase; OX, oxidase; MOT, Motility; CHAINS, cells in chains; SPORE LOC, spore location in cell; T, terminal; ST, Subterminal; C, Central; NO_3 RED, reduction of nitrate to nitrite; GROWTH TEMP, temperature range for growth; ANAER, growth under anaerobic conditions; ADH, arginine dihydrolase; VP, Voges–Proskauer (acetoin production); CIT, citrate utilization; LEC, Lecithinase; GLU, glucose; MAL, maltose; FRU, fructose; SUC, sucrose; RIB, ribose; TRE, trehalose; MNE, mannose; SAL, salicin; CELL, cellobiose; STA, starch; GLYG, glycogen; TUR, turanose; NAG, *N*-acetyl-glucosamine; CAS, casein; GEL, Gelatin.

and face). After an incubation period of 2 to 7 days (range of 1 to 19 days), a painless or pruritic initial papule develops at the site of inoculation.[287] Cutaneous lesions are generally single, but sometimes two or more lesions may be present. The lesion is surrounded by an area of edema and erythema due to growth of the organism and the elaboration of edema toxin locally. The lesion rapidly evolves into a vesicle or bullous lesion that is less than 4 cm in diameter, and the fluid within the vesicle becomes black

from hemorrhage into the lesion. This lesion eventually ulcerates and develops a black-bottomed, central eschar with surrounding edema. Frequently, small, pearl-like satellite lesions may appear surrounding the central

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eschar. Eschars of cutaneous anthrax are characteristically painless, although the surrounding edematous reaction may be painful. The eschar usually dries up and sloughs over the next 10 to 14 days. On biopsies, eschars usually show massive edema with necrosis and lymphocytic infiltrates, with focal hemorrhage and no abscess formation.[710] Large, deep eschars may require surgical removal with subsequent skin grafting once the localized inflammation and edema subside 4 to 6 weeks later.[288] Localized or generalized painful lymphadenopathy may be observed, depending on the extent and severity of the initial infection, and constitutional symptoms (e.g., fever, malaise, headache) may also be present. In severe cases, a toxemic shock picture may present, with hypothermia, cutaneous inflammation with extensive edema, leukocytosis with a predominance of polymorphonuclear leukocytes, hypoalbuminemia, and elevated liver enzymes.[288] Rare complications of cutaneous anthrax include airway obstruction due to massive neck edema, bacteremia, meningitis, and deep-tissue necrosis with scar tissue formation.[286,574,1036,1095] Cutaneous anthrax may resolve spontaneously in some cases, but the mortality associated with untreated cutaneous anthrax may be as high as 10% to 20%. With treatment, mortality is less than 1%.[1193]

Oropharyngeal anthrax and **gastrointestinal anthrax** are acquired by ingestion of inadequately cooked foods (usually meats) containing either spores or vegetative bacilli.[77,998,1075] Both of these forms of infection may become systemic and, like the pulmonary form, have a high mortality if untreated. The incubation period of gastrointestinal anthrax is 2 to 7 days; in one large outbreak, the infection was associated with ingestion of contaminated beef, and the incubation period was 42 hours.[1027]Oropharyngeal anthrax usually presents as an oral or esophageal ulcer, with regional lymphadenopathy and edema.[1028] Eschars may be noted on the posterior pharynx, tonsillar areas, or the hard palate. Progression of the infection results in dysphagia and a severe sore throat. Gastrointestinal anthrax involves primarily the terminal ileum and cecum (Image Plate 14-2C). Symptoms begin with fever, nausea, malaise, and vomiting and progress rapidly to an acute abdomen, sepsis and bloody diarrhea. A few days after onset, cloudy or outright purulent ascites that is teeming with anthrax bacilli may develop.[22] The mucosal surfaces of the gastrointestinal tract show extensive necrosis, edema, and mesenteric lymphadenitis, and anthrax bacilli can be cultured from the gastrointestinal tissues and inflammatory infiltrates. Profound

illness due to loss of blood, electrolyte imbalance, and toxic shock may develop.[569] Mortality can be as high as 60% to 100% if not aggressively treated. Death usually results from overwhelming sepsis, and intestinal blockage with subsequent intestinal perforation.[1028] Gastrointestinal anthrax has been reported in the United States in a patient who presented with abdominal cramps, nausea, vomiting, and hypotension. The patient went on to develop massive ascites, nodular hemorrhagic mesenteric lesions, small bowel necrosis, and retroperitoneal abscesses and hematomas. Unfortunately, respiratory failure ensued that required intensive support, along with antimicrobial chemotherapy and administration of anthrax immune globulin.[187,605]

Pulmonary or inhalational anthrax results from inhalation of *B. anthracis* endospores.[998] In the past, this infection was seen in persons whose occupations involved handling animal hides contaminated with anthrax spores. Although this situation no longer obtains due to animal control guidelines and vaccinations, occasional reports of inhalational anthrax still appear now and then from endemic regions. Following inhalation, the spores reach the lungs, where they are phagocytosed and cleared by macrophages of the reticuloendothelial system. The macrophages migrate to the hilar and mediastinal lymph nodes, where the spores germinate and mature into vegetative bacilli.[514] The organisms multiply and produce toxins, causing edema and hemorrhagic necrosis of the mediastinum, which is evident on chest films as marked mediastinal widening. From the substernal area, the organisms invade the pleural space, resulting in hemorrhagic pleural effusions.[514,1210] The macrophages harboring the organisms are killed and the vegetative bacteria escape into the general circulation where they continue to multiply. Hematogenous dissemination seeds distant sites, leading to metastatic lesions throughout the body, including the gastrointestinal tract and CNS. Anthrax meningitis is a fulminant complication that can follow cutaneous, gastrointestinal, or inhalational anthrax. This complication may develop in up to 30% to 40% of patients with inhalational anthrax and has a mortality of 100%.[514,634] Most patients do not survive long enough to manifest signs and symptoms of meningitis; patients are usually lucid until systemic shock supervenes, resulting in coma and death. The incubation period of inhalational anthrax is 1 to 4 days, followed by the sudden onset of flu-like symptoms, such as fever, chills, myalgias, substernal pain and a nonproductive, dry cough, and nausea with vomiting. After 3 or 4 days of this prodromal illness, fulminant disease follows, with the development of dyspnea, hypotension, an elevated hematocrit, and mental status alterations reflecting overwhelming toxemia, often progressing to coma and death in 24 to 48 hours. On chest roentgenogram, infiltrates, pleural effusions, and widening of the mediastinum may be noted; histologic examination of lung tissues at autopsy usually show focal necrotizing, hemorrhagic pneumonitis and necrosis of peribronchial and mediastinal lymph nodes with no abscess formation or consolidation. Gastrointestinal

involvement results in abdominal pain, hematemesis, and melena. The mortality associated with this form of the disease exceeds 80% to 90% if left untreated.

Injectional Anthrax. This newly described clinical presentation of anthrax infection was first recognized in an injection drug user in Norway who skin-popped heroin into his right buttock.[941] Within 4 days, extensive swelling and erythema involved the entire right gluteal region. The patient was placed on anti-staphylococcal therapy and discharged until he returned 4 days later with meningitis and shock, swelling, edema, and erythema involving the gluteal region, the thighs, and lower abdomen. Unlike cutaneous inoculation anthrax, papules, vesicles, and classical eschars were not observed. Surgery revealed no abscesses but extensive tissue edema was present, and the CSF and intraoperative wound specimens all grew *B. anthracis*. The patient died within 3 days of admission while on high-dose penicillin plus chloramphenicol. This seemingly isolated scenario was not reported again until December of 2009, when two intravenous drug users were hospitalized in the United Kingdom with *B. anthracis* isolated from their blood

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cultures. By the end of 2010, there were 47 confirmed cases with 13 deaths reported in Scotland, 5 cases with 4 deaths in the United Kingdom, and 2 cases with 1 death reported from Germany.[78,127,893,898] The clinical presentation of injectional anthrax is quite variable. In most patients, soft tissue infection is evidenced by swelling in the area of drug injection after 1 to 10 days. Surgical examinations of infected tissues show edema, necrosis of the fatty tissue, and diffuse bleeding; in some cases patients developed deep wounds requiring wide excisional debridement or, in the case of a groin infection, a fasciectomy due to fasciitis and peritonitis. Constitutional symptoms consisting of chills, fever, and nausea were also seen in some patients. Injectional anthrax has a mortality rate as high as 34%; this is partly due to the increased likelihood of recurrent shock with this form of disease and failure to consider this uncommon diagnosis. The heroin involved in these outbreaks was suspected of being contaminated because it was smuggled into Europe from anthrax-enzootic regions in Afghanistan, Iran, and Turkey.

Antimicrobial Susceptibility and Treatment of *B. anthracis* Infections. *B. anthracis* is susceptible to several antimicrobial agents, including tetracyclines, macrolides, clindamycin, aminoglycosides, fluoroquinolones, carbapenems, rifampin, and the first-generation cephalosporins (cephalothin, cefazolin). *B. anthracis* is resistant to the second- and third-generation cephalosporins (i.e., cefotaxime, ceftriaxone, ceftazidime), monobactams (i.e., aztreonam), and trimethoprim–sulfamethoxazole.[174,216,766,1128] Resistance to penicillin, ampicillin, and second-

generation cephalosporins was detected in 11.5% of 96 *B. anthracis* isolates from animals and the environment in France from 1994 through 2000.[174]

Treatment regimens for anthrax differ depending on the clinical presentation and the severity of disease.[183,184,1057] For naturally acquired (e.g., occupational) cutaneous infections, oral penicillin is the drug of choice. The recommended agent is amoxicillin (adults, 500 mg every 8 hours; children, 80 mg/kg/day every 8 hours). Unless there is clear evidence that the infection was naturally acquired, the possibility of anthrax infection being acquired by intentional release of spores must be kept in mind. The use of penicillin and ampicillin is controversial and these agents should not be used alone because of constitutive or inducible β-lactamase production by some strains of *B. anthracis*. The recommended treatment for gastrointestinal and inhalational anthrax is intravenous (IV) ciprofloxacin (400 mg every 12 hours) or IV doxycycline (100 mg every 12 hours), plus addition of another intravenous agent (i.e., ampicillin, imipenem, clindamycin, clarithromycin, rifampin, vancomycin). Once clinically indicated, oral therapy (ciprofloxacin, 500 mg twice daily; doxycycline 100 mg twice daily) can be substituted. Treatment of children uses the same agents with dosages adjusted for age and weight. In the event that the patient develops meningitis, the fluoroquinolone regimen should be used instead of doxycycline due to superior penetration into the CNS; in such instances, when the second IV agent is added, it should be able to penetrate into the CNS as well.[72] In most cases, 10 to 14 days of intravenous antimicrobial chemotherapy is sufficient, followed by oral therapy. Since the US anthrax mailings in 2001, it was recommended that treatment be continued for up to 60 days in case the patient harbored spores that may germinate into vegetative cells later.[189]

Patients being treated for anthrax also require intensive supportive care, and newer adjunctive treatment regimens are available to address these issues. Patients with inhalational and injectional anthrax often develop recurring pleural effusions that contain large numbers of anthrax bacilli. Frequent thoracentesis and drainage of accumulating pleural fluids is recommended and has been shown to increase survival.[514,546] Anthrax immune globulin (AIG) is also available as an adjunctive therapeutic agent. This material is made by plasmaphoresis of persons who were immunized with anthrax vaccine (see below) and is available from Cangene Corporation in Canada. AIG contains predominantly antibodies against the PA and should be used along with antimicrobial chemotherapy for treatment of severe disease (primarily inhalational anthrax).[1175] Human monoclonal antibodies against the PA of *B. anthracis* are also available through consultation with the CDC. These novel therapeutic agents were developed following passage of the Project Bioshield Act in 2004, which allows the FDA to approve anti-biothreat agents and drugs that show efficacy only in animal models and, based on these data, would have reasonable

benefit in humans.[327] This legislative measure also established the Strategic National Stockpile, which stores medical supplies and drugs as a protective measure in case of an attack. These agents may have utility as preexposure prophylactic agents and as postexposure therapy for inhalational anthrax.[732] Three anti-PA monoclonal antibodies have been developed and tested that have demonstrated protective efficacy in animal models of inhalational anthrax. These include Raxibacumab (human recombinant IgG monoclonal antibody [Human Genome Sciences]), Anthim (an affinity-enhanced monoclonal antibody [Elusys Therapeutics]), and Valortim (human monoclonal antibody [PharmAthene/Medarex]).

Prevention of Anthrax. The first-generation human vaccine to be licensed for use in the United States is BioThrax (formerly Anthrax Vaccine Adsorbed (AVA), Bioport, Lansing, MI), a cell-free formulation prepared from a culture filtrate of a toxigenic, nonencapsulated, avirulent strain of *B. anthracis* (strain V770-NP-1-R) adsorbed to aluminum salts as an adjuvant.[112,127] This culture filtrate contains PA, LF, and EF, with PA being the major component. Following the initial injection, five more doses are given over the next 18 months to complete a six-dose regimen. After receipt of the six-injection series, annual booster immunizations are given to maintain a protective level of antibodies.[874] The AVA vaccine is recommended for those who work directly with *B. anthracis* in the laboratory, those who work with animal products (e.g., hides and furs) imported from areas of anthrax endemicity, those who work with animals and animal products within endemic regions, and military personnel who are deployed to high-risk regions of the world. In addition, anthrax vaccine may be used as preexposure prophylaxis for those individuals with quantifiable risks of exposure to anthrax and, in conjunction with CDC, may be used along with antibiotics for postexposure prophylaxis of inhalational anthrax. The vaccine is well tolerated and appears to be

safe.[231,429] Work is proceeding on the development of new vaccines, with the most promising candidate antigen being highly purified, recombinant PA (rPA).[487,577] Two vaccines using rPA have been manufactured in the United States and the United Kingdom. The US product was tested and found to be immunogenic and safe, but the instability of the vaccine on storage precluded its ability to be stockpiled for future use. Work on third-generation rPA-based vaccines is proceeding, with the examination of different adjuvants, novel inoculation and administration strategies, and use of candidate vaccine antigens other than rPA.[60,137,548,581,1059]

Bacillus cereus

Virulence Factors of *B. cereus*. *B. cereus* produces several potential virulence factors in addition to the toxins associated with gastrointestinal infections (see below), and these factors are thought to play a role in nongastrointestinal infections.[130] Virulence factors include three hemolysins and three phospholipases. The hemolysins are called cereolysin, hemolysin II, and hemolysin III, respectively. Cereolysin is similar to streptolysin O (SLO) in being a heat-labile protein that is activated by thiols and inhibited by cholesterol. Hemolysin II is a hemolytic toxin that is also a lethal toxin in mice, and hemolysin III causes osmotic lysis of erythrocytes by transmembrane pore formation. Phospholipases include phosphatidylinositol hydrolase, phosphatidylcholine hydrolase, and a sphingomyelinase. The phospholipases cleave lipid moieties that serve to anchor cell-surface proteins, compromise cell membrane integrity, and abrogate regenerative capacities of injured cells. *B. cereus* strains also produce at least three different β-lactamases, extracellular collagenases, and membrane-bound proteases.

***B. cereus* Gastroenteritis.** *B. cereus* is found in the soil and contaminates many agricultural products. Several different food items may become contaminated with vegetative cells or spores of *B. cereus,* including pastas, rice, milk products, grains, spices, vegetables, meat, chicken, and seafoods.[988] Some *B. cereus* strains are able to grow at low temperatures, so even refrigerated food are subject to spoilage by this organism.[395] The presence of spores in food products is another issue, since spore germination occurs at 65°C to 75°C, which is similar to the range used for high temperature-short duration pasteurization methods for dairy products. The highly resistant spores can also withstand exposure to γ-radiation. Biofilm formation by some strains of *B. cereus* makes it difficult to eradicate these and other organisms from pipes, vats, and other containers used in food processing and manufacturing.[858] Contaminated foods, dairy products, and hospital- or pharmacy-prepared supplements and infant formulas represent potential risks for hospitalized patients, particularly children and immunosuppressed patients. Since food-borne illness due to this organism is not reportable, exact figures on incidence and prevalence are not available for most parts of the world. Between 1973 and 1985, *B. cereus* was responsible for 17.8% of the food-borne outbreaks in Finland, 11.5% in the Netherlands, 2.2% in Canada, 0.8% in Scotland, 0.7% in England and Wales, and 0.7% in Japan. The incidence in the United States is also quite low, with only 1.3% of bacterial food poisoning cases being attributable to *B. cereus* between 1972 and 1982. It is estimated that over 27,000 cases of food-borne illnesses in the United States were caused by *B. cereus*.[739,823]

Enterotoxin-producing strains of *B. cereus* are known to cause acute, self-limited gastroenteritis in humans, usually following ingestion of contaminated foods. This

syndrome follows two clinical forms, depending on the type of toxin produced by the organisms present in the suspect food. Short-incubation illness (or emetic food poisoning) is characterized by the onset of symptoms within 1 to 6 hours after ingestion of contaminated foods, with the predominant symptoms being nausea, vomiting and abdominal cramping. Symptoms generally abate within 10 to 24 hours of onset. Incriminated foods associated with the emetic type of illness include Asian fried rice dishes, cream and milk products, pastas, and reconstituted infant formula. The responsible heat-stable exotoxin produced by these *B. cereus* strains is a cyclic dodecadepsipeptide called **cereulide**, which has a molecular weight of about 1,165 Da and is very heat-stable.[12,13] With rice dishes, the rice is usually cooked, cooled, and then held at room temperature, during which time the vegetative cells outgrow and produce cereulide, which is not destroyed by reheating. This molecule is also stable in the presence of acids, alkalis, and proteases and is nonantigenic. Cereulide causes swelling of the mitochondria and uncoupling of oxidative phosphorylation.[755] In mice, cereulide causes liver pathology and in cell cultures, this toxin causes mitochondrial swelling and eventually apoptosis.[834] Cereulide poisoning causing fulminant liver failure was reported as a cause of death in a patient in 1997.[706] High levels of cereulide were found in the liver, bile, small intestine and blood of the patient, and on the cooking utensils and pan used to reheat the contaminated food. The gene for cereulide has been identified and a PCR assay was designed for its detection.[303]

Long-incubation *B. cereus* food poisoning occurs 8 to 16 hours following ingestion, and is characterized predominantly by the onset of profuse watery diarrhea, nausea, tenesmus, and lower abdominal cramps.[694] Symptoms usually abate after 12 to 14 hours. Foods associated with this type of illness include meat and vegetable dishes, cakes, sauces, and dairy products. Four different heat-labile enterotoxins are associated with this clinical presentation including two protein complexes (hemolysin BL [HBL] and nonhemolytic enterotoxin [NHE]), and two enterotoxic proteins (enterotoxin T and cytotoxin K).[988] HBL and NHE are composed of three proteins each, and the genetic loci encoding for either HBL or NHE are organized in discreet and separate operons located on the chromosome of *B. cereus* (and occasionally *B. thuringiensis*).[162] HBL is an exotoxin that has hemolytic activity ("H") and is composed of three proteins: a 37.8-kDa binding component ("B") and two proteins (L1, 38.5 kDa, and L2, 43.2 kDa) that have cytolytic and dermonecrotic properties.[81] Genes encoding the B component (*hblA*), L1, (*hblD*), and L2 (*hblC*) are arranged in a tandem operon adjacent to each other.[957] All three components of HBL are required for biologic activity. HBL causes hemolysis of the red blood cells from a variety of mammalian species, including guinea pig, swine, sheep, rabbit, human, and horse blood. HBL binds to the cell membrane of eukaryotic cells, forming pores that result in leakage of the cell contents. HBL is also dermonecrotic, causes increases in vascular

permeability,

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and causes accumulation of fluids, villi necrosis, submucosal edema, and lymphocytic infiltration in the rabbit ileal loop assay.[82] HBL from different strains of *B. cereus* demonstrate a high degree of molecular heterogeneity, differ in the extent and species host range for hemolysis, and usually have similar biologic activities.[83] NHE is a 41-kDa antigenic protein that is associated with 39.8-kDa and 36.5-kDa proteins that are encoded by a three-gene operon.[432] This tripartite toxin is dermonecrotic, cytotoxic, and alters membrane permeability.[697] Other workers have characterized and reported on other enterotoxins from various *B. cereus* strains, but HBL and NHE are considered to be the toxins responsible for *B. cereus* diarrheal illness resulting from food poisoning. Some isolates of *B. cereus* may produce both the emetic toxin and the enterotoxin complexes. Intoxication associated with *B. cereus* and its toxins may be avoided by adequate cooking of foods, particularly meats, and proper refrigerated storage. *B. thuringiensis* has also been isolated in association with gastroenteritis outbreaks and has demonstrated cytotoxicity that is similar to that caused by enterotoxigenic *B. cereus*.[542] On rare occasions, other *Bacillus* species (e.g., *B. subtilis*, *B. licheniformis*) may also cause gastroenteritis and food poisoning.

Opportunistic Infections Caused by B. cereus, *Other* Bacillus *Species, and* Paenibacillus *Species*

Because of their ubiquity in the environment, the recovery of *Bacillus* species from clinical specimens has most frequently been considered as nuisance contamination. However, the increasing frequency of reports in the literature of *Bacillus* species as agents of disease in compromised hosts suggests that these organisms should not be dismissed as contaminants in all cases. Serious *Bacillus* and *Paenibacillus* species infections have been associated with operative procedures, immunosuppression, traumatic wounds, burns, hemodialysis, and parenteral drug abuse. Isolates recovered from nongastrointestinal infections produce a variety of putative virulence factors, including hemolysins, necrotizing exotoxins, and phospholipases. Analyses of large series of cases involving significant *Bacillus* infections indicate their involvement in the clinical syndromes discussed below. *B. cereus* is well recognized as an agent of gastroenteritis and is the *Bacillus* species that is most frequently recovered as an opportunistic agent from compromised hosts. Box 14-2 details infections caused by nonanthrax *Bacillus* species other than *B. cereus* plus infections caused by *Paenibacillus* species.

***B. cereus* Bacteremia and Endocarditis.** Bacteremia and endocarditis with *B. cereus* is usually seen in intravenous drug users, patients with valvular heart disease, those with prosthetic valves or cardiac implants (e.g., pacemakers), or patients who are immunosuppressed due to cancer or chemotherapy.[2,171,233,409,498] Endocarditis is a rare event, but in the setting of parenteral (usually heroin) drug abuse, it is a well-recognized complication. In these cases, organisms probably originate from contaminated injection paraphernalia or from the heroin. Endocardial infection usually involves the tricuspid valve, with septic emboli to the lungs being noted on chest films. Drug users with *B. cereus* endocarditis will have compatible clinical findings and multiple positive blood cultures a short time after parenteral drug administration. *Bacillus* bacteremia may also reflect nosocomial contamination of implanted intravascular catheters. In these cases, removal of catheter hardware often will result in clearance of the organisms from the bloodstream. Sometimes a source of *B. cereus* bacteremia cannot be pinpointed, and polymicrobial bacteremia with *B. cereus* and *S. epidermidis*, members of the *Enterobacteriaceae*, or *P. aeruginosa* is well documented.[65] Premature and very low birth weight infants are also at risk for serious infections with *B. cereus*; organisms in these cases are usually isolated from both blood and CSF cultures or from endotracheal aspirates.[498,668] *B. cereus* has also been a cause of catheter-related sepsis in children with various neoplastic disease receiving parenteral chemotherapy.[170]

***B. cereus* Infections in Compromised Hosts.** *B. cereus* infections in immunocompromised patients have included fulminant bacteremia and sepsis, CNS infections (meningitis, meningoencephalitis, brain abscess), lower respiratory tract infections with empyema, penetrating wound infections, postsurgical infections, and burn wound infections.[45,65,402,646,712,833,964] CNS infections occur as a result of spinal anesthesia, fistula repair, spread from a contiguous focus of infection, or from contaminated ventriculoperitoneal shunts.[204,402,716] *B. cereus* infection of premature and very low birth weight infants usually involves both the bloodstream and the CNS and outcomes in these babies are usually poor.[498,668,712] Pulmonary infections, including necrotizing pneumonitis, pseudomembranous tracheobronchitis, pneumonia, and acute respiratory failure with sepsis have occurred primarily in patients with leukemias, hepatocellular carcinoma, and in premature neonates.[353,757,1062] Reasons for immunosuppression in these patients have included severe alcohol abuse, leukemia, lymphoma, induction chemotherapy, meningioma, rheumatoid arthritis, systemic lupus erythematosus, severe burns, diabetes, severe combined immunodeficiency disease, and AIDS. Lee and colleagues[656] reported a fatal case of bacteremia, spontaneous bacterial peritonitis and necrotizing fasciitis due to *B. cereus* in a 47-year-old man whose underlying conditions included cirrhosis due to chronic

hepatitis B infection and esophageal varices. Fatal outcomes in many of these patients reflect generalized immunosuppression resulting from immunologic disease, cytotoxic chemotherapies used for treatment, and the use of indwelling devices used for life support (e.g., total parenteral nutrition) and receipt of therapies.

***B. cereus* Ocular Infections.** Endophthalmitis and other ocular infections with *B. cereus* ordinarily occur following penetrating eye injuries (exogenous infection) or as a consequence of bacterial seeding of the posterior segment of the eye during bacteremic episodes resulting from indwelling devices, transfusions. contaminated drug injection paraphernalia, or contaminated drugs (endogenous infection).[258,728,756,1003,1121] Penetrating eye injuries involving *B. cereus* often occur in association with specific occupations (metalworkers, construction crews) and settings (rural areas, farming accidents).[258,720] In penetrating injuries and/or

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BOX 14-2

Members of the Genus *Bacillus* (Other Than the *B. anthracis* and *B. cereus*) and *Paenibacillus* Isolated From Human Infections

Species	Infections	References
Bacillus circulans	*B. circulans* has been isolated from blood cultures as a cause of endocarditis, pericarditis, catheter-related bacteremia, and fatal sepsis in immunocompromised patients (e.g., patients receiving anti-neoplastic chemotherapy, bone marrow and solid organ transplant recipients). This organism has also been isolated as a cause of peritonitis in a patient receiving chronic ambulatory peritoneal dialysis and as a cause of endogenous endophthalmitis and wound infections.	21, 65, 98, 170, 422, 452, 618, 1090
Bacillus coagulans	This organism is a rare cause of bacteremia in patients with cancer.	65
Bacllis hackensackii	This new species was isolated from a single blood culture from a 22-year-old woman with abdominal pain and tenderness.	516

Bacillus licheniformis	*B. licheniformis* has been isolated as a cause of Hickman, Broviac, and central venous catheter-related bacteremias. This organism was also isolated from a cluster of bloodstream infections in patients with hematological malignancies; the source of the organism was traced to contaminated cotton used during skin disinfection. *B. licheniformis* has also been associated with endophthalmitis and brain abscess following penetrating orbital injuries, and has been recovered from peritoneal fluid in patients receiving CAPD. *B. licheniformis* is also a rare cause of prosthetic valve endocarditis and has been isolated from a parotid gland abscess.	120, 469, 480, 556, 665, 689, 730, 833, 841, 1105
Bacillus megaterium	This *Bacillus* species has been isolated from a cutaneous anthrax-like ankle lesion and as a cause of delayed-onset lamellar keratitis in a male patient 2 weeks after LASIK surgery.	298, 897
Bacillus pantothenticus	*B. pantothenticus* was isolated from the blood and a liver abscess of a 44-year-old immunocompetent man.	790
Bacillus pumilis	*B. pumilis* has been isolated from blood cultures of septic neonates, as a cause of central venous catheter-related sepsis in a child on total pareneteral nutrition, and from primary cutaneous infections resembling cutaneous anthrax in three shepherds.	65, 89, 596, 1101
Bacillus sphaericus	*B. sphaericus* was isolated from a cancer patient with bacteremia.	65
Bacillus subtilis	*B. subtilis* is a rare cause of bacteremia in patients with cancer, head tauma, and pyelonephritis. In some cases, bacteremia was due to a *B. subtilis* strain that was administered to the patients as a probiotic. This organism was also associated with two cases of severe hepatotoxicity following consumption of Herbalife nutritional products contaminated with *B. subtilis*.	65, 812, 923, 1058
Bacillus thuringiensis	This member of the *B. cereus* group has industrial and commercial applications due to production of insecticidal proteins. Like *B. cereus*, this organism also produces hemolysins, proteases, and, occasionally, enterotoxins. *B. thuringiensis* has been isolated from blood cultures, soft tissue infection (periorbital cellulitis), burn wounds,	250, 403, 482, 622, 856

	periodontal and pulmonary infections.	
Paenibacillus alvei	This organism was isolated from blood cultures and from an infected hip arthroplasty of a 26-year-old woman with sickle cell disease.	902
Paenobacillus cineris	This species, originally isolated from soil in Antarctica, was isolated from a sputum specimen of a 25-year-old man during a acute exacerbation of his cystic fibrosis.	645, 685
Paenibacillus hongkongensis	This organism was isolated from one of four sets of blood cultures collected from a 9-year-old boy with neutropenic fever who had undergone surgery, radiation, and several cycles of chemotherapy for treatment of a medulloblastoma.	1102
Paenibacillus konsidensis	This organism was isolated from two sets of blood cultures collected from a 75-year-old man with persistent fevers, hematemesis, and hypotension.	609
Paenibacillus larvae	*P. larvae* is known to cause American foulbrood, a severe disease of honey bees, and this organism can be cultured from honey. Bacteremia with this organism occurred in five intravenous drug users who were injecting methadone opioid that was mixed with honey to be used as an oral medication. The clinical course of bacteremia with *P. larvae* was benign in three patients, but one patient developed spontaneous bacterial peritonitis, and another developed pulmonary emboli.	928

865

Paenibacillus macerans	*P. macerans* was isolated from blood cultures of eight neonates in the intensive care unit. None of these infants were infected, as evidenced by the absence of fever and leukocytosis. Infection control identified that the pseudobacteremia was due to contamination of the rubber stoppers of the blood culture bottles.	809
Paenibacillus massiliensis	This species was recovered from a blood culture of a 13-year-old boy with acute lymphoblastic leukemia who received chemotherapy and subsequently underwent a stem cell transplant. At the time the blood culture was collected, the patient was neutropenic and had cutaneous	950

	and hepatic graft-versus-host disease.	
Paenibacillus polymyxa	This species was isolated from a blood culture from a 93-year-old woman who suffered a cerebral infarction.	797
Paenibacillus provencensis	Isolated as a contaminant from the CSF of a 54-year-old man with Whipple's disease	949
Paenibacillus sanguinis	This species was isolated from a blood culture of a 49-year-old man who was receiving chemotherapy and radiation for epidermoid carcinoma of the oropharynx.	950
Paenibacillus sputi	Isolated from the sputum of a 60-year-old woman who was being screened for tuberculosis infection	594
Paenibacillus thiaminolyticus	*P. thaminolyticus* was isolated from blood cultures of an 80-year-old man with a history of subtotal colectomy with ileostomy for colon cancer. The patient also had hypertension, congestive heart failure, Parkinson's disease, and end-stage renal disease requiring hemodialysis. The patient had an indwelling Permacath, which was thought to be the source of the bacteremia.	832
Paenibacillus timonenesis	This organism was isolated from a blood culture collected from a 75-year-old woman who presented with chronic interstitial nephropathy requiring hemodialysis.	950
Paenibacillus urinalis	This organism has been isolated as a contaminant from a urine specimen of a 36-year-old woman with suspected tuberculosis.	949

retained foreign bodies within the orbit, periorbital edema and corneal inflammation lead to vitreal infection that spreads to the retina, resulting in loss of visual acuity within 12 to 24 hours after the injury. Diagnosis is made by immediate anterior-chamber paracentesis and collection of blood cultures.[728,756] Fever and leukocytosis are usually present. Endophthalmitis may be complicated by spread of the organisms to contiguous anatomic sites, including the brain and CNS. Rare cases of *B. cereus* keratitis and endophthalmitis have also been reported in association with contaminated contact lens care systems and following cataract surgery and LASIK procedures.[293,953] This syndrome follows a rapid clinical course and may result in loss of vision requiring enucleation of the eye.[258] Because these infections are rapidly

destructive, a high index of suspicion and aggressive therapy (e.g., vancomycin, ceftazidime, aminoglycosides) administered systemically and intraocularly is required.[756] In the clinical laboratory, Gram's stains of material aspirated from aqueous and vitreous humor specimens should be interpreted carefully, and the isolation of a *Bacillus* species from these specimen must be reported to the physician and not dismissed as a contaminant. The rapidly destructive course of intraocular *B. cereus* infections has been attributed to the production of toxins and destructive enzymes by the causative organism.

***B. cereus* Skin, Bone, and Soft Tissue Infections.** *B. cereus* may cause posttraumatic cutaneous and subcutaneous infections, necrotizing fasciitis, cellulitis, myonecrosis, and acute and chronic osteomyelitis.[442,743,990] These infections are often assumed to be due to clostridia, until culture results prove otherwise. Posttraumatic wound infection, soft tissue infection, necrotizing fascitis, and osteomyelitis caused by *B. cereus* have been documented in individuals who had sustained severe trauma such as automobile accidents or gunshot wounds.[552,779,1206] Intravenous drug use, underlying hematologic malignancies, or treatment with immunosuppressive therapies are risk factors for the development of these infections.[442,960] The presence of underlying disease (e.g., leukemia, sickle cell disease, myelodysplastic syndrome) in individuals suffering trauma also predisposes them to more complicated *B. cereus* infections and superinfections, including fasciitis and myonecrosis.[960] *B. cereus* wound infections may be highly destructive to tissues and may requiring wide excisional debridement or amputation of affected limbs to prevent spread of the infection.[743] In intravenous drug users, soft tissue infection with *B. cereus* can lead to bacteremic episodes that seed distant sites, and unusual infections such as acute vertebral osteomyelitis have been reported as a result of hematogenous spread. *Bacillus* species may also cause significant infections in burn patients. The tissue destruction that is seen with severe wound infections caused by *Bacillus* species is related to production of extracellular enzymes that have histolytic and histotoxic properties (e.g., hemolysins, phospholipases, proteases).

Nosocomial Infections. Outbreaks of *Bacillus* gastroenteritis have been reported in health care facilities

and in other institutional settings. In these cases, implicated foods have been beef stews, fried rice, chicken, and processed turkey. Other nosocomial outbreaks of *Bacillus* infections have involved common-source spread from contaminated reservoirs in the environment.[730] These sources have included contaminated

hemodialyzers, bronchoscopes, Ommaya reservoirs, manual ventilation balloons, multiple unit injectables, and contaminated diapers, gloves, and surgical bandages.[166,489,924,1143,1235] In 2001, pseudobacteremia in a neonatal intensive care unit caused by *Paenibacillus macerans* was traced to contaminated blood culture bottles.[809] *Bacillus* species have also been isolated from peritoneal fluids in cases of nosocomial peritoneal dialysis-associated peritonitis. Pseudo-bacteremias caused by *Bacillus* species have also been traced to contaminated stoppers of blood culture bottles and have been temporally related to environmental disruption, such as hospital construction.[683]

Laboratory Safety, Specimen Collection, and Processing

If anthrax is suspected, the states' public health laboratory and the CDC should be notified immediately. Specimens that may be collected include material from cutaneous lesions, blood cultures, and any other potentially infected tissues. Laboratory safety is of utmost importance when working with any materials thought to contain *B. anthracis*. All specimens and cultures must be examined and carefully processed in a class II biologic safety cabinet. All precautions should be taken to minimize the generation of aerosols from the potentially infected material. Laboratory personnel must wear protective coats, gowns, masks, and gloves when manipulating specimens. This apparel must be discarded or autoclaved before re-use. After completion of specimen processing and inoculation of plated and tubed media, all surfaces in the biologic safety cabinet and the open benchtops must be disinfected with 5% sodium hypochlorite and all instruments used for specimen processing must be autoclaved. In addition, laboratory personnel who work with spore suspensions or contaminated animals carcasses and tissues must be appropriately immunized.

Specimens for isolation of *B. anthracis* should reflect the mode of acquisition and the consequent pathology of the infection. For the diagnosis of cutaneous anthrax, specimens of serous fluids or a biopsy obtained from beneath the surface of the eschar should be collected along with blood cultures. Diagnosis of suspected inhalational anthrax necessitates collection of sputum specimens and blood cultures. Gastric aspirates and fecal specimens may be submitted to aide in the diagnosis of gastrointestinal anthrax along with blood cultures. For suspected *B. cereus* gastroenteritis, food that is epidemiologically linked to the illness should be collected. Since *B. cereus* may be present in the stool of healthy individuals and since there is no typing system for this organism readily available to clinical laboratories, the recovery of *B. cereus* from the stools of ill patients does not provide sufficient evidence to ascribe an etiologic role to the isolates. It may be worthwhile, however, to recover the organism from the feces of individuals in an outbreak situation provided similar specimens from appropriately matched controls are demonstrated not to contain the organism. Isolates from implicated foods and from the stool of patients can be sent to

a reference laboratory (e.g., the state health department laboratory or the CDC) for identity confirmation and strain typing. A stool specimen of sufficient volume (25 to 50 g) should be collected in a sterile, leak-proof container. Shipment of the food, stool specimens, and/or isolates to the state health department or the CDC should be undertaken after prior notification and discussion of the outbreak situation with these agencies.

Isolation and Identification of the "Bacillus cereus *Group"*

Members of the genus *Bacillus* that are recovered from clinical specimens usually grow well and sporulate on sheep blood and chocolate agars incubated at 37°C under aerobic conditions. Sporulation may be stimulated by subculture onto nutrient agar supplemented with $MnSO_4$ (5 μg/mL final concentration) and subsequent incubation. On Gram's stains from blood cultures, other broth media, and occasionally solid media, *Bacillus* and related species may appear as gram-negative bacilli, resulting in the mistaken impression that the organism is a gram-negative, nonfermentative bacillus. Inoculation of broth media and preparation of a Gram-stained smear after a few hours of growth may reveal the gram-positive nature of the organisms. Intracellular and cell-free spores do not stain by the Gram's technique but may be visualized with the malachite green stain. A smear of the organism is made on a slide, heat-fixed, and stained with 10% malachite green for 45 minutes. The smear is washed, counterstained with safranin for 30 seconds, and observed under oil immersion. The spores will appear green, while vegetative cells are stained pink to red. Unstained, spores appear as slightly refractile, clear, round-to-oval structures within and outside of vegetative cells (Image Plate 14-2D).

On SBA, colonies of *B. cereus* and *B. thuringiensis* are usually large with a matte or ground glass texture and most strains are β-hemolytic (Image Plate 14-2H). These colonies have a butyrous consistency with entire, crenate or fimbriate edges. In contrast, colonies of *B. anthracis* are usually smaller, gray-white, rough-surfaced, nonhemolytic, and have a tenacious consistency such that, when the colonies are touched with an inoculating loop, they form standing peaks resembling beaten egg whites (Image Plate 14-2E, 14-2F, and 14-2G). Under the dissecting microscope, numerous undulating, comma-shaped outgrowths consisting of filamentous chains of bacilli surround the *B. anthracis* colony, giving the appearance of a "Medusa head" from Greek mythology (Image Plate 14-2F). Colonies of *B. mycoides* are flat, nonhemolytic, and have a sinuous or rhizoid appearance that resembles some clostridia. On Gram-stained smears, cells of the *B. cereus* group (including *B. anthracis*) are 3 to 8 μm long and 1.2 to 1.4 μm wide gram-positive bacilli that occur singly or in chains. Individual cells have squared-off or concave ends. Oval spores may be observed centrally or subterminally and the cells are not swollen in the areas where the spore is located. Phase-contrast microscopy of smears of *B. thuringiensis*

may show crystals adjacent to the spore (parasporal crystals). These crystals, which consist of the naturally produced insecticidal toxin, may also be observed by staining a smear of the organism with malachite green.

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All members of the *B. cereus* group produces lecithinase on egg-yolk agar and hydrolyze casein, starch, and gelatin. *B. thuringiensis* and some *B. cereus* and *B. mycoides* strains are arginine dihydrolase-positive; *B. anthracis* is arginine dihydrolase-negative. Indole is not produced by any of the *B. cereus* group, and most strains reduce nitrate to nitrite. *B. anthracis* is also nonmotile in hanging drop preparations or in semisolid motility medium, while *B. cereus*, *B. thuringiensis*, and *B. mycoides* are motile. In addition, *B. anthracis* strains are often encapsulated in infected tissues, as demonstrated with an India ink wet prep or with the M'Fadyean capsule stain.[1051] Formation of capsular material can be stimulated by subculture of the organism on nutrient agar containing 0.7% $NaHCO_3$ and incubation at 37°C overnight. The capsule of *B. anthracis* is composed of poly-D-γ-glutamic acid and it functions to prevent phagocytosis. *B. anthracis* may also be definitively identified and differentiated from the other *Bacillus* species by gas chromatographic whole-cell fatty acid analysis or by susceptibility to theγ-bacteriophage. Biochemical characteristics for identification of *B. anthracis*, *B. cereus*, *B. thuringiensis* and the other members of the *B. cereus* group are presented in Table 14-4.

B. anthracis is one of several potential agents of bioterrorism. The CDC and members of the LRN have determined that all *Bacillus* species recovered in the clinical laboratory be screened for detection of possible *B. anthracis*. Agents that fit the screening criteria are forwarded to state public health laboratories for definitive identification. In general, these characteristics include Gram's stain and colony morphology, presence or absence of hemolysis, production of catalase, and determination of motility. At the referral laboratories, the identity of presumptive *B. anthracis* strains is confirmed by whole-cell fatty acid analysis, susceptibility to γ-bacteriophage, or real-time PCR methods. The sentinel laboratory characteristics for screening of *Bacillus* isolates for possible *B. anthracis* are presented in Box 14-3. Additional characteristics that may be helpful include the capsule stain (performed directly on clinical material) and a β-lactamase test. Most nonanthrax *Bacillus* species are nonencapsulated and resistant to penicillin due to production of β-lactamase enzymes. The chromogenic cephalosporin disk test (Nitrocefin, BD Microbiology Systems) can be used for the latter test.

Identification of *Bacillus* species and related species can be accomplished by observation of cellular morphology, the shape, position, and appearance of endospores, and the determination of biochemical characteristics using conventional

tests.[920] Spore shapes and locations are usually determined by Gram's stain or a spore stain, and cell motility is determined by stab inoculation of semisolid motility medium. Carbohydrate acidification can be assessed with semisolid CTA medium containing 1% carbohydrates. Nitrate reduction and production of indole are detected by inoculation of indole nitrate broth and development with the appropriate reagents, respectively. Gelatin, casein, and starch hydrolysis are determined by inoculation of tubed or plated conventional media. Miniaturized kit systems like API 20E (includes cupules for arginine dihydrolase [ADH], indole, nitrate reduction, and gelatin hydrolysis) and the API 50 Carbohydrate (API 50CH) may also be helpful for differentiation of *B. anthracis* from other *B. cereus* group organisms, and for identifying other *Bacillus* and related species.[684] The Vitek system (bioMérieux, Inc.) has a stand-alone *Bacillus* identification card, and the Biolog system (Biolog, Inc., Hayward, CA) also has a robust database of *Bacillus* species.[61] Fatty acid methyl-ester analysis using the Microbial Identification System (MIDI) (Microbial Identification, Inc., Newark, DE) is also available for identification of these organisms. The aerobic

bacteria library of the MIDI system includes some members of the *B. cereus* group (i.e., *B. cereus*, *B. thuringiensis*, *B. mycoides*), several other environmental *Bacillus* species, and some members of the genus *Paenibacillus*. MIDI also has a Biodefense Library that includes *B. anthracis* and other potential agents of bioterrorism. *Bacillus* species have also been identified using a variety of chemotaxonomic methods, including pyrolysis mass spectroscopy, Fourier-transform infrared spectroscopy, and polyacrylamide gel electrophoresis. MALDI-TOF has also been used to identify *B. anthracis* and other members of the *B. cereus* group.[589,640]

BOX 14-3

Sentinel Laboratory Screening Tests for Presumptive Identification of Bacillus anthracis

Category	Characteristics
Safety	Biosafety level 2 for processing of clinical specimens; Biosafety level 3 laboratory practices for all culture manipulations that may produce aerosols (mincing, cutting, vortexing, etc.); Perform all work in a biologic safety cabinet
	Rapidly growing colonies, 2–5 mm in diameter after incubation for

Colonial characteristics	18–24 hours at 35°C–37°C Non-pigmented, dry, "ground glass" surface; flat or slightly convex; Irregular edges with "comma-shaped" projections ("Medusa's head" colony); Sticky, tenacious consistency such that, when colonies are touched with a loop, the colonial growth will stand up like beaten egg whites No hemolysis on SBA
Microscopic characteristics	Large, gram-positive bacilli, single and/or in chains; May become gram-variable after 72 hours; May be encapsulated in clinical material and in blood culture media; Terminal/subterminal spores do not swell the vegetative cell; Spores are not present in clinical material unless exposed to low CO_2levels, such as that found in ambient air; higher CO_2 levels will inhibit spore formation
Key characteristics	Nonhemolytic, nonmotile, catalase-positive; spores present when cultured aerobically without CO_2; β-lactamase-negative

Because of the potential role of *B. anthracis* in bioterrorism, much work has been done to develop methods for direct detection of the organism in both environmental and clinical specimens. While cultural methods are fairly reliable, isolation and identification of potential *B. anthracis* is time consuming. Various affinity-based methods, such as antibody-based enzyme immunoassays directed against the BclA surface glycoprotein, the poly-D-glutamic acid capsule, the *B. anthracis* S-layer, or extracellular antigen-1 (EA-1) have been developed.[620,1086,1087,1177] In addition to surface antigens of *B. anthracis*, assays for detection of the three toxin protein antigens—PA, EF, and LF—have also been exploited using antibodies against these antigens as ligands.[977] Unfortunately, these assays lack both sensitivity and specificity. EIAs for detection of *B. anthracis* spores have a detection limit of 10^5 to 10^6 spores, which was 100- to 1,000-fold less sensitive than that claimed by the manufacturer.[598] Molecular methods such as PCR have also been proposed for direct detection, but care must be taken to select gene targets and to design primer pairs and probes that are specific for *B. anthracis*. Gene targets for molecular assays have included the pXO1 and pXO2 plasmids of *B. anthracis*, but pXO1- and pXO2-like plasmids are found in various nonvirulent *B. cereus* strains and therefore affect the specificity of these real-time PCR assays. *B. cereus* strains that cause anthrax-like illness are also detected by

plasmid-specific PCR identification tests.[507,508,600,601] Several workers have examined *B. anthracis* chromosomal genes in order to design specific molecular assays, but, again, these efforts have been hampered by the presence of genes with similar nucleotide sequences in other *B. cereus* group organisms.[118] Certain chromosomal genes and "signature" nucleotide sequences, such as the BA-813 sequence, the BA-5449 sequence, and the *rpoB* gene, were initially thought to represent *B. anthracis*-specific genotypic markers and, therefore, useful for detection and identification.[608] However, subsequent studies have detected counterpart genes with similar nucleotide sequences in *B. cereus* strains. It has also been discovered that some *B. thuringiensis* strains share *rpoB* polymorphisms with *B. anthracis*.[1244] In 2008, Antwerpen and colleagues[42]identified a genomic marker that has not yet been found in *B. cereus* or other nonanthrax *Bacillus* species. The sensitivity and specificity of this sequence for detection and identification of *B. anthracis* is currently being evaluated.

Molecular methods have also been used for strain typing, and molecular epidemiology of *B. cereus* group isolates associated with food-borne outbreaks and clusters of apparent nosocomially transmitted infections.[680,729,920,1099] PFGE, AFLP, and multilocus sequence typing (MLST) have been shown to have insufficient discriminatory power to resolve *B. anthracis* of various origins.[483,575] Variable number tandem repeats (VNTRs) found in open reading frames of *B. anthracis* genes and intergenic areas on the *B. anthracis* chromosome and on the pX01 and pXO2 plasmids has been used to devise a VNTR typing method called multiple locus VNTR analysis or MLVA.[576,1137] Between 8 and 25 loci can be used to discriminate among *B. anthracis* isolates using this method.[678,1145]

Antimicrobial Susceptibility Testing of Bacillus *Species*

The CLSI M45-A2 approved guideline for susceptibility testing of infrequently isolated or fastidious bacteria includes a procedure for microbroth antimicrobial susceptibility testing of *Bacillus* species, not *B. anthracis*.[214] The method uses cation-adjusted Müeller–Hinton broth and an inoculum prepared from a direct colony suspension equivalent to a 0.5 McFarland turbidity standard. The tests are incubated at 35°C in ambient air for 16 to 20 hours. Clindamycin, the fluoroquinolones, and vancomycin are suggested for primary testing. Breakpoints are available for penicillin, ampicillin, first- and second-generation cephalosporins, imipenem, gentamicin, amikacin, erythromycin, tetracycline, ciprofloxacin, levofloxacin, trimethoprim–sulfamethoxazole, rifampin, and vancomycin.[214] Most *Bacillus* species are penicillin- and cephalosporin-resistant due to production of broad-pectrum β-lactamase enzymes.

Corynebacterium Species

Introduction and Taxonomy

The genus *Corynebacterium* contains many species and includes the classical pathogen, *C. diphtheriae*, the causative agent of diphtheria. While diphtheria is relatively uncommon in developed countries due to widespread vaccination, it is a cause of significant morbidity and mortality in resource-limited countries, and sporadic outbreaks are occasionally reported in the United States. Many of the organisms in the genus *Corynebacterium* constitute part of the normal flora of the skin and the upper respiratory tract.[1166] These same species, as well as other *Corynebacterium* species and related coryneform bacteria, have become recognized as significant agents of human disease, particularly in compromised hosts and patients with indwelling medical devices. Application of molecular (i.e., 16S rRNA and rDNA sequencing, nucleic acid hybridization), chemotaxonomic (i.e., peptidoglycan analysis, cellular fatty acid studies, menaquinone characterization) and expanded phenotypic methods (i.e., kit identification systems, organic compound assimilation tests, detection of preformed glycosidase or aminopeptidase enzymes) to the characterization of gram-positive bacilli has resulted in the description of many new *Corynebacterium* species isolated from human clinical specimens, from animals, and from the environment. Analysis of unnamed coryneform isolates has resulted in their assignment to previously described genera (e.g., the genera *Corynebacterium*, *Arcanobacterium*, and *Actinomyces*) and to new genera (e.g.,

Turicella, *Cellulosimicrobium, Dermabacter*). Some CDC coryneform group isolates belong to genera not previously associated with human infections (e.g., *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Microbacterium*). Genera that are related to *Corynebacterium* species include the nocardioform organisms (*Nocardia*, *Streptomyces*, *Oerskovia*, *Rhodococcus*, *Gordona*, *Dietzia*, *Tsukamurella*), the facultative and anaerobic actinomycetes (*Actinobaculum*, *Actinotignum*, *Actinomyces*), anaerobic "diphtheroids" (*Propionibacterium*, *Propioniferax*), and the acid-fast bacilli (*Mycobacterium*). Chemotaxonomic and phenotypic descriptions of the genus *Corynebacterium* and related coryneform genera found in human clinical specimens are presented in Table 14-5. Some of these genera will be discussed in this chapter, while others (e.g., *Propionibacterium* species) are described in subsequent chapters.

Genus *Corynebacterium* include pleomorphic, club-shaped, gram-positive bacilli that are catalase-positive, nonmotile, non–spore-forming, and non–acid-fast. Most *Corynebacterium* species contain mycolic acids, arabinose, and galactose in their cell

walls.[1220] The peptidoglycan of *Corynebacterium* species contains ***meso*-diaminopimelic acid** (*meso*-DAP) as the constituent di-amino acid. Distinctive cellular fatty acids and menaquinones are also found in the cell membranes of these organisms (Table 14-5). Classical descriptions of this genus include their tendency to form "picket fence" and "Chinese letters" arrangements on Gram-stained smears (Image Plate 14-3A). Most species are facultative and fermentative in their metabolism of carbohydrates, although a few species are non-fermentative and/or utilize carbohydrates oxidatively. Some species also require lipids for optimal growth (lipophilism). This requirement is suggested by growth on lipid-containing media (e.g., SBA) and either poor or no growth on commercially available chocolate agar, which contains hemin powder instead of lysed erythrocytes. Lipophilism is best demonstrated by enhanced growth of these species on medium containing 0.1% to 1% Tween-80 in comparison with the same medium lacking Tween-80 (Image Plate 14-3B).

The application of "research" techniques to the characterization of coryneform organisms has also created problems for the clinical laboratory, where resources for genetic/chemotaxonomic identification methods are limited and phenotypic identification is the norm. Two clinical isolates may produce identical results on phenotypic testing (e.g., an API Coryne), but when genetic and chemotaxonomic methods are applied, these isolates may be different (e.g., different cellular fatty acids, different di-amino acids in the peptidoglycan, etc.) Because of limited phenotypic characterization of many environmental genera (e.g., *Microbacterium* species), the assignment of clinical isolates to these genera is more difficult and, unfortunately, less accurate. As a result of scientific progress, the interpretation of clinical case reports regarding individual *Corynebacterium* or coryneform species has become problematic because isolates described in these reports may not have been rigorously characterized.

Identification of Corynebacterium *Species and Coryneform Bacteria*

Identification of *Corynebacterium* species and other coryneform bacteria was originally performed with phenotypic tests like other organisms in the clinical laboratory. With the application of molecular methods, the number of species in the genus *Corynebacterium* and related genera has expanded so rapidly that, by necessity, the number of phenotypic tests required for identification has increased as well. Conventional tests are performed as for other organisms (e.g., enteric gram-negative bacilli) with some modifications. Requirements for lipid are determined by inoculating the organism as a lawn onto a non-lipid-containing medium (e.g., Müeller–Hinton agar, BHI agar, Tryptic soy agar) and placing a drop of 0.1% Tween-80 onto the inoculum. After incubation, lipophilic strains will show enhance growth in the area of Tween deposition and poor or no growth in areas away from the lipid (Image Plate 14-3B).

Nonlipophilic strains will grow over the entire agar surface. Conventional identification media (i.e., carbohydrate utilization tests) may also require supplementation with 3 to 4 drops of 0.1% to 1% Tween-80 (per 5 mL of broth media) for identification of lipophilic strains. Gas-liquid chromatography for detection of volatile and nonvolatile products of glucose fermentation is also helpful. All taxa produce small to moderate amounts of acetic, lactic, and succinic acids from glucose, but others may produce propionic acid. This characteristic is often helpful in separating strains that have very similar biochemical profiles (e.g., separation of *C. amycolatum* [propionate-positive] from *C. striatum* [propionate-negative]).[723] Box 14-4 lists the currently described *Corynebacterium* species and their clinical significance, and Table 14-6, which starts on page 885, presents the phenotypic characteristics for the identification of *Corynebacterium* species.

Commercial products that address identification of coryneform gram-positive bacilli are the **API Coryne** (bio Merieux, Inc.), the **RapID CB-Plus** (Remel Laboratories), and the **Vitek 2 Anaerobe/*Corynebacterium* card** (bioMérieux, Inc.).

API Coryne. The API Coryne is a microcupule gallery identification kit comprised of 11 enzymatic tests, 8 carbohydrate fermentation tests, and a fermentation control cupule. A heavy organism suspension (#6 or greater McFarland standard) is prepared in water and placed in the enzyme test cupules (NIT through GEL). A 0.5 mL aliquot of this suspension is then placed in the GP medium (enriched medium with phenol red indicator) provided; this is used to fill the carbohydrate control and test cupules (O through GLYG). Each of the latter cupules is overlaid with sterile mineral oil. The strip is incubated for 24 to 48 hours in a non-CO_2 incubator. Reagents A and B are added to the nitrate cupule (NIT) and ZYM A and ZYM B reagents are added to the enzyme cupules (PYZ through β-NAG); these reactions are read after 10 minutes. ESC, URE, GEL, and the carbohydrate tests are read directly without the addition of reagents. From the pattern of reactions on the strip, a biocode is generated and interpreted by consulting the online database. The API Coryne database has been updated to accommodate some newly described taxa and to reflect changes in taxonomy, but nomenclature continues to change. Funke et al.[384] evaluated version 2.0 with 390 strains representing all 49 taxa included in the new database, plus 17 strains belonging to taxa not included in the database. The kit identified 90.5% of the strains belonging to included taxa,

TABLE 14-5 Characteristics of the Genus *Corynebacterium* and Other Coryneform Gram-Positive Bacilli

Genus	Cell Morphology	Catalase	Motility	Pigment	Growth	CHO Metabolism	Mycolic Acids	Peptidoglycan Di-Amino Acid	Menaquinones	Cellular Fatty Acids	Comments
Actinobaculum	Straight to slightly curved slender rods; may show some branching	–	–	None	Fac/Ana	Ferm	Absent	L-Lysine, L-Ornithine	MK-10(H4)	C16:0, C18:1(9C, cycl), C18:0	Four species, including former *Actinomyces suis*
Actinomyces	Rods, filamentous, branching	V	–	None; occasionally pink/red	Fac	Ferm	Absent	L-Lysine, L-Ornithine	MK-10(H_4)	C16:0, C18:1(9C, cycl), C18:0	CDC group 1, group 2, "group 1-like", and group E strains, plus other previously described *Actinomyces* species
Arcanobacterium	Short, irregular rods	V	–	None	Fac	Ferm	Absent	L-Lysine	MK-9(H_4)	C16:0, C18:1(9C, cycl), C18:0	Five species, including former *Actinomyces bernardiae*
Arthrobacter	Rod-to-coccus cycle	+	V	Variable	Aer	Oxid	Absent	L-Lysine	MK-8, MK-9, MK-9(H_2)	C15:0ai, C17:0ai, C15:0i	Some CDC group B-1 and B-3 strains
Brevibacterium	Rod-to-coccus cycle	+	–	Dirty white, yellow, orange, purple	Aer	Oxid (inert)	Absent	*meso*-DAP	MK-8(H_2), MK-7(H_2)	C15:0ai, C17:0ai, C16:0i	Some CDC group B1 and B-3 strains
Cellulomonas	Irregular rods; coccal forms	+	V	Yellow	Fac	Ferm/Oxid	Absent	L-Ornithine	MK-9(H_4)	C15:0ai, C16:0	CDC group A-3 (*C. hominis*)
Cellulosimicrobium	Irregular rods; rod-to-coccus cycle	+	–	Yellowish white	Fac	Ferm	Absent	L-Lysine	MK-9(H_4)	C15:0ai, C16:0i, C16:0, C16:0i	Includes *Cellulosimicrobium cellulans* (formerly *Oerskovia xanthineolytica*) and *Cellulosimicrobium funkei* (formerly *Oerskovia turbata*)
Corynebacterium	Rods, club-shaped bacilli	+	–	None, gray/white	Fac	Ferm/inert	V(C22–C38)	*meso*-DAP	MK-9(H_2), MK-8(H_2)	C18:1, C16:0, C18:0	*Corynebacterium* species
Dermabacter	Short bacilli or coccobacilli	+	–	None	Fac	Ferm	Absent	*meso*-DAP	MK-9, MK-8, MK-7	C17:0ai, C15:0ai, C16:0i	CDC groups 3 and 5
Exiguobacterium	Short, irregular rods with rod-to-coccus cycle	+	+	Pale orange, yellow	Fac	Ferm	Absent	L-Lysine	MK-7	C17:0i, C15:0i, C16:0	Include *E. acetylicum* resembles some CDC group A-4 strains
Leifsonia	Pleomorphic rods that fragment into shorter rods and cocci; motile via peritrichous flagella	+	+	Yellowish to deep yellow with age	Aer	Oxid, ferm (so CHO's)	Absent	DL-Diaminobutyric acid	MK-11, MK-10	C15:0,ai, C17:0,ai, C16:0i	Includes *L. aquatica* (formerly "*Corynebacterium aquaticum*" and other environmental bacteria
Microbacterium	Irregular, thin rods; some coccal forms	+	V	None-to-yellow	Aer	Oxid/weakly ferm	Absent	L-Lysine	MK-12, MK-11, MK-10	C15:0ai, C17:0ai, C16:0i	Some CDC group A-4 and A-5 strains and former *Aureobacterium* species

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Oerskovia	Coccoid to rudimentary filaments; vegetative but no aerial "hyphae"	+	V	Yellow	Fac/Aer	Ferm	Absent	L-Lysine	MK-9(H_4)	C15:0, C16:0, C15:0i, C17:0ai	Includes *O. jenensis* (from insects) and *O. paurometabola* (from soil)
Propionibacterium	Irregular rods with branching or "bifid" ends; some coccoid forms	V	–	White/gray-white	Ana/Fac	Ferm	Absent	LL-DAP	MK-9(H_4)	C15:0, C15:0ai, C17:0ai	Usually grow better under anaerobic conditions, especially on primary isolation; produce major amount of lactic acid from glucose
Propioniferax	Irregular rods	+	–	None	Aer	Ferm	Absent	LL-A_2pm	MK-9(H_4)	C15:0ai, C16:0i	Includes single species *Propioniferax innocua* (formerly *Propionibacterium innocuum*)
Rothia	Irregular rods with rudimentary branching	+	–	White, gray-white	Fac	Ferm	Absent	L-Lysine	MK-7	C15:0ai, C17:0ai, C16:0	Includes white and black colony variants and "*Rothia*-like" CDC fermentative coryneform group 4; other human isolate in the genus is *R. mucilaginosa*
Turicella	Irregular rods	+	–	None	Aer	Inert	Absent	*meso*-DAP	MK-10(H_2), MK-11(H_2)	C18:1, C16:0, C18:0	Single species (*T. otitidis*); phenotypically similar to *C. auris* and *C. afermentans*

Fac, facultative; Ana, anaerobic; Aer, aerobic.

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BOX 14-4

Members of the Genus *Corynebacterium* Isolated From Human Clinical Specimens

Species	Clinical Significance and Identification
Corynebacterium accolens	*C. accolens* has been isolated from human clinical specimens, including wound drainage, endocervical specimens, sputum, and throat swabs.[798] Cases of native mitral and aortic valve endocarditis, breast abscess, and pelvic osteomyelitis caused by *C. accolens* have been reported.[37,209,1204] *C. accolens* is a lipophilic species that forms small (i.e., less than 0.5 mm in diameter), gray, smooth, transparent, nonhemolytic colonies on SBAafter 48 hours incubation. It reduces nitrate but does not hydrolyze esculin or produce urease. Like most other corynebacteria, most *C. accolens* strains are pyrizinamidase-positive; however, alkaline phosphatase is not produced. Glucose and ribose are fermented, and some strains will also produce acid from sucrose and/or mannitol. Isolates of this species are susceptible to penicillin, cephalosporins, erythromycin, clindamycin, tetracycline, and the aminoglycosides, and are resistant to sulfamethoxazole.[209,798]
Corynebacterium afermentans subsp. *afermentans, Corynebacterium afermentans* subsp. *lipophilum*	Organisms belonging to CDC group ANF-1 are pleomorphic gram-positive bacilli that do not produce acid from any carbohydrates, hence the name ANF, meaning "absolute nonfermenter." Riegel and coworkers[931] examined the genetic and biochemical characteristics of 11 ANF-1 strains and proposed a new species, *Corynebacterium afermentans*, containing two subspecies, *C. afermentans* subsp. *afermentans* and *C. afermentans* subsp. *lipophilum*.[931] The former subspecies grows equally well in both the absence or presence of 1% Tween-80, although the colonies acquire a beige pigmentation on Tween-containing media. The latter subspecies forms very small colonies on SBA after 24 hours, but produces large, beige colonies on Tween 80-supplemented SBA. These organisms are biochemically inert except for positive pyrazinamidase (PYZ) and alkaline phosphatase (PAL) reactions. Phenotypically, *C. afermentans* subsp. *afermentans* resembles *Corynebacterium auris* and *Turicella otitidis*, both of which are associated with otitis media. All produce the same biocode on the API Coryne (biocode 2100004). These three organisms are differentiated based on colony morphology, CAMP test, DNase and leucine arylamidase (LAP) activities. *C. afermentans* subsp. *lipophilum* also produces the same API biocode and is the only lipophilic species that is CAMP test-positive. The isolate was susceptible to ampicillin, vancomycin, cefazolin, ceftriaxone, gentamicin, erythromycin, ciprofloxacin, and imipenem, and was resistant to clindamycin, and trimethoprim-sulfamethoxazole. *C. afermentans* subsp. *lipophilum* also has been described as the cause of a brain and a liver abscess in a previously healthy 39-year-old man and lung abscess with empyema in a patient with AIDS.[299,762] *C. afermentans* subsp. *afementans* has also been documented as a cause of sepsis following neurosurgery.[621]
Corynebacterium amycolatum	In 1988, Collins and associates[218] reported a new species of *Corynebacterium* isolated from human skin. Although *meso*-DAP, arabinose, and galactose were present in the cell wall, corynemycolic acids found in most *Corynebacterium* species were absent. Despite this departure from an important chemotaxonomic genus characteristic, 16S rRNA sequencing supported inclusion of this organism in genus *Corynebacterium*. The organism was named *Corynebacterium amycolatum* ("without mycolic acids") and accepted as a valid *Corynebacterium* species in 1988. *C. amycolatum* is regarded as an emerging pathogen in immunocompromised patients. This organism has been documented as an agent of sepsis in patients with underlying hematological malignancies and neutropenic fever.[8,264] Native valve endocarditis involving *C. amycolatum* has occurred in patients with preexisting valvular disease or as a nosocomial complication from contaminated peripheral access devices (e.g., left subclavian hemodialysis catheters, IV catheters).[85,248,254,264,607,1234] Oteo and coworkers[829] described three cases of *C. amycolatum* bacteremia in three patients. All had underlying disease (e.g., laryngeal carcinoma, chronic bronchitis, diabetes, deep venous syndrome, adenocarcinoma of the stomach) and bacteremia developed as a complication of pneumonia, infection of a hip fracture following open reduction/internal fixation, empyema, and hemothorax. *C. amycolatum* has been involved in catheter-related and surgical wound infections, mastitis, cardioverter lead-electrode infection, and septic arthritis following vascular graft sepsis.[211,852,1144] *C. amycolatum* was reported as the cause of fatal sepsis in a premature infant and recurrent peritonitis associate with continuous ambulatory periotoneal dialysis.[97,200,1234] Isolates of *C. amycolatum* show a range of MICs for the β-lactams, macrolides, clindamycin, aminoglycosides, quinolones, and rifampin. *C. amycolatum* strains are susceptible to tigecycline, vancomycin, tetracycline, linezolid, and quinupristin-dalfopristin, but macrolides and clindamycin usually demonstrate poor activity against this organism.[338,418,967] About 40% of 101 *C. amycolatum* strains tested in a 1996 study were resistant to β-lactam antimicrobial agents (MICs > 64 μg/mL).[380] In addition, the

	MICs of ciprofloxacin, chloramphenicol, clindamycin, and erythromycin at which 50% of the isolates were inhibited were all greater than the susceptibility breakpoints for staphylococci. Resistance to gentamicin was also seen in some isolates. *C. amycolatum* strains that show high-level fluoroquinolone resistance have single or multiple mutations in the *gryA* gene, resulting in MICs of >32 μg/mL for these agents.[418,1016,1234] Macrolide resistance in *C. amycolatum* is associated with methylase enzymes encoded by the *erm(X)* gene.[828]
	C. amycolatum characteristically produces flat, dry, whitish-gray, matte or waxy colonies on SBA. Confluent areas of growth on agar media may become "wrinkled-looking" on prolonged incubation (see Image Plate 14-3C). This colony morphology is easily differentiated from those of *C. minutissimum* or *C. striatum*, which grow as gray-white, moist colonies. *C. amycolatum* is nonlipophilic, pyrazinamidase- and alkaline phosphatase-positive and most strains also reduce nitrate to nitrite. Acid is produced from glucose and ribose, and most strains also ferment maltose and sucrose. Some isolates may be urease-positive and PYR-positive. *C. amycolatum* strains produce propionic acid as the major end product of glucose metabolism. *C. amycolatum* has likely been misidentified as *C. striatum, C. xerosis, C. minutissimum*, and CDC group I-1 by clinical laboratories for many years.[372,1185,1247] Table 14-6 shows the additional tests that can be used to differentiate *C. amycolatum* from these other corynebacteria.[912,1185,1188,1247] Common API Coryne profile numbers for *C. amycolatum* (i.e., 3100325, 3100125, 7100125, 3100365, 4100325, 3040121) usually provide a cross-call of *C. amycolatum/C. striatum*, necessitating additional tests for definitive identification (see Image Plate 14-3D, and 14-3E). Letek and colleagues described a real-time PCR assay based on the use of various primer pairs for amplification of the cell division gene, *divIVA*, that was able to specifically differentiate *C. amycolatum* from *C. striatum, C. minutissimum*, and *C. xerosis*.[670]
Corynebacterium appendicis	*C. appendicis* is a recently described species that was isolated from a postsurgical wound in a patient with appendicitis.[1230] Genotypically, this new species is most closely related to *C. afermentans*, *C. coyleae*, and *C. lipophiloflavum*. *C. appendicis* is a lipophilic species that is pyrazinamidase-, alkaline phosphatase- and urease-positive and produces acid from glucose and maltose only within 7 days (API Coryne reactions). Lactic acid is the major product from glucose fermentation.
Corynebacterium argentoratense	*C. argentoratense* is the name assigned to four isolates that were recovered from throat cultures of patients with tonsillitis, but the role of these bacteria in disease is not known. *C. argentoratense* is closely related to members of the "*C. diphtheriae* group" (i.e., *C. diphtheriae, C. pseudotuberculosis,* and *C. ulcerans*) and to *C. kutscheri*. Because of the genetic similarity to the "*C. diphtheriae* group", these strains were examined for the presence of the *tox* gene of the β-corynephage, but this gene was not detected. *C. argentoratense* is nonlipophilic, forms creamy gray-white, nonhemolytic colonies on SBA, and rapidly produces acid from glucose, fructose, and occasionally ribose.[937] Nitrate is not reduced and urea, esculin, and gelatin are not hydrolyzed. Pyrazinamidase is produced, but most strains are alkaline phosphatase-negative. This organism resembles *C. coyleae* except that *C. coyleae* is strongly CAMP test-positive and acidifies ribose, while *C. argentoratense* is CAMP test-negative and is ribose fermentation-variable. Like *C. amycolatum*, *C. argentoratense* produces propionic acid from glucose fermentation.
Corynebacterium atypicum	The description of *C. atypicum* corresponds to a single isolate obtained from an undisclosed human source.[461] The isolate was nonlipophilic, nonhemolytic, and did not hydrolyze esculin, gelatin, or starch, and was LAP-positive. Unlike most *Corynebacterium* species, both pyrazinamidase and alkaline phosphatase tests are negative. Acid is produced from glucose, maltose, sucrose, and ribose. This new species, like *C. amycolatum* and *C. kroppenstedtii*, lacks cell wall mycolic acids. Like *C. glucuronolyticum*, this species produces β-glucuronidase, with other enzymatic tests on the API Coryne being negative. The unique API code for this atypical species is 0200365.
Corynebacterium aurimucosum	*C. aurimucosum* has been isolated from human clinical specimens, including blood cultures from a patient with bronchitis, bone and joint infections, and diabetic foot wounds.[414,948,1229] The organism grows as nonlipophilic, sticky, slightly yellow colonies on SBA. On the API Coryne, the pyrazinamidase and alkaline phosphatase tests are positive and acid is produced from glucose, maltose, and sucrose, resulting in API Coryne biocode 2100125. Lactic acid is the principal product of glucose fermentation. Genotypically and phenotypically, *C. aurimucosum* closely resembles *C. minutissimum*. The API Coryne profile, 2100125, is the same for both species, except that *C. aurimucosum* produces yellow colonies and is hippurate hydrolysis-positive, while *C. minutissimum* grows as moist, gray-white colonies and is hippurate-negative. In 2004, Daneshvar et al.[253] reported on several charcoal-black-pigmented isolates from female genital tract specimens that were provisionally designated as CDC fermentative coryneform

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	group 4. These isolates were related at the species level to *C. aurimucosum* and *C. nigricans*, which was originally isolated from female genital tract specimens. Black-pigmented variants of *C. aurimucosum* appear to be specific to the female lower genital tract and are associated with complications during pregnancy (e.g., spontaneous abortion).[1118] Because of these isolates and their close relationship to *C. aurimucosum*, the description of this organism has been amended to include not only the original colony types ("sticky, slightly yellow") but also these charcoal-black pigmented variants. *C. nigicans* is a later synonym of *C. aurimucosum*.
Corynebacterium auris	In 1995, Funke and coworkers[373] reported isolation of 10 strains that were identified as CDC group "ANF-1-like", but were distinct from both *C. afermentans* subspecies and *T. otitidis* by cellular morphology and colonial appearance. Colonies of these isolates were dry, weakly adherent to the agar, and became slightly yellow with time, while colonies of both *C. afermentans* and *T. otitidis* were creamy, smooth, and gray-white. These strains were also phenotypically similar to both *C. afermentans* subsp. *afermentans* and *T. otitidis*, but could be differentiated by substrates in the Biolog identification system. Genetic analysis confirmed that the 10 strains were a new *Corynebacterium* species and the name *Corynebacterium auris* ("auris" referring to the ear) was proposed. *C. auris* grows as nonlipophilic, nonhemolytic, dry, adherent, yellowish colonies on SBA and, as mentioned, produces reactions in common phenotypic tests that are identical to *C. afermentans* subsp. *afermentans* and *T. otitidis*. However, both *C. auris* and *T. otitidis* are strongly CAMP test-positive, while *C. afermentans* is CAMP test-variable. In addition, *C. afermentans* subspecies and *C. auris* are DNase-negative, while *T. otitidis* is DNase-positive. LAP activity can be detected in *T. otitidis* and *C. auris*, while *C. afermentans* subsp. *afermentans* is LAP-negative.[913] On the API Coryne, the numerical biocode for *C. auris* is 2100004.
Corynebacterium bovis	This lipophilic species is associated mainly with cattle, although rare human infections have been documented.[4,95,247] *C. bovis* is a lipophilic species that is pyrazinamidase-negative, alkaline phosphatase- and urease-positive, and produces acid from glucose only. Rare strains are also weakly maltose-positive, although this reaction is negative on the API Coryne. The API Coryne profile for *C. bovis* is 0101104.
Corynebacterium canis	Like *C. freiburgense*, this organism was isolated from a dog bite wound along with *Bacteroides* species and *Prevotella* species, so this organism is likely found in the mouths of dogs.[363] This species forms dry, beige-white, adherent colonies with irregular, convoluted edges on SBA. The organism produces a variety of enzymes including pyrazinamidase, alkaline phosphatase, and α- and β-glucosidase. Urease, pyrrolidonyl arylamidase (PYR), α- and β-galactosidase, β-glucuronidase, and *N*-acetyl-β-d-glucosaminidase are not produced. *C. canis* produces acid from glucose, maltose, fructose, sucrose, trehalose, starch and glycogen, but not from lactose, ribose, xylose, mannitol, sorbitol, or amygdalin. The isolate was susceptible to all antimicrobial agents.
Corynebacterium confusum	*C. confusum* has been isolated from a plantar abscess, calcaneal osteomyelitis, and from blood.[377] The blood culture isolate was from a non-immunocompromised patient during a febrile episode following back surgery. This organism is nonlipophilic and produces a delayed acidic reaction from glucose (i.e., the GLU cupule on the API Coryne was positive only after 48–72 hours incubation). Failure to prolong incubation of the API Coryne strip past 24 hours resulted in identification of these strains as *C. propinquum* (see below), but these strains did not hydrolyze tyrosine (*C. propinquum* is tyrosine hydrolysis-positive). *C. confusum* reduces nitrate to nitrite, produces pyrazinamidase and alkaline phosphatase, does not hydrolyze esculin or urea, and is CAMP test-negative. All API enzymatic tests are negative. On the API Coryne, codes generated by this organism are 3100304 or 3100104, indicating that some strains are also ribose-positive. In the original report, strains of *C. confusum* were susceptibile to the penicillins, cephalosporins, aminoglycosides, tetracyclines, quinolones, and glycopeptides, and were variably susceptible to rifampin, and the macrolides.[377] All strains were resistant to aztreonam.
Corynebacterium coyleae	*C. coyleae* has been recovered from pleural fluid, pancreatic abscess, breast abscesses, genitourinary tract specimens, and blood cultures of patients with fevers of unknown origin secondary to underlying conditions (i.e., AIDS, surgery, diabetes, non-Hodgkin's lymphoma).[336,382,1078] Colonies of *C. coyleae* are nonlipophilic, nonhemolytic on SBA, creamy to sticky in consistency, and about 1 mm in diameter after 24 hours of incubation. The organism is pyrazinamidase-, alkaline phosphatase-, and CAMP test-positive, and produces acid from glucose, ribose, fructose, and mannose. Most strains produce API Coryne biocode numbers 2100304 or 6100304. The latter biocode is the same as that for *C. jeikeium*, but *C. coyleae* is nonlipophilic and does not display multiple resistance to antimicrobial agents. *C. coyleae* strains are susceptible to β-lactams, gentamicin, imipenem, rifampin, tetracycline, vancomycin, and linezolid. Many strains are resistant to clindamycin, erythromycin, and the fluoroquinolones.

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Corynebacterium diphtheriae	See text
Corynebacterium durum	*C. durum* has been recovered from throat cultures of healthy individuals and from sputum and lavage specimens of immunocompromised hosts (i.e., neoplasms, leukemia, renal failure).[932,1166] On culture, this nonlipophilic organism produces small, beige colonies that are adherent to the agar, although not all isolates have this appearance. On Gram's stains, the cells are long, pleomorphic, and sometimes filamentous, somewhat resembling the cellular morphology of *C. matruchotii*, but lacking the characteristic "whip handle" of the latter species. Most strains of *C. durum* are negative for alkaline phosphatase and PYR. Esculin is hydrolyzed weakly and urea hydrolysis is slowly positive or negative. *C. durum* is also one of the few *Corynebacterium* species that strongly ferments both mannitol and galactose, which helps to differentiate this organisms from *C. matruchotii*, which does not ferment these carbohydrates. On the API Coryne strip, *C. durum* produces unique numerical profiles (i.e., 3000135, 3001135, 3040135, 3400115, 3400135, 3400305, 3400325, 3400335, 3040325, 3040335, 3440335, 3441335) even though this organism is not included in the data base of that system.[71] Data on the antimicrobial susceptibility of this species are not available.
Corynebacterium falsenii	*C. falsenii* was described in 1998 by Sjoden and colleagues[1031] at the University of Goteborg, Sweden.[1031] Isolates were obtained from blood, and CSF cultures. Two of the blood culture isolates were from patients with malignant lymphoma and lymphocytic leukemia, respectively. These isolates were nonlipophilic and developed a distinct yellow pigmentation after 72 hours; prolonged incubation beyond this time resulted in more intense pigmentation. The isolates did not reduce nitrate or hydrolyze esculin. All four strains fermented glucose slowly, while a single strain also produced a slow acidic reaction with maltose. Conventional urea was hydrolyzed after overnight incubation, and the urease cupule of the API Coryne became positive only after 48 hours incubation. These fermentative, nonlipophilic strains were genetically and chemotaxonomically distinct and the name *C. falsenii* was proposed. Since the description of *C. falsenii* from human clinical specimens, similar strains also have been isolated from the mouths of eagles along with a second nonlipophilic isolate (*C. aquilae*).[331] On the API Coryne, the biocodes for *C. falsenii* include 2101104 and 2101304. Some API Coryne biocodes for this organism are identical to those for *C. jeikeium* (2100304, 2100324), but *C. falsenii* strains will be yellowish and nonlipophilic. *C. falsenii* isolates are susceptible to cephalothin, ciprofloxacin, erythromycin, gentamicin, imipenem, rifampin, tetracycline, cefetamet, and ceftibuten and resistant to aztreonam. MICs for penicillin range from 0.25 to 0.50 μg/mL. In 2010, *C. falsensii* was isolated from the blood of a 13-month-old infant.[1085] This isolate was susceptible to penicillin, clindamycin, doxycycline, gentamicin, linezolid, meropenem, rifampin, and vancomycin and resistant to ceftriaxone, erythromycin, and trimethoprim-sulfamethoxazole.
Corynebacterium freiburgense	This species is probably found in the oral cavity of dogs, as the only isolate was obtained from a dog bite wound on the forearm of a 57-year-old female. This organism was isolated along with *Pasteurella multocida*, *Prevotella* species, and α-hemolytic streptococci.[367] Colonies are beige-white, dry, convoluted with irregular edges, and adherent to the agar surface. After 5 days, the colonies assume a "spoked wheel" morphology. Pyrazinamidase, urease, PYR, gelatinase, and α-glucosidase are not produced, while β-glucosidase and β-galactsidase are produced. Acid is produced from glucose, maltose, sucrose, lactose, and ribose, but not from mannitol, sorbitol, xylose, raffinose, trehalose, or starch. Acid production from lactose and the positive β-galatosidase reaction are unusual among *Corynebacterium* species. This isolate was susceptible to penicillin, cefotaxime, ciprofloxacin, doxycycline, erythromycin, gentamicin, linezolid, meropenem, rifampin, and vancomycin.
Corynebacterium freneyi	*C. freneyi* has been isolated from blood, wounds, abscesses, and a sperm specimen.[55,911] In 2008, Funke and Frodl reviewed 18 additional *C. freneyi* isolates;13 isolated from vaginal swab specimens, with the rest being from ear discharge, human skin, a nasal culture, a wound culture, and a varicose ulcer.[365] *C. freneyi* is genotypically related to *C. xerosis* and *C. amycolatum* and can be phenotypically differentiated from them by carbon source assimilations and certain biochemical characteristics. Colonies of this organism are white-gray or yellowish, dry, wrinkled, 0.5–1 mm in diameter after 24 hours growth, nonhemolytic, and nonlipophilic. Like *C. xerosis*, isolates of *C. freneyi* are α-glucosidase-positive. Pyrazinamidase and alkaline phosphatase are produced and reduction of nitrate to nitrite is variable. *C. freneyi* is able to grow at 20°C, ferments glucose at 42°C, and does not acidify ethylene glycol, while *C. xerosis* strains do not grow at 20°C and do not ferment glucose at 42°C; some strains acidify ethylene glycol. The ability of *C. freneyi* to grow at 20°C also aids in the differentiation of it from *C. amycolatum*, which is not able to grow at this temperature. *C. striatum* and *C. minutissimum* may also resemble *C. freneyi*, but *C. striatum* grows at 20°C, ferments glucose at 42°C, and acidifys ethylene glycol. *C. minutissimum* ferments glucose at 42°C, but does not grow at 20°C and does not acidify ethylene glycol.

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	On the API Coryne, *C. freneyi* yields a biocode of 3110325, which corresponds to a *C. striatum/C. amycolatum* cross-call; these species can be separated by the tests described above. *C. freneyi* is susceptible to doxycycline, linezolid, gentamicin, meropenem, rifampin, vancomycin, and penicillin.[365]
Corynebacterium glucuronolyticum ("Corynebacterium seminale")	*C. glucuronolyticum* is a nonlipophilic coryneform that has been isolated from man with genitourinary tract infections and from semen specimens of patients with prostatitis.[359,393] In 2001, this organism was isolated as a cause of urethritis in an 18-year-old man.[393] This organism has also been found in the semen of boars and in uterine and vaginal secretions of sows.[271] This organism produces gray-white to slightly yellow, nonhemolytic colonies on SBA (see Image Plate 14-4G and 14-4H). While some phenotypic test results are variable (i.e., nitrate reduction, esculin hydrolysis, urease), most isolates are strongly CAMP test-positive and β-glucuronidase (β-GUR)-positive (see Image Plates 14-5A and 14-5B). On GLC of spent culture media, this species also produces propionic, lactic, and succinic acids from glucose. Strains of this organism are usually susceptible to β-lactams, aminoglycosides, rifampin, and vancomycin; some strains are resistant to tetracycline, doxycycline, erythromycin, clindamycin and certain quinolones (e.g., ciprofloxacin, norfloxacin). Another group of investigators also have described this organism and named it "*Corynebacterium seminale*," but the name *C. glucuronolyticum* has been given precedence.[935]
Corynebacterium hansenii	The description of *C. hansenii* is based on a single strain that was isolated from an aerobic culture of pus from surgical debridement of a liposarcoma.[914] Phenotypically, this species closely resembles *C. freneyi* and *C. xerosis*. This species is nonlipophilic and forms small, dry, rough, yellow-pigmented colonies on agar media. It is pyrazinamidase-positive, alkaline phosphatase-negative, and does not hydrolyze urea, gelatin, or esculin. Acid is produced from glucose, maltose, sucrose, and ribose. The API Coryne profile of this species was 2000325. Unlike *C. freneyi* and *C. xerosis*, the α-glucosidase reaction is negative. This isolate was susceptible to all antimicrobial agents except tetracycline.
Corynebacterium imitans	*C. imitans* has been recovered from pharyngeal cultures of patients with suspected diphtheria in Poland.[361] In addition to the index case, a 5-month-old boy, seven additional patients were subsequently reported, all of whom had contact with the index case. A "*C. diphtheriae*-like" organism was recovered from the index patient and three of the other patients. All strains were negative for diphtheria toxin by both animal challenge and the Elek method. Two independent PCR methods also failed to demonstrate the presence of any portion of the *tox* gene in any of the isolates. Because the isolates did not produce diphtheria toxin, the authors felt that they were not the cause of the patients' illness and that they had been misdiagnosed, although person-to-person transmission of the organisms had likely occurred.[361] *C. imitans* is a nonlipophilic species that can easily be distinguished from *C. diphtheriae*. Unlike *C. diphtheriae*, *C. imitans* is weakly pyrazinamidase-positive, α-glucosidase-negative, and CAMP test-positive, while *C. diphtheriae* strains are pyrazinamidase-negative, α-glucosidase-positive, and CAMP test-negative. All four isolates in the initial report produced identical profile numbers on the API Coryne (2100324). *C. imitans* could be differentiated from *C. striatum* and from *C. minutissimum/C. amycolatum* by maltose fermentation and the CAMP test, respectively. Lactate and succinate are produced as the sole products of glucose fermentation by *C. imitans*, while both *C. amycolatum* and *C. diphtheriae* produce mostly propionic acid. All four of the isolates described in the original report were susceptible to antimicrobial agents except aztreonam, clindamycin, erythromycin, and azithromycin.[361]
Corynebacterium jeikeium	*C. jeikeium* (formerly group JK) is one of the most commonly isolated *Corynebacterium* species from human infections.[540] This lipopophilic species colonize the axillar, groin, rectum, and perineum of hospitalized patients and may also be recovered from the hospital environment.[822,1048,1100] Infections with *C. jeikeium* are seen predominantly in immunocompromised hosts with hematologic and solid organ malignancies, other underlying diseases (including AIDS), indwelling medical devices, integumentary barrier breaks, neutropenia, and therapy with broad-spectrum antimicrobial agents.[282,1046,1050,1142,1176] *C. jeikeium* is a well documented cause of community- and nosocomially-acquired bacteremia and sepsis, native and prosthetic valve endocarditis.[85,771,947] Other less common clinical presentations described for *C. jeikeium* include, meningitis/ventriculitis, cardiovascular device-related infections, pneumonia, pyelonephritis, osteomyelitis, peritonitis, otitis media, and cutaneous/soft tissue infections.[79,121,265,434,550,827,1191,1231] After 24 hours of incubation, colonies of *C. jeikeium* on SBA are small (0.5–1 mm in diameter), nonhemolytic, and gray-white (Image Plate 14-4D). Growth on commercially available chocolate agar is usually scant due to the absence of lipids in current chocolate agar formulations. Unlike several other species, this organism is strictly aerobic. In the absence of supplementation of the growth media with Tween 80, acidification of the glucose and ribose test cupules in the API Coryne is often weak after 24 hours. The organism is pyrazinamidase- and alkaline phosphatase-positive, and acidifies glucose, ribose, galactose, and some strains also acidify maltose. All of the enzymatic tests on the API Coryne are negative for *C. jeikeium* (see Image Plate 14-4E and 14-4F).

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	C. jeikeium was the first *Corynebacterium* species that was documented as being resistant to multiple antimicrobial agents. Isolates of this species often show resistance to β-lactams (penicillins, cephalosporins, imipenem) and aminoglycosides and variable susceptibility to the macrolides, tetracyclines, rifampin, and the fluoroquinolones.[551,722,773,974] *C. jeikeium* is susceptible to vancomycin, tigecycline, daptomycin, linezolid, and quinupristin/dalfopristin.[282,338,418,635] Multiple antibiotic resistance in *C. jeikeium* is believed to be chromosomal rather than plasmid-associated. However, inducible, transposon-associated macrolide-lincosamide-streptogramin (MLS) resistance has been demonstrated in *C. jeikeium*.[828,946]
Corynebacterium kroppenstedtii	The description of *C. kroppenstedtii* is based on a single human isolate that was recovered from the sputum of an 82-year-old woman with pulmonary disease.[219] Cell wall analysis revealed the presence of *meso*-DAP and arabinoglactan, and 16S rRNA sequence analysis supported the inclusion of this organism in the genus *Corynebacterium*. Recent studies suggest a role for this organism in the pathogenesis of granulomatous mastitis in women.[91,852,933,1097] *C. kroppenstedtii* is a lipophilic species that produces gray, non-pigmented, slightly dry or smooth colonies on blood agar. Colonies are usually less than 0.5 mm in diameter after 24 hours of incubation. This organism is esculin-positive, urease- and nitrate reductase-negative, and CAMP test-negative. Acid is produced from glucose, sucrose, and maltose (weak reaction). API Coryne biocodes for this strain are 2040105 or 2040125, depending upon the interpretation of the maltose reaction. *C. kroppenstedtii* does not grow at 42°C. In vitro testing of the single isolate demonstrated susceptibility to penicillin, cefuroxime, clindamycin, erythromycin, and vancomycin.[219]
Corynebacterium lipophiloflavum	During a search for microorganisms involved in the pathogenesis of BV, Funke and colleagues[371] isolated a previously undescribed coryneform organism from the vagina of a 32-year-old woman with this clinical diagnosis. This isolate fulfilled the initial criteria for being a member of the genus *Corynebacterium*, was intensely yellow-pigmented, and displayed a lipid requirement for growth. The organism was oxidative in its metabolism, did not produce acid from carbohydrates, and was urease-positive after 24 hours in urease broth. The API Coryne produced the same profile number as *C. urealyticum* (profile #2101004). However, *C. urealyticum* is rapidly urease-positive (i.e., Christianson's urea slants become noticeably positive within minutes) and is not pigmented. In addition, this new isolate was broadly susceptible to β-lactams, macrolides, clindamycin, and aminoglycosides, while *C. urealyticum* is usually resistant to many of these agents.
Corynebacterium macginleyi	This species was proposed following a genetic examination of 51 lipophilic coryneform isolates (13 reference strains and 38 clinical isolates) that exhibited heterogenous biochemical patterns.[936] Isolates of *C. macginleyi* have been recovered predominantly from ocular specimens of patients with conjunctivitis, corneal ulcers, or postoperative endophthalmitis.[29,341,405,555,1072,1158] *C. macginleyi* has also been isolated from a tracheostomy site of a patient with laryngeal carcinoma, the urine of a patient with a long-term indwelling catheter, and from blood cultures of patients with catheter-related sepsis.[276,285,781,1159] *C. macginleyi* is also a rare cause of endocarditis.[884] On SBA, the organism forms pinpoint colonies after 48 hours; on SBA supplemented with 0.1% Tween 80, large, reddish beige colonies are produced. Higher concentrations of Tween-80 (i.e., 1% vs. 0.1%) may be somewhat inhibitory to the growth of some strains. *C. macginleyi* is alkaline phosphatase-positive, but is pyrazinamidase-negative. Acid is produced from glucose, sucrose, and mannitol and propionic acid is not produced from glucose fermentation. *C. macginleyi* isolates are usually susceptible to a broad range of antimicrobial agents, although some isolates may be resistant to fluoroquinolones, clindamycin, erythromycin, and/or the aminoglycosides.[1158] Some isolates of *C. macginleyi* have demonstrated high-level resistance to the fluoroquinolones due to point mutations in the *gyrA* gene.[302]
Corynebacterium massiliense	This new *Corynebacterium* species was isolated from hip joint fluid from an 84-year-old man who had previously undergone arthroplasty.[745] After 48 hours growth on SBA, colonies are grayish, glistening, and 0.5–1 mm in diameter (lipophilic). Urea, esculin, and gelatin are not hydrolyzed and nitrate is not reduced to nitrite. This species is weakly pyrazinamidase-positve and does not form acid from glucose, maltose, sucrose, xylose, or ribose on the API Coryne. This isolate was susceptible to amoxicillin, doxycycline, gentamicin, ofloxacin, clindamycin, trimethoprim-siulfamethoxazole, erythromycin, and rifampin.
Corynebacterium matruchotii	*C. matruchotii* is part of the oral flora of humans and animals and has been isolated on rare occasions from cases of endophthalmitis and corneal ulcers in compromised patients. This organism is also a documented veterinary pathogen.[979] Although this organism may appear on Gram's stain as typical coryneform bacteria, they may also appear as a "whip and handle" form on staining and may also show

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	rudimentary branching. Colonies of *C. matruchotii* are also atypical compared with other coryneforms, being small and flat. *C. matruchotii* is nonlipophilic, produces pyzazinamidase but not alkaline phosphatase, reduces nitrate to nitrite, and is usually esculin-positive. Acid is produced from glucose, maltose, sucrose, and ribose. Recent analysis of several ATCC strains of *C. matruchotii* revealed that some were actually members of the recently described species *C. durum*.[71] Although they are not included in the API Coryne data base, bona fide *C. matruchotii* strains produce API Coryne biocodes 7000325, 7010325, or 7050325. The original ATCC strain of *C. matruchotii* (ATCC #43833) has also been found to be distinct from true *C. matruchotii* strains by 16S rDNA sequence analysis. This strain is now called "*C. matruchotii*-like strain" and yields biocode 2140325 on the API Coryne.[71]
Corynebacterium minutissimum	*C. minutissimum* is a nonlipophilic species of uncertain clinical significance because most case reports do not contain sufficient phenotypic data to rule out other species, particularly *C. amycolatum*.[1185] Historically, *C. minutissimum* is associated with erythrasma, a superficial skin infection characterized by small, brownish-red macular patches, generally in the intertriginous areas. Case reports implicating "*C. minutissimum*" as an etiologic agent include endocarditis, meningitis, recurrent breast abscesses, costochondral abscess, bacteremia, postdiscectomy/vertebral fusion surgical wound infection, pyelonephritis with bacteremia, catheter-related sepsis, and CAPD-associated peritonitis.[15,43,64,175,246,333,955] Van-Bosterhaut and colleagues[1139] reported the isolation of multidrug-resistant strains of "*C. minutissimum*" from blood cultures in a 60-year-old man undergoing treatment for lymphocytic leukemia, a 50-year-old hemodialysis patient, and a 77-year-old woman on peritoneal dialysis for chronic renal failure. These isolates were probably *C. amycolatum*, since propionic acid was produced from glucose fermentation and the organism was multidrug-resistant. True *C. minutissimum* strains grow as smooth, moist, gray-white, nonhemolytic colonies on SBA, unlike the waxy colonies of *C. amycolatum*.[1247] Phenotypically, this organism closely resembles *C. aurimucosum*. Acid is produced from glucose and maltose; most strains also acidify sucrose. No acid is produced from glycogen, inulin, lactose, mannose, ribose, sorbitol, trehalose or xylose. Pyrazinamidase, alkaline phosphatase, and leucine arylamidase are positive. *C. minutissimum* strains also produce acetoin, are PYR-negative, and are rarely CAMP test-positive. *C. minutissimum* strains are DNase-positive and produce lactic and succinic acid from glucose, while *C. amycolatum* strains are DNase-negative and produces lactic and propionic acid from glucose.[1185,1247] Isolates of *C. minutissimum* may be multiresistant to antimicrobial agents, including β-lactams, macrolides, and fluroquinolones.[1125]
Corynebacterium mucifaciens	*C. mucifaciens* has been recovered from human blood, wound, ear and nasal specimens, and joint fluid specimens.[157,375,775] This organism was also isolated from blood cultures of a 50-year-old man with cavitary pneumonia.[284] Colonies of this species are unusual in that they are nonlipophilic, glistening, slightly yellowish, and very mucoid, a characteristic that is uncommon among the corynebacteria. On the API Coryne, this organism produces profile numbers that correspond to several lipophilic species (i.e., *C. jeikeium, C. bovis*, CDC group G) or the nonlipophilic fermentative species *C. striatum* (Table 14-10). *C. mucifaciens* produces pyrazinamidase and alkaline phosphatase and does not hydrolyze esculin, urea, or gelatin. The oxidative metabolism displayed by this species is likely responsible for failure to rapidly acidify carbohydrates present on the API Coryne gallery. *C. mucifaciens* produces acid oxidatively from glucose and occasionally from sucrose; acid production from fructose, mannose, and glycerol is useful to differentiate this species from *R. equi*. The eight strains of *C. mucifaciens* described in the original report were also examined for antimicrobial susceptibility; seven were susceptible to β-lactams (cefazolin, penicillin), and all were susceptible to the glycopeptides and aminoglycosides. The susceptibilities of these strains to the tetracyclines, the macrolides (erythromycin, azithromycin, clarithromycin), clindamycin, and the quinolones were variable. All eight strains were resistant to aztreonam, cefetamet, ceftibuten, and fosfomycin.
"Corynebacterium nigricans" (C. aurimucosum)	*"C. nigricans"* is an unusual black-pigmented *Corynebacterium* species that has been isolated from the human female genital tract. These isolates have been associated with complications of pregnancy, including spontaneous abortion and preterm labor.[1014,1015] Morphologically, colonies of *C. nigricans* are black, adherent and tend to "dig into" the agar. This organism must be differentiated from gray-black-pigmented strains of CDC group 4, which resemble *Rothia* species more closely than corynebacteria. Colonies of *"C. nigricans"* are about 2–3 mm in diameter after 48 hours incubation and are nonlipophilic. This species ferments glucose, maltose, and sucrose; glycogen and/or ribose are fermented by some strains. While pyrazinamidase and alkaline phasphatase reactions are variable, the reduction of nitrate and hydrolysis of esculin, urea, and gelatin are negative. On the API Coryne, *"C. nigricans"* produces biocodes 0000125, 2000125, and 2100327. These codes correspond to low-confidence identifications for *C. striatum, C. amycolatum, C. minutissimum,* and CDC groups G and F-1, but the colony

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	morphology of *C. nigricans* is distinctive enough that identification is not a problem. Strains of CDC group 4 bacteria reduce nitrate and hydrolyze both esculin and gelatin.[996] "*C. nigricans*" is also related at the species level to black-pigmented variants of *C. aurimucosum*.[253]
Corynebacterium pilbarense	This nonlipophilic species is based on a single isolate obtained from an anaerobic BACTEC bottle that was inoculated with an ankle aspirate from a man with gout in Pilbara, West Australia (hence the organism name).[44] This organism is urease- and nitrate reduction-negative, and does not hydrolyze esculin, hippurate, or gelatin. Acid is produced from glucose, sucrose, and ribose, but not from maltose, lactose, mannitol, sorbitol, xylose, raffinose, trehalose, or glycogen. Pyrazinamidase and alkaline phosphatase reactions are positive.
Corynebacterium propinquum	*C. propinquum* includes strains formerly belonging to CDC group ANF-3.[930] This nonlipophilic species has been isolated from the respiratory tract, a traumatic elbow wound, and from blood cultures associated with native aortic valve endocarditis.[864,963] Colonies on SBA are 1–2 mm in diameter after 24 hours of incubation and are off-white and dry with entire edges. Esculin and urea are not hydrolyzed, and strains variably produce pyrazinamidase and alkaline phosphatase. Like *C. pseudodiphtheriticum, C. propinquum* is asaccharolytic, but the two may be differentiated by the production of urease by *C. pseudodiphtheriticum. C. propinquum* can be differentiated from *C. afermentans* subsp. *afermentans* by the ability of the former to reduce nitrate and from *C. afermentans* subsp. *lipophilum* by both nitrate reduction and the lack of a lipid requirement.
Corynebacterium pseudodiphtheriticum	Increasing numbers of case reports suggest that *C. pseudodiphtheriticum* is an important emerging pathogen, particularly among immunocompromised hosts. While considered a member of the normal human oropharyngeal flora, respiratory tract infections associated with this organism include exudative pharyngitis, bronchitis, bronchiolitis, necrotizing tracheitis, tracheobronchitis, pneumonia, and lung abscess.[16,148,199,354,454,717] In compromised hosts with these infections, underlying diseases or predisposing factors have included permanent tracheostomy secondary to laryngeal carcinoma, chronic obstructive pulmonary disease, diabetes mellitus, coronary artery disease, intubation and mechanical ventilation secondary to a motor vehicle accident, end-stage renal disease, cirrhosis, tuberculosis, and HIV infection.[153] In some cases, respiratory tract infection with *C. pseudodiphtheriticum* may mimic respiratory diphtheria.[539] In 2010, an outbreak of *C. pseudodiphtheriticum* infection was documented among 13 children with cystic fibrosis (CF) at two treatment centers in France.[114] The children presented with cough, rhinitis, and exacerbations of their chronic disease. Molecular techniques demonstrated that about 20% of CF patients at the centers were colonized with *C. pseudodiphtheriticum*. Native and prosthetic valve endocarditis caused by *C. pseudodiphtheriticum* have also been reported, and most native valve infection have occurred in the setting of preexisting valvular damage.[777,1197] Suppurative cervical lymphadenitis, vertebral discitis following nasal turbinate surgery, postarthroscopy arthritis, cutaneous and wound, and eye infections caused by *C. pseudodiphtheriticum* have also been reported.[314,484,637,672,1213] *C. pseudodiphtheriticum* is nonlipophilic and grows as smooth, white colonies on SBA (Image Plate 14-5C). Pyrazinamidase and nitrate reductase reactions are positive, and the alkaline phosphatase reaction is variable. This organism is urease-positive after 24 hours and does not ferment or oxidize carbohydrates (Image Plate 14-5D). Like *C. afermentans* subspecies and *C. auris, C. pseudodiphtheriticum* does not produce acid from carbohydrates, but is easily differentiated from these species by its positive urease and nitrate reductase reactions. *C. pseudodiphtheriticum* isolates are generally susceptible to β-lactam agents, vancomycin, and aminoglycosides, but strains that are resistant to clindamycin, erythromycin, tetracycline, and the quinolones have been found. In a study of 58 strains of *C. pseudodiphtheriticum*, 52 were erythromycin-resistant and 6 were susceptible. The macrolide, lincosamide, and streptogramin B (MLSB)-constitutive resistance phenotype was found in 89.7% of the strains. The presence of the *erm(X)* gene that is associated with the MLSB resistance phenotype was confirmed by PCR.[820]
Corynebacterium pseudotuberculosis	*C. pseudotuberculosis* causes caseous lymphadenitis (CLA or "cheesy gland") in sheep, cattle, and other small ruminants.[1198] Most human infections associated with *C. pseudotuberculosis* have been reported among rural sheep-herders in Australia or in butchers, and have presented with either acute or chronic lymphadenitis.[136,855] In human infections, surgical drainage and excision of the affected lymph node(s) was necessary, and most of the patients had persistent or recurrent drainage from the wound site for prolonged periods. *C. pseudotuberculosis* is a member of the "*C. diphtheriae* group" *C. pseudotuberculosis* can be experimentally transformed into a diphtheria toxin-producing species by infection and lysogenization with the β-corynephage, but naturally occurring lysogenized strains have not been isolated. *C. pseudotuberculosis* is pyrazinamidase-negative and produces cystinase, as evidenced by

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	the production of brown halos around colonies on modified Tinsdale medium. *C. pseudotuberculosis* is urease-positive, nitrate reductase and alkaline phosphatase-variable and differs from *C. ulcerans* in that it does not produce acid from glycogen or trehalose and does not hydrolyze gelatin. Like *C. ulcerans* and *A. haemolyticum*, *C. pseudotuberculosis* is reverse CAMP test-positive. In the report on 10 patients with lymphadenitis due to *C. pseudotuberculosis* published by Peel and colleagues,[855] all isolates produced API Coryne profile #0111324, indicating positive reactions for alkaline phosphatase, α-glucosidase, urease, glucose, maltose, and ribose. *C. pseudotuberculosis* are generally susceptible to penicillins, macrolides, tetracyclines, cephalosporins, quinolones, and rifampin; most are resistant to aminoglycosides, nitrofurans, and polymyxin.[561]
Corynebacterium resistens	*C. resistans* was described in 2005 based on five isolates from blood, abscess, and bronchial aspirate specimens.[830] The organism derives its name from its antimicrobial susceptibility pattern. All isolates had MICs beyond clinically achieveable levels for penicillin (MIC > 64 μg/mL), cefazolin (MIC > 64 μg/mL), cefepime (MIC > 64 μg/mL), imipenem (MIC > 32 μg/mL for all but one isolate, MIC ≤ 0.13 μg/mL), amikacin (MIC ≥32 μg/mL), clindamycin (MIC > 16 μg/mL), ciprofloxacin (MIC > 16 μg/mL), and minocycline (two isolates, MIC 16 μg/mL; two isolates, MIC 8 μg/mL; one isolate, MIC 0.25 μg/mL). MICs for vancomycin and teicoplanin were 2 μg/mL and ≤0.5 μg/mL, respectively, for all five isolates. On the API Coryne, only the pyrrolidonyl arylamidase (PYR) and alkaline phosphatase reactions are positive, with negative reactions for nitrate reduction, pyrazinamidase, esculin, urease, gelatin, glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, and glycogen (API Coryne profile number 4100004).
Corynebacterium riegelii	*C. riegelii* has been recovered in urine specimens from women with symptoms of urinary tract infection and from blood cultures.[374] Urine isolates were recovered from women of ages 21 to 62 years with no underlying diseases. The organisms were present in significant numbers (i.e., >100,000 cfu/mL). Isolates of *C. riegelii* are nonlipophilic and produce white, glistening sticky colonies on SBA (Image Plate 14-5E). Isolates are strongly urease-positive (like *C. urealyticum*), with positive reactions on Christensen's urea slants being noted within 5 minutes after inoculation (Image Plate 14-5F). An unusual characteristic of this species is that strains produce acid slowly from maltose, but no acid is formed from glucose. Atypical reactions occur with conventional media (e.g., CTA-based carbohydrates) and with the API Coryne strip (Image Plate 14-5G and 14-5H). API Coryne profile numbers for *C. riegelii* are 0101224, 2001224, and 2101224. Strains of *C. riegelii* are susceptible to penicillin, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, rifampin, tetracycline, and vancomycin and resistant to cefetamet, ceftibuten, and fosfomycin.[374]
Coynebacterium sanguinis	*C. sanguinis* has only been recovered from blood cultures to date.[369] Colonies of this organism on SBA are 1–2 mm in diameter after 48 hours incubation, smooth, dry and yellowish. Pyrazinamidase, alkaline phosphatase, and PYR tests are positive, and the CAMP test is negative. The organism slowly ferments glucose and ribose (on the API Coryne) but no other carbohydrates. *C. sanguinis* is negative for nitrate, urease, esculin, and the CAMP test. On the API Coryne, all isolates produced the biocode number 6100304.
Corynebacterium simulans	*C. simulans* has been isolated from a foot abscess, a lymph node biopsy, a boil, and a prosthetic joint infection.[176,1181] This organism is a nonlipophilic fermentative species that is phenotypically similar to *C. minutissimum* and *C. striatum*, but phylogenetically distinct based on 16S rRNA sequences. *C. simulans* strains grow as nonlipophilic, gray-white, glistening, creamy colonies that are 1–2 mm in diameter after 48 hours. *C. simulans* is nitrate positive and maltose-negative (*C. minutissimum* is nitrate-negative and maltose-positive) and does not ferment ethylene glycol or grow at 20°C (*C. striatum* ferments ethylene glycol and grows at 20°C). This is the only *Corynebacterium* species that is able to reduce nitrate to nitrite and then further reduce nitrite to nitrogen gas, so a negative nitrate test must be challenged with zinc to confirm this reaction. In addition, some strains may be catalase-negative. On the API Coryne, *C. simulans* produces several biocodes. Data on the antimicrobial susceptibility of *C. simulans* are not available.
Corynebacterium singulare	The description of *C. singulare* is based on two human isolates (one from semen, the other from blood).[939] These two isolates possessed all the key chemotaxonomic characteristics of the genus *Corynebacterium* and resembled *C. minutissimum* by morphologic and biochemical characteristics. Colonies are convex, entire, creamy, and white-gray. Unlike *C. minutissimum*, however, the two isolates were urease-positive. Examination of other urease-positive taxa within the genus confirmed that the two isolates were indeed distinct from them by several characteristics, including increased fermentative ability, failure to reduce nitrate, and the absence of β-glucuronidase. The organism can be differentiated

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	from *C. amycolatum* by its failure to produce propionic acid from glucose. On the API Coryne, the organism generates a unique octal code (biocode 6101125).
Corynebacterium sputi	This species encompasses a single isolate from the sputum of a patient with pneumonia.[1228] This organism is lipophilic and is genotypically most closely related to *C. hansenii*. *C. sputi* is urease-positive, but hippurate, esculin, and gelatin are not hydrolyzed and nitrate is not reduced. Acid is produced from glucose, but not from maltose, sucrose, lactose, mannitol, sorbitol, ribose, trehalose, xylose, or starch. The API Coryne biocode for this organism is 2011104.
Corynebacterium striatum	*C. striatum* has been found in cattle, as part of the normal flora of the human nasal passages, and on human skin. In 1980, the first case of human pleuropulmonary infection with *C. striatum* was reported in a 79-year-old man with chronic lymphocytic leukemia.[133] The organism was isolated from the sputum, two sets of blood cultures, and a pleural fluid culture. Since then, *C. striatum* had been associated with several human infections and has been isolated from several types of clinical specimens. In 1994, Wolde Rufael and Cohn reported the first patient with native valve endocarditis caused by *C. striatum*.[1202] Since then, *C. striatum* has been documented as a significant agent of both native valve and prosthetic valve endocarditis and pacemaker-associated endocarditis.[103,125,259,725,727,821,1001,1106,1115] *C. striatum* is an important agent of sepsis and bacteremia in immuncompromised patients, including those with lymphoblastic lymphoma with autologous bone marrow transplantation, myelodysplasia with allogeneic bone marrow transplantation, amyotrophic lateral sclerosis, chronic renal failure, colorectal cancer, diabetes, and HIV infection.[197,719,831,1123] *C. striatum* is also a significant cause of respiratory tract infections, including community-acquired and nosocomial pneumonia, bronchitis, and empyema, particularly in those with chronic obstructive pulmonary disease.[916,1094,1205] Isolates of *C. striatum* has been recovered from patients with vertebral osteomyelitis, septic arthritis and synovitis, breast abscess, meningitis, CSF shunt infections, pancreatic abscess, peritoneal dialysis catheter exit site infection, and CAPD-associated peritonitis.[75,102,105,125,232,243,1060,1189] Evidence from studies conducted in intensive care units indicate that that *C. striatum* may occasionally be transmitted nosocomially.[534,663,831,916] *C. striatum* isolates are nonlipophilic and grow as gray-white, moist, smooth, nonhemolytic colonies with entire edges on SBA (Image Plate 14-6A). Colonies are about 1–2 mm in diameter after 24 hours' incubation. Pyrazinamidase and alkaline phosphatase are produced, and urea, esculin, and gelatin are not hydrolyzed.[723] Most strains reduce nitrate and produce acid from glucose, sucrose, fructose, and galactose only (Image Plate 14-6B). *C. striatum* strains produce lactic and succinic acids from glucose, which is helpful in differentiating isolated from *C. amycolatum*. A 1995 examination of 31 clinical isolates found that all strains were susceptible to both imipenem and vancomycin; most strains were susceptible to ampicillin, cephalosporins, and aminoglycosides, and most were resistant to clindamycin, erythromycin, and tetracycline. However, this organism has demonstrated increasing resistance to antimicrobial agents since that time. Several case reports have described isolates of *C. striatum* that are multiresistant to β-lactams (including penicillin, ampicillin, ceftriaxone, cefotaxime, and cefipime), macrolides, tetracyclines, fluoroquinolones.[105,197,534,725,821,916,1094,1106] Susceptibility to the carbapenems (imipenem, meropenem) is variable.[831] The *C. striatum* isolate from the patient with endocarditis described by Mashavi and colleagues was susceptible only to vancomycin.[727] *C. striatum* isolates are usually susceptible to vancomycin, rifampin, linezolid, tigecycline, and daptomycin.[725,967,1001,1094] A *C. striatum* isolate from a a 56-year-old man with endocarditis, which was complicated by diabetes mellitus and end-stage renal disease, demonstrated the presence of a mixed daptomycin-susceptible and -resistant population by Etest.[1095] The susceptible subpopulation had a daptomcyin MIC of < 0.094 μg/mL, while the resistant subpopulation had a daptomycin MIC of >256 μg/mL by broth microdilution testing. The resistant subpopulation emerged after the patient had received daptomycin for two 6-week treatment courses.
Corynebacterium sundsvallense	*C. sundsvallense* has been recovered from blood cultures, a vaginal specimen, and a groin-wound culture.[217] The Gram's stain appearance of this organism is unique in that knobs or "bulges" may be noted on the ends of some of the bacilli. The organism is nonlipophilic, and produces buff-to-yellow, sticky, adherent colonies on SBA. The organism slowly ferments glucose, maltose, and sucrose, but not lactose, mannitol, ribose, xylose, or amygdalin. Nitrate is not reduced and esculin is not hydrolyzed, but urease is produced and the α-glucosidase reaction is positive. Lactic and succinic acids are the major products of glucose fermentation. Antimicrobial susceptibility data on this organism are not available.
Corynebacterium thomssenii	The species description of this organism is based on two similar isolates that were recovered from pleural fluid specimens of a 56-year-old man with chronic renal failure, stroke and pneumonia.[1246] *C. thomssenii* is a slow-growing, nonlipophilic organism that produces sticky, adherent colonies that

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	are less than 0.5 mm in diameter even after 48 hours incubation. The organism is urease-positive, DNase-positive, and both nitrate and esculin hydrolysis-negative. Acid is slowly produced from glucose, maltose, and sucrose. Unlike all other *Corynebacterium* species, isolates of this species are strongly *N*-acetyl-β-glucosaminidase-positive, producing the API Coryne profile number 2121125. Isolates of *C. thomssenii* are susceptible to ampicillin, cefazolin, gentamicin, erythromycin, clindamycin, tetracycline, ciprofloxacin, rifampin, and vancomycin; isolates areresistant to aztreonam, cefetamet, ceftibuten, and fosfomycin.
Corynebacterium timonense	This species was isolated from five of six sets of blood cultures from a patient with pacemaker-associated endocarditis.[745] This species is nonlipophilic and forms shiny yellow colonies 1–2 mm in diameter after 24 hours' incubation. Esculin is weaky hydrolyzed, but gelatin and urea are not hydrolyzed. On the API Coryne, pyrazinamidase and alkaline phosphatase are positive, and acid is produced from glucose and ribose, but not from maltose, sucrose, lactose, mannitol, xylose, or glycogen. This isolate was susceptible to amoxicillin, doxycycline, gentamicin, ofloxacin, clindamycin, trimethoprim-siulfamethoxazole, and rifampin, and resistant to erythromycin. The organism is named for Hopital de la Timone, the hospital in Marseille, France where the type strain was isolated and characterized.
Corynebacterium tuberculostearicum	*C. tuberculostearicum* is a species that was described years ago, but never validly published. This organism was formally proposed as a new species in 2004 based on isolates from clinical specimens (e.g., bone marrow, lymph nodes, blood, urethral specimen, skin, peritoneal fluid), foods (canned tuna), and the environment.[342] This lipophilic species is urease-, esculin-, and gelatin hydrolysis-negative. Acid is produced from glucose and ribose, but not from mannitol, sorbitol, lactose, xylose, glycogen, starch, or inulin. Acid production from maltose, sucrose, trehalose, and *N*-acetyl-glucosamine is variable. API Coryne biocodes (6100324, 6100325, 7100325, 6100305, 2100325) correspond to *Corynebacterium* CDC group G in the API profile index.
Corynebacterium tuscaniae	This species is based on a single isolate recovered from six sets of blood culture from an 85-year-old woman with gastric carcinoma and endocarditis.[929] This species possesses mycolic acid in its cell walls and is pyrazinamidase- and alkaline phosphatase-positive. Urease is not produced and esculin, gelatin, and hippurate are not hydrolyzed. Acid is produced from glucose and maltose, but not from, ribose, lactose, xylose, mannitol, sucrose or glycogen. The API Coryne profile number for this speies is 2100124. The organism name refers to Tuscany, the Italian region where the strain was isolated.
Corynebacterium ulcerans	*C. ulcerans* is a nonlipophilic member of the "*C. diphtheriae* group," along with *C. diphtheriae* and *C. pseudotuberculosis*.[938] Most *C. ulcerans* isolates (and some isolates of *C. pseudotuberculosis*) produce diphtheria toxin. In one study, 25 of 37 *C. ulcerans* strains and 1 of 14 *C. pseudotuberculosis* strains produced positive Elek tests for diphtheria toxin.[441] In addition, all the Elek test-positive strains and two Elek test-negative strains produced positive signals when hybridized with a DNA probe for the tox structural gene for diphtheria toxin. Restriction endonuclease fragment analyses have shown that *C. diphtheriae* and *C. ulcerans* strains share considerable homology on Southern blots; these homologous fragments include the integration site for the β family of corynephages. Wong and Groman also demonstrated that the diphtheria toxins produced by strains of *C. ulcerans* and *C. pseudotuberculosis* were similar in molecular weight and immunological structure, in enzymatic ADP-ribosylating activity, and in the regulation of their synthesis by iron.[1207] In the United Kingdom, a survey of 102 patients with infections caused by toxigenic corynebacteria, 59 were due to toxigenic *C. ulcerans*.[1172] *C. ulcerans* causes mastitis in cattle, nonhuman primates, and other animals, and has been recovered from unpasteurized cows' milk and other dairy products.[128] *C. ulcerans* has also been isolated from cats and dogs, and the ribotypes of feline and canine isolates suggest that these animals may be reservoirs for human infections.[93,239,275,639] Human infections have generally occurred in rural areas in individuals with histories of animal exposure or ingestion of raw dairy products. *C. ulcerans* has been isolated from patients with illnesses indistinguishable from classical diphtheria, with and without the presence of both cardiac and neurologic sequelae.[477,572,889,1109] Severe pulmonary infections due to *C. ulcerans* have been reported in immunocompromised and immunocompetent hosts.[1098] In 1996, a case of respiratory diphtheria due to toxigenic *C. ulcerans* was reported in a 54-year-old woman from Terre Haute, Indiana.[181] The patient, who had never been immunized against diphtheria, was treated with ceftriaxone, erythromycin, and 40,000 international units (IU) of equine diphtheria antitoxin with a rapid clinical response. Culture of clinical specimens (throat swabs and fragments of diphtheritic pseudomembrane) confirmed the presence of *C. ulcerans* and toxigenicity of this isolate was demonstrated by both the Elek immunoprecipitation assay and by a PCR assay for diphtheria toxin. Toxin-producing isolates of *C. ulcerans* have also caused cases of cutaneous "diphtheria".[1171,1172] In 2002, Wellinghausen and colleagues[1192] described a fatal case of necrotizing sinusitis in an elderly man due to toxigenic *C. ulcerans*. *C. ulcerans* has also

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	been documented as a cause of peritonitis related to continuous ambulatory peritoneal dialsysis.[597] Colonies of *C. ulcerans* are gray-white, dry, and waxy appearing with a slight diffusely β-hemolytic area under and surrounding individual colonies. The organism is nonlipophilic and colonies are 1–2 mm in diameter after 24 hours of incubation. *C. ulcerans* is pyrazinamidase-negative and positive for alkaline phosphatase and urease production. Acid is produced from glucose, maltose, mannose, arabinose, ribose, and glycogen. Like *C. pseudotuberculosis*, *C. ulcerans* is reverse CAMP test-positive and produces propionic acid as a product of glucose fermentation. *C. ulcerans* isolates are susceptible to penicillin, ampicillin, cephalothin, erythromycin, clindamycin, tetracycline, and gentamicin.
Corynebacterium urealyticum	*C. urealyticum* was formerly called CDC coryneform group D-2; in 1992, the name *Corynebacterium urealyticum* was formally proposed and adopted.[872] Normally a saprophytic organism found on the skin, this species has been associated with acute and chronic urinary tract infections, urolithiasis, renal cysts, and ureteral stenosis.[291,1021,1049] This organism causes similar infections in dogs and cats as well.[59,138] *C. urealyticum* is highly associated with a clinical condition called alkaline encrusted cystitis. The latter is a chronic condition seen in compromised hosts with preexisting bladder injury, where ulcerative inflammation results from the deposition of ammonium-magnesium phosphate crystals (struvite and apatite crystals) on the bladder wall.[261,554] This condition is caused by organisms that are able to rapidly split urea, such as *Proteus* species. Complicated urinary tract infections and pyelonephritis caused by *C. urealyticum* are seen primarily in immunocompromised patients and in kidney transplant patients with postsurgical complications.[14,291,349,690,835] In addition to its role as a urinary tract pathogen, *C. urealyticum* has also been isolated occasionally from blood cultures as a cause of bacteremia, and native and prosthetic valve endocarditis.[308,1049] Other infections associated with *C. urealyticum* include pericarditis, osteomyelitis, wound/soft tissue infections, and nosocomial infecti ons.[201,318,335,814,958,1046,1047] *C. urealyticum* is a lipophilic coryneform that grows slowly on SBA, producing small (<0.5 mm in diameter after 24 hours incubation) gray, nonhemolytic colonies on SBA. It is pyrazinamidase and alkaline phosphatase-positive, asaccharolytic, and rapidly urease-positive. Urea agar slants begin to turn positive immediately upon inoculation. Molecular approaches for detection and identification of *C. urealyticum* have also been described.[1021] *C. urealyticum* strains are usually multidrug-resistant to β-lactam, aminoglycoside, and macrolide antimicrobial agents; occasional strains may be β-lactam-susceptible.[337,418,974,1242] Fluoroquinolones show irregular activity against *C. urealyticum*. Isolates that are resistant to the quinolones and rifampin may be selected during therapy with these drugs. *C. urealyticum* strains are generally susceptible to tigecycline, linezolid, rifampin, quinupristin/dalfopristin and vancomycin.[337] *C. urealyticum* is known to produce biofilms, and studies on the antimicrobial susceptibility of *C. urealyticum* have shown that organisms grown in biofilms have higher MICs and MBCs to fluoroquinolones and vancomycin, than planktonic (free-floating) organisms.[1045] MICs of erythromycin for biofilm-trapped organisms were more than 8,000 times higher than those for planktonic organisms. This ability may contribute to the difficulties encountered in eradicating the organism from patients with chronic *C. urealyticum* urinary tract involvement.
Corynebacterium ureicelerivorans	The species description of this organism is based on a single isolate that was recovered from a blood culture.[1224] Subsequently, this organism was isolated from blood cultures of five patients with underlying diseases (e.g., colon cancer, diabetes, acute abdomen, leukemia, diverticulitis) and from ascites fluid of a patient with cirrhosis.[337] *C. ureicelerivorans* is a lipophilic species that is rapidly and strongly urease-positive (positive reaction in 60 seconds) and hippurate-positive. Esculin and gelation are not hydrolyzed and nitrate is not reduced. Acid is formed from glucose on the API Coryne after 72 hours, with weak acidic reactions being noted for ribose and xylose. For most isolates, the API biocode number is 6101104 after 72 hours, which fits the profile of *C. bovis*. *C. ureicelerivorans* is susceptible to β-lactams, gentamicin, rifampin, tetracycline, linezolid, and vancomycin and often resistant to the fluoroquinolones, erythromycin, clarithromycin, azithromycin, and clindamcyin.
Corynebacterium xerosis	Substantial data now indicate that most clinical isolates of "*C. xerosis*" are actually *C. amycolatum* (see discussion of this organism above).[372] True *C. xerosis* produce granular, yellowish colonies, contain mycolic acids in their cell walls, are LAP- and α-glucosidase-positive, and produce lactic acid from glucose fermentation, while strains of *C. amycolatum* produce dry waxy, whitish-gray colonies, lack mycolic acids, are negative or weakly positive for LAP, are α-glucosidase-negative and produce propionic acid from glucose fermentation.[372,1185] Unlike *C. striatum*, true *C. xerosis* isolates do not hydrolyze tyrosine and ferment sugars slowly (72–96 hours). The literature contains several case reports regarding *C. xerosis* as the etiologic agent in a variety of types of human infections. In these reports, the isolates were oftem found to be multiply-resistant to antimicrobial agents. These multiresistant strains were actually *C. amycolatum* and not *C. xerosis*, since bona fide strains of the latter species are quite rare and are

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	known to be broadly susceptible. Because of its infrequent isolation in the clinical laboratory, *C. xerosis* was deleted from the version #2 of the API Coryne data base. However, certain biocodes—2110325 and 3110325—do correspond to true isolates of *C. xerosis*.[838] True human infections with *C. xerosis* are rare and the organism has been recovered from clinical specimens obtained from animals.[838,1152] *C. xerosis* strains are susceptible to most antimicrobial agents.
Corynebacterium Group F1	CDC coryneform group F1 is a lipophilic, fermentative coryneform that can be separated into two phenotypically indistinguishable DNA homology groups. As a urease-positive, lipid-requiring coryneform, CDC group F1 closely resembles the newly named lipid-requiring species *C. macginleyi*.[936] However, CDC group F1 strains are pyrazinamidase-positive, alkaline phosphatase-negative, and ferment glucose, maltose, and sucrose, while *C. macginleyi* strains are pyrazinamidase-negative, alkaline phosphatase-positive, and ferment glucose and sucrose but not maltose. CDC coryneform group F1 has been recovered most frequently from the urogenital tract. In 1992, an isolate of the CDC group F1 was reported from a 40-year-old man with struvite stone formation.[281] The organism was present in the urine in significant numbers ($>10^8$ cfu/mL) and was susceptible to tetracycline. Treatment of the patient with doxycycline resulted in resolution of the infection and cessation of stone formation.
Corynebacterium group G	CDC coryneform group G has been isolated from vitreous humor specimens, blood, cerebrospinal fluid, and the genitourinary tract.[936] Isolates belonging to group G are lipophilic, and growth is stimulated Tween-80 (Image Plate 14-6C and 14-6D). In 1983, Austin and Hill reported a 40-year-old man with prosthetic valve endocarditis, which unfortunately was fatal, caused by a CDC group G microorganism.[54] The isolate was susceptible to ampicillin, cephalothin, cefamandole, cefoxitin, gentamicin, and vancomycin. Group G coryneforms were also isolated from blood cultures of a 74-year-old man undergoing maintenance hemodialysis due to chronic renal failure.[583] This isolate was also uniformly susceptible to antimicrobial agents.
Corynebacterium groups F2 and I	CDC groups F2 and I have been shown to correspond to strains of *C. amycolatum*.[323,708,1185]

with additional tests being required for correct identification of 55.1% of all strains tested. Only 5.6% of isolates were not identified and 3.8% were misidentified. The current API Coryne database is found at apiweb.biomerieux.com is version 3. The API Coryne requires a heavy inoculum to prevent false-negative reactions and longer incubation may be necessary to detect carbohydrate fermentation reactions.[378] Reports on new *Corynebacterium* species often include API Coryne tests as part of the species description. Seven-digit API biocodes only provide identifications for species in the database, and new species may be misidentified unless additional tests are performed. However, some newer species do generate unique biocodes.

RapID CB-Plus. The RapID CB-Plus employs the cuvette format similar to other Remel identification kits and includes 18 single-test wells. To inoculate the system, a suspension of the organism grown on 5% SBA (tryptic soy, Columbia, or BHI base) equivalent to a #4 McFarland turbidity standard is prepared in 2 mL of RapID inoculation fluid. The suspension is poured or pipetted into the cuvette and all 18 wells are simultaneously inoculated by manual manipulations of the cuvette. The system is incubated for 4 to 6 hours at 35°C to 37°C in a non-CO_2 incubator. Nitrate reagents A and B are added to the NIT well, and a second reagent (RapID CB-Plus Reagent) is

added to the arylamidase test wells. Thirty seconds to 1 minute after reagent addition, the color reactions in the wells are read and a numeric microcode is generated. The RapID CB-Plus Code Compendium is consulted for the identification. Evaluations of RapID CB-Plus have reported that the kit correctly identifies 88.5% to 95% of *Corynebacterium* species, particularly those species commonly encountered in the clinical laboratory (i.e., *C. amycolatum, C. jeikeium*, *C. striatum*, *C. urealyticum*, *C. minutissimum*, *C. pseudodiphtheriticum,* and CDC group G).[379,528] Isolates of some newly described taxa (e.g., *Dermabacter hominis*, *Microbacterium* species, *Turicella otitidis*, *Cellulomonas* species, *Brevibacterium casei*, and *Arthrobacter cumminsii*) were also correctly identified. Difficulties with this system were related to the interpretation of some of the carbohydrate acidification and aminopeptidase reactions.

Vitek 2 Anaerobe/*Corynebacterium* Card. The ANC card is a 64-well biochemical test card that is used with the Vitek 2. The card is inoculated by the Vitek 2 instrument and incubation takes place within the machine. Identifications are usually available within 6 hours after inoculation of the card. The database of the ANC card includes corynebacteria, (*C. amycolatum*, *C. diphtheriae*, *C. jeikeium*, *C. pseudodiphtheriticum*, *C. striatum*, *C. ulcerans*, and *C. urealyticum*), some *Actinomyces* species (*A. israelii*, *A. meyeri*), and *Arcanobacterium* species (*A. haemolyticum*, *A. pyogenes*) in addition to clinically significant gram-positive and gram-negative anaerobic bacteria. Evaluations indicate that the Vitek 2 ANC card is able to adequately identify those corynebacteria that are included in the database.[915]

MALDI-TOF MS. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF

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TABLE 14-6 Phenotypic Characteristics for Identification of *Corynebacterium* Species Isolated From Humans

Species	LIP	HEM SBA	NO3	PYZ	PAL	ESC	URE	GEL	CAMP	LAP	PYR	α-GAL	β-GAL	α-GLU	β-GLU	β-GUR	NAGA
C. accolens	+	–	+	V+	–	–	–	–	–	NA	V	NA	–	–	–	–	–
C. afermentans subsp. *afermentans*	–	–	–	+	+	–	–	–	V	–	–	–	–	–	–	–	–
C. afermentans subsp. *lipophilum*	+	–	–	+	+	–	–	–	V	–	–	–	–	–	–	–	–
C. amycolatum	–	–	V+	+	+	–	V–	–	–	NA	V–	–	–	–	–	–	–
C. appendicis	+	–	–	+	+	–	+	–	NA	–	–	–	–	–	–	–	–
C. argentoratense	–	–	–	+	V–	–	–	–	–	V	–	NA	–	–	–	–	–
C. atypicum	–	–	–	–	–	–	–	–	NA	+	–	–	–	–	–	+	–
C. aurimucosum	–	–	–	+	+	–	–	–	NA	+	–	–	–	–	–	–	–
C. auris	–	–	–	+	+	–	–	NA	+	NA	–	NA	–	–	–	–	–
C. canis	–	–	+	+	+		–	–	–	+	–	–	–	+	+	–	–
C. confusum	–	–	+	+	+	–	–	–	–	V	–	–	–	–	–	–	–
C. coyleae	–	–	–	+	+	–	–	NA	+	+	V	–	–	–	–	–	–
C. diphtheriae gravis	–	–	+	–	–	–	–	V–	–	NA	–	–	–	+	–	–	–
C. diphtheriae intermedius	+	–	+	–	–	–	–	V–	–	NA	–	–	–	+	–	–	–
C. diphtheriae mitis	–	–	+	–	–	–	–	V–	–	NA	–	–	–	+	–	–	–
C. diphtheriae belfanti	–	–	–	–	–	–	–	V–	–	NA	–	–	–	+	–	–	–
C. durum	–	–	+	+	–	+	V+w	–	NA	NA	–	NA	V	–	NA	–	–
C. falsenii	–	–	–	+w	+	–	+sl	–	–	V	–	–	–	–	–	–	–
C. freiburgense	–	–	+	–	–	–	–	–	–	+	–	–	+	–	+	–	–
C. freneyi	–	–	V	+	+	–	–	–	NA	NA	–	NA	–	+	–	–	–
C. glucuronolyticum	–	–	V	+	V–	V	V+	–	+	+	–	–	–	–	–	+	–
C. hansemii	–	–	–	+	–	–	–	–	NA	NA	–	NA	–	–	NA	–	–
C. imitans	–	–	–	+w	+	–	–	–	+	–	–	–	–	–	–	–	–
C. jeikeium	+	–	–	+	+	–	–	–	–	NA	–	–	–	–	–	–	–
C. kroppenstedtii	+	–	–	+	–	+	–	–	NA	+		–	–	–	–	–	–

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C. lipophiloflavum	+	–	–	+	+	–	+24h	NA	NA	+	–	–	–	–	–	–	–
C. macginleyi	+	–	+	–	+	–	–	–	NA	NA	–	NA	–	–	–	–	–
C. massiliense	–	–	–	+w	+	–	–	–	NA	+	–	–	–	–	–	–	–
C. matruchotii	–	–	+	+	–	–	–	–	–	NA	+	NA	–	V	–	–	–
C. minutissimum	–	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–
C. mucifaciens	–	–	–	+	+	–	–	–	–	–	V	–	–	–	–	–	–
C. nigricans	–	–	–	V	V	–	–	–	–	NA	–	NA	–	–	–	–	–
C. pilbarense	–	–	–	+	+	–	–	–	NA	+	+	–	–	–	–	–	–
C. propinquum	–	–	V	V	V	–	–	–	–	NA	V	NA	–	–	–	–	–
C. pseudodiphtheriticum	–	–	+	+	V	–	+	–	–	NA	V	NA	–	–	–	–	–
C. pseudotuberculosis	–	β	–	–	V	–	+	–	–	NA	–	NA	–	V	–	–	–
C. resistens	+	–	–	–	+	–	–	–	–	+	+	–	–	–	–	–	–
C. riegelii	–	–	–	V	V	–	+Rapid	–	–	+	–	–	–	–	–	–	–
C. sanguinis	–	–	–	+	+	–	–	–	–	NA	+	–	–	–	–	–	–
C. simulans	–	–	+	V	+	–	–	–	–	+	–	–	–	–	–	–	–
C. singulare	–	–	–	+	+	–	+	–	NA	NA	+	NA	–	–	–	–	–
C. sputi	+	–	–	+	–	–	+	–	NA	+	–	–	–	–	–	–	–
C. striatum	–	–	+	+	+	–	–	–	V	NA	–	–	–	–	–	–	–
C. sundvallanse	–	–	–	V	V	–	+	–	–	V	–	–	–	+	–	–	NA
C. thomssenii	–	–	–	+	+	–	+	–	–	+	–	–	–	–	–	–	+
C. timonense	–	–	–	+	+	+w	–	–	NA	+	–	–	–	–	–	–	–
C. tuberculostearicum	+	–	V	+	V	–	–	–	NA	V	NA	–	–	–	–	NA	NA
C. tuscaniae	–	–	–	+	+	–	–	–	–	NA	–	NA	–	–	–	–	–
C. ulcerans	–	β	–	–	+	–	+	+	Rev+	NA	–	NA	–	+	–	–	–
C. urealyticum	+	–	–	+	V	–	+Rapid	–	–	NA	–	–	–	–	–	–	–
C. ureicelerivorans	+	–	–	+	+	–	+	–	NA	+	+	–	–	–	–	–	–
C. xerosis	–	–	V	+	+	–	–	–	–	NA	–	NA	–	+	–	–	–
CDC group F1	+	–	V+	+	–	–	+	–	–	NA	–	NA	–	–	–	–	–
CDC group G	+	–	V	+	+	–	–	–	–	NA	V	NA	–	–	–	–	–

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Species	GLU	MAL	SUC	LAC	MNTL	MANN	XYL	ARAB	SBTL	RIB	GLYG	AMYG
C. accolens	+	–	V	–	–	+	–	–	–	+	–	NA
C. afermentans subsp. *afermentans*	–	–	–	–	–	–	–	NA	–	–	–	NA
C. afermentans subsp. *lipophilum*	–	–	–	–	–	–	–	NA	–	–	–	NA
C. amycolatum	+	V+	V+	–	–	NA	–	NA	NA	+	–	NA
C. appendicis	+sl	+sl	–	–	–	NA	–	–	–	–	NA	–
C. argentoratense	+	–	–	–	–	NA	–	NA	NA	V	–	NA
C. atypicum	+	+	+	–	–	NA	–	NA	NA	+	–	NA
C. aurimucosum	+	+	+	–	–	–	–	–	–	–	–	–
C. auris	–	–	–	–	–	NA	–	NA	NA	–	–	NA
C. canis	+	+	+	–	–	+	–	–	–	–	+	NA
C. confusum	+	–	–	–	–	NA	–	–	–	+	–	–
C. coyleae	+	–	–	–	–	+	–	–	NA	+	–	–
C. diphtheriae gravis	+	+	–	–	–	NA	–	NA	NA	+	+	NA
C. diphtheriae intermedius	+	+	–	–	–	NA	–	NA	NA	+	–	NA
C. diphtheriae mitis	+	+	–	–	–	NA	–	NA	NA	+	–	NA
C. diphtheriae belfanti	+	+	–	–	–	NA	–	NA	NA	+	–	NA
C. durum	+	+	+	–	V+	NA	–	NA	NA	+	–	–
C. falsenii	+sl	V+sl	–	V	–	NA	–	–	–	+	–	–
C. freibugense	+	+	+	+	–	+	–	–	–	+	–	–
C. freneyi	+	+	+	–	–	NA	–	NA	NA	+sl	–	NA
C. glucuronolyticum	+	V	+	NA	–	NA	V	NA	NA	+	–	–
C. hansenii	+	+	+	NA	NA	NA	NA	NA	NA	+	NA	NA
C. imitans	+	+	+w	–	–	+	–	+	–	+	–	–
C. jeikeium	+	V	–	–	–	NA	–	NA	NA	V+	–	–

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C. kroppenstedtii	+	+w	+	–	–	NA	–	NA	NA	–	–	NA
C. lipophiloflavum	–	–	–	–	–	NA	–	NA	NA	–	–	NA
C. macginleyi	+	–	+	–	V	NA	–	NA	NA	+	–	NA
C. massiliense	–	–	–	–	–	NA	–	NA	NA	–	–	NA
C. matruchotii	+	+	+	–	–	NA	–	NA	NA	+	–	NA
C. minutissimum	+	+	+	–	–	–	–	–	–	–	–	–
C. mucifaciens	+	–	V	–	–	+	–	–	–	V	–	–
C. nigricans	+	+	+w	–	NA	+	–	NA	NA	V	V	NA
C. pilbarense	+	–	+	–	–	–	–	–	–	+	–	–
C. propinquum	–	–	–	–	–	NA	–	NA	NA	–	–	NA
C. pseudodiphtheriticum	–	–	–	–	–	NA	–	NA	NA	–	–	NA
C. pseudotuberculosis	+	+	–	–	–	+	–	NA	NA	+	–	NA
C. resistems	–	–	–	–	–	NA	–	NA	NA	–	–	NA
C. riegelii	–	+sl	–	–	–	–	–	–	–	+	–	–
C. sanguinis	+sl	–	–	–	–	NA	–	NA	NA	+	–	NA
C. simulans	+	–	+	–	–	+	–	–	–	V	–	–
C. singulare	+	+	+	–	–	NA	–	NA	NA	–	–	NA
C. sputi	+	–	–	–	–	–	–	NA	–	–	–	NA
C. striatum	+	–	V+	–	–	NA	–	NA	NA	–	–	NA
C. sundvallanse	+	+	+	–	–	NA	–	NA	NA	–	–	–
C. thomssenii	+sl	+sl	+sl	–	–	+	–	–	–	–	–	–
C. timonense	+	–	–	–	–	NA	–	NA	NA	+	+	NA
C. tuberculostearicum	+	V	V	–	–	+	–	NA	–	+	–	NA
C. tuscaniae	+	+	–	–	–	NA	–	NA	NA	–	–	NA

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C. ulcerans	+	+	–	–	–	+	–	+	NA	+	+	NA
C. urealyticum	–	–	–	–	–	NA	–	NA	NA	–	–	NA
C. ureicelerivorans	+	–	–	–	–	–	+w	–	–	+w	–	NA
C. xerosis	+	+	+	–	–	NA	–	NA	NA	+	–	NA
CDC group F1	+	+	+	–	–	NA	–	NA	NA	V–	–	NA
CDC group G	+	V	V	–	–	NA	–	NA	NA	+	–	NA

+, positive reaction; –, negative reaction; V, variable reaction; +sl, slow positive reaction; V+, variable reaction, most strains positive; V–, variable reaction, most strains negative; V+w, variable reaction, most strains weakly positive; Rev+, reverse CAMP test positive; NA, data not available; LIP, lipid requirement; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; GEL, gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase.
GLU, Glucose; MAL, Maltose; SUC, Sucrose; LAC, Lactose; MNTL, Mannitol; MANN, Mannose; XYL, Xylose; ARAB, Arabinose; SBTL, Sorbitol; RIB, Ribose; GLYG, Glycogen; AMYG, Amygdalin.

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MS) has also been used to identify *Corynebacterium* species, including *C. diphtheriae*. Alatoom and colleagues[20] evaluated the Bruker Biotyper MALDI-TOF MS for identification of 92 clinical *Corynebacterium* species isolates in comparison with 16S rRNA and *rpoB* gene sequencing. Of these 92 isolates, 80 (87%) were correctly identified to species level, except for *C. aurimucosum* strains, which were misidentified as the closely related species, *C. minutissimum*. In another study,

MALDI-TOF MS was able to rapidly identify all 90 strains of *C. diphtheriae* tested; these investigators proposed a testing algorithm using MALDI-TOF MS for rapid identification, followed by real-time PCR for *tox* gene detection, and the Elek test for detecting production of diphtheria toxin.[616] MALDI-TOF MS has also been used in individual case reports to confirm the identity of isolates that were provided by conventional biochemical testing or by the API Coryne identification kit.[1201]

Antimicrobial Susceptibility Testing of Corynebacterium *Species and Coryneform Bacteria*

CLSI first addressed antimicrobial susceptibility testing of coryneform bacteria in their 2005 standard for testing of infrequently isolated or fastidious bacteria, which was published in its second edition in 2010.[214] The approved standard is a microbroth dilution procedure that uses cation-adjusted Müeller–Hinton broth with lysed horse blood (2.5% to 5% v/v). For testing of daptomycin, the medium must be supplemented with calcium (50 μg/mL). The inoculum is prepared from a direct colony suspension that is equivalent to a 0.5 McFarland turbidity standard. Microbroth dilution panels are incubated at 35°C in ambient air for 24 to 48 hours. Results for antimicrobial agents that demonstrate resistance can be reported after 24 hours, while isolates demonstrating susceptible results for the β-lactams should be incubated for an additional 24 hours before reporting the results. The agents recommended for primary testing include penicillin, erythromycin, gentamicin, and vancomycin, but breakpoints defining susceptible, intermediate, and resistant categories are available for certain cephalosporins, carbapenems, gentamicin, erythromycin, ciprofloxacin, tetracycline, doxycycline, clindamycin, trimethoprim-sulfamethoxazole, rifampin, quinupristin-dalfopristin, and linezolid. Isolates that should be tested include those from sterile body sites, deep-tissue specimens, and prostheses-associated infection.

Members of the Genus Corynebacterium *Isolated From Humans*

The genus *Corynebacterium* contains species that have been isolated from human clinical specimens, animals, and the environment. *C. diphtheriae*, a classical pathogen, is the cause of diphtheria. Other species, such as *C. jeikeium*, *C. striatum*, *C. pseudodiphtheriticum*, and *C. urealyticum* are isolated more frequently than the other *Corynebacterium* species and behave primarily as opportunistic agents in immunocompromised and/or debilitated patients.[318] *C. amycolatum* is the most commonly isolated species in the clinical laboratory and may represent a skin contaminant or a significant agent of infection (Image Plate 14-3C).[263,284,579] Box 14-4 describes the clinical significance of *Corynebacterium* species that have been isolated from human clinical specimens and includes additional information on cultural

characteristics of these organisms. Table 14-6 presents the phenotypic biochemical characteristics of *Corynebacterium* species isolated from human clinical specimens.

Corynebacterium diphtheriae

Epidemiology. *C. diphtheriae* is the cause of the classic disease diphtheria. This infection is relatively uncommon in the United States, although it is still seen in developing countries. The frequency of infection in the United States has decreased markedly since the introduction and widespread use of effective diphtheria toxoid vaccines and the introduction of universal childhood immunization in the late 1940s. During the late 1960s and into the 1970s, diphtheria was still endemic in the Pacific Northwest and in the southwestern United States, with the last major outbreak of diphtheria in the United States occurring in the Seattle, Washington area in the 1970s.[198] Between the early 1980s through 2010, only 55 cases of diphtheria were reported to the CDC.[113] The importation of toxigenic *C. diphtheriae* from certain regions of developing countries where diphtheria remains endemic poses a constant threat and has accounted for most cases of diphtheria in industrialized countries in recent years. In developing countries, implementation of the World Health Organization's Expanded Program on Immunization for children during the 1970s was successful in achieving high levels of vaccination. With the passage of time, however, large portions of the populations targeted in earlier vaccination efforts became susceptible to infection due to waning immunity. During the 1980s and 1990s, sporadic outbreaks of diphtheria were reported in Germany, Sweden, China, Thailand, the Sudan, Jordan, and Ecuador. Epidemic diphtheria re-appeared in the New Independent States (NIS) of the former Soviet Union beginning in 1990 in the Russian Federation.[1162] This event occurred in part from unnecessary vaccination contraindications in regions of the NIS that resulted in inadequate vaccination coverage for children. Large population movements during the dissolution of the former Soviet Union resulted in disruptions of health services, and inadequate resources to maintain supplies of vaccine and antitoxin set the stage for the emergence and spread of diphtheria.[180] By 1994, all NIS regions were involved in the emergent epidemic, and more than 90% of all diphtheria cases reported from 1990 through 1995 were from this part of the world.[181] By 1998, the Newly Independent States of the former Soviet Union reported more than 150,000 cases of diphtheria to the WHO in Geneva.[283,410,592,805] Intensive control measures, including mass vaccination campaigns targeting persons aged 23 years or more, plus efforts to administer a four-dose DTaP series by age 2 years to more than 95% of the eligible populations has succeeded in bringing the epidemic under control in most regions.[1173,1240] More recently, outbreaks of diphtheria have occurred in Haiti and the Dominican Republic, and recent cases diagnosed in the United States have occurred in travelers returning

from Haiti.[185] Despite the success of widespread immunization, diphtheria continues to be a

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lethal, resurgent infectious agent in many parts of the world, including Africa (Algeria, Angola, Egypt, Ethiopia, Guinea, Niger, Nigeria, Sudan, Zambia, other sub-Saharan countries), Central and South America (Bolivia, Brazil, Columbia, Dominican Republic, Ecuador, Haiti, Paraguay), Europe (Albania, Armenia, Azerbaijan Belarus, Estonia, Georgia, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Moldova, Russia, Tajikistan, Turkmenistan, Ukraine, Uzbekistan), Asia/South Pacific (Bangladesh, Bhutan, Myanmar, Cambodia, China, India, Indonesia, Laos, Malaysia, Mongolia, Nepal, Pakistan, Papua New Guinea, the Philippines, Thailand, Vietnam), and the Middle East (Afghanistan, Iran, Iraq, Saudi Arabia, Syria, Turkey, and Yemen).

While historical evidence suggests that humans constitute the sole reservoir of *C. diphtheriae*, recent investigations have identified nontoxigenic *C. diphtheriae* as a cause of infections in animals. *C. diphtheriae* was isolated from both ears of a cat with bilateral otitis, ataxia, and anorexia.[457] An investigation of the cat's household and the veterinary staff caring for the animal yielded another isolate from the ear of a second cat. All the strains produced black colonies with brown halos on Tinsdale agar, produced the same identification code on the API Coryne, belonged to biotype belfanti, were sucrose fermentation-negative, and did not produce diphtheria toxin. PCR testing demonstrated the presence of the gene for the toxin A subunit, but not the gene for the toxin B subunit. The isolates were susceptible to all agents, including ampicillin, the cephalosporins, the fluoroquinolones, the carbapenems, erythromycin, clindamycin, gentamicin, daptomycin, linezolid, and vancomycin. Sequencing of the *rpoB* gene suggested that these feline strains may represent a novel *C. diphtheriae* subspecies.[457] *C. diphtheriae* biotype *belfanti* has been isolated from a bovine skin lesion and biotype *gravis* was isolated from a wound of a horse.[238,485]

Diphtheria Immunizations. Currently, the Immunization Practices Advisory Committee of the Public Health Service recommends immunization with DTaP (diphtheria toxoid, tetanus toxoid, and pertussis acellular vaccine) for all persons at least 6 weeks old but less than 7 years old. The vaccination series consists of three doses administered at 2, 4, and 6 months of age, with a minimum of 4 weeks between doses. The first booster dose is given at 15 to 18 months of age and should be administered at least 3 months after the third DTaP dose. A second booster dose is recommended for children ages 4 to 6 years, and adolescents ages 11 through 18 years should be given a single booster dose of TdaP if they have completed the primary immunization series. After this, boosters of Td vaccine should be given at 10-year

intervals.[188] Travelers to diphtheria-endemic areas should be up to date with all immunizations, and information on inoculations for travel can be found at http://www.cdc.gov/travel. In addition, persons convalescing from clinical diphtheria should also receive toxoid immunization since clinical infection does not necessarily induce protective levels of antitoxin. Because of the failure of most adults to receive booster injections, a susceptible adult population currently exists; 10% to 60% of individuals greater than 30 years of age have inadequate levels of circulating antibodies against diphtheria toxin. Because of this, outbreaks of diphtheria continue to be reported.

Diphtheria: Pathogenesis and Clinical Presentation. *C. diphtheriae* is the classical prototype of the toxigenic microorganism. Virulence of the organism is related almost entirely to the production of diphtheria toxin. Diphtheria toxin is a 58,342 Da polypeptide containing 535 amino acid residues. The toxin is composed of two fragments, fragment A (21,500 Da) and fragment B (37,200 Da). Fragment B, containing the receptor binding/translocation domains, allows the passage of the toxin molecule through the cell membrane of the target cells. Fragment A, the biologically active part of the molecule, catalyzes the transfer of the adenosine diphosphate ribose (ADPR) moiety from nicotinamide adenine dinucleotide (NAD) to elongation factor 2 (EF-2), a soluble protein that is required for the translocation of peptidyl-transfer RNA from the acceptor to the donor site on the eucaryotic ribosome. This adenoribosylation inactivates EF-2 and protein synthesis in the affected cell is inhibited. The structural gene (*tox* gene) that codes for diphtheria toxin resides on the DNA of a corynephage (bacteriophage) called the β-corynephage.[512] Upon infection of a *C. diphtheriae* strain with the *tox* + corynephage, the nucleic acid containing the *tox* gene becomes integrated into the bacterial chromosome (lysogeny) and replicates with chromosomal replication. Most nontoxigenic isolates of *C. diphtheriae* and most *tox*$^-$ corynephages do not contain any detectable *tox*-related DNA sequences. Only *C. diphtheriae* strains that are lysogenized by a β-phage containing the *tox* gene are capable of producing diphtheria toxin. *C. ulcerans* and *C. pseudotuberculosis* (the "*C. diphtheriae* group" organisms) are also capable of carrying the β corynephage and producing diphtheria toxin.

Respiratory tract diphtheria is primarily transmitted among humans via direct contact or by sneezing or coughing. Carriage of the organism on the skin and asymptomatic carriage of the organisms in the upper respiratory tract are common sources of transmission to other susceptible individuals. The onset of disease is insidious, and during an incubation period of 2 to 7 days, the organism multiples locally in the posterior naso- and oropharynx. Initial symptoms consisting of a sore throat, difficulty swallowing, malaise, and a low-grade fever may be present during this time. As infection progresses, there is an accumulation of organisms, fibrin, and

inflammatory cells to produce the characteristic grayish white **diphtheritic pseudomembrane**, which may eventually cover the tonsils, pharynx, larynx, and posterior nasal passages (Image Plate 14-3F).[455] Extension of the pseudomembrane anteriorly may lead to involvement of the soft palate and the uvula. In severe cases, the pseudomembrane may extend into the trachea and bronchi, resulting in airway obstruction. The pseudomembrane is strongly adherent to the oropharyngeal mucosa, and attempts to remove portions of pseudomembrane usually result in bleeding. Cervical and submandibular lymphadenopathy is also usually present. Further extension of the pseudomembrane into the posterior nasopharynx and nasal passages is associated with a serosanguinous or purulent discharge from the nose that is highly infectious. Middle ear involvement may result from contiguous extension of the infectious process. Extension of respiratory tract infections to the skin of the face (i.e., nose, ears, cheeks) and neck also occurs occasionally. Inoculation of the organisms into the eyes may lead to conjunctivitis with

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or without involvement of the cornea. Satellite lesions may also occur in the esophagus, stomach, or lower airways.

Locally multiplying bacteria synthesize and release diphtheria toxin, which is absorbed systemically. The toxin has direct toxic effects on the heart, the central and peripheral nervous system, liver, and kidneys. A diffuse myocardiopathy may result in 20% to 70% of patients. These symptoms may appear insidiously or cataclysmically, resulting in circulatory collapse and acute congestive heart failure. Neurologic complications and neuropathies may appear in 20% to 75% of patients and usually correlate with the severity of the illness. These complications may initially include paralysis of the soft palate and posterior pharyngeal wall, followed by oculomotor and ciliary paralysis. The latter contributes to the risk of oropharyngeal aspiration and pneumonia. Fatty degenerative changes and focal necrosis in the kidneys, liver, and adrenals may also complicate severe infections. In general, the myocardiopathy and neuropathy occur later and are less severe following cutaneous diphtheritic infections than those following respiratory tract infection. Death may ensue from cardiac failure or paralysis of the diaphragm.

C. diphtheriae may also cause a primary cutaneous infection, where toxin is also absorbed systemically. Skin lesions may resemble several other skin conditions, including folliculitis, impetigo, pyoderma, abscess, or seborrheic dermatitis. Other pyogenic organisms, such as *Streptococcus pyogenes*, may be co-isolated with *C. diphtheriae*. Cutaneous diphtheria has also been reported following the bites of insects, such as spiders, infection of preexisting wounds (e.g., surgical wounds, pyoderma, eczema, impetigo, dermatitis) and following tattooing.[455,760,995] An

ulcerative lesion called **ecthyma diphtheriticum** is the initial lesion. This lesion begins as a vesicle or pustule containing a straw-colored or serosanguinous fluid. After this material drains, the lesion becomes a punched-out ulcer a few millimeters to a few centimeters in size. The margins of the lesion are elevated and undermined. Diphtheritic skin lesions are usually painful and subsequently become covered with a dark, adherent pseudomembranous eschar or scab. This pseudomembrane eventually falls off, leaving a beefy, hemorrhagic base that oozes serosanguinous fluid. As the initial lesion develops, the surrounding skin becomes red and edematous, with the formation of satellite bullae surrounding the ecthyma diphtheriticum lesion. Most of the cutaneous lesions are on the arms, hands, feet or lower legs. Although cutaneous lesions are much less likely to cause serious systemic toxicity, they do pose a significant risk of spread to the environment and to other individuals. Lesions are often co-infected by other pathogens (e.g., *S. aureus*, β-hemolytic streptococci).

Reports of severe infections caused by nontoxigenic *C. diphtheriae* have increased substantially since the late 1980s and into the present. This increase has been noted in Canada, throughout Europe (including England, Wales, Poland, France, Italy, Germany, Switzerland), and Australia.[357,448,693,848,901,945] Isolates involved in these infections are often clonal (i.e., have the same multilocus sequence type [MLST]) Infections include cutaneous and wound infections, bacteremia, and endocarditis. Cutaneous infection with nontoxigenic *C. diphtheriae* have been found particularly among homeless persons in urban areas, and are associated with homelessness, intravenous drug use and alcoholism.[448,693,848] Skin lesions are characterized by chronic, weeping, non-healing ulcers that are often co-infected with other skin and soft tissue pathogens. In a study of cutaneous infections performed in Australia, cultures from more than 50% of patients grew both *S. aureus* and β-hemolytic streptococci along with *C. diphtheriae*.[421] Nontoxigenic bloodstream isolates are usually biotype *gravis* or *mitis*, and, in regions where outbreaks have occurred, ribotyping and MLST have demonstrated different invasive clones residing in each outbreak region (e.g., ST130 in France, ST82 in New Caledonia, MLST8 in Poland).[322,1243] Bloodstream infection with a nontoxigenic *C. diphtheriae* was reported in a 23-year-old man from Ohio with acute myelogenous leukemia.[1201] The source was not documented, but the patient had pharyngitis on presentation and also had a triple-lumen Hickman catheter in place for chemotherapy. While *C. diphtheriae* endocarditis is rare, case reports documenting endocarditis due to nontoxigenic isolates have increased substantially since 1980. *C. diphtheriae* endocarditis occurs in those with underlying or preexisting cardiac disease (e.g., congenital heart disease, mitral regurgitation, rheumatic fever), or with prosthetic valves or other hardware (e.g., aortic homograft).[742,787,986] In 2011, Muttaiyah and colleagues[787] reported on 10 cases of endocarditis due to nontoxigenic strains that occurred in Auckland, New Zealand, over a 14-year period. The patients

ranged in age from 4 to 62 years, 8 of the 10 had preexisting cardiac abnormalities, and only 3 of the 10 patients required surgical intervention for valve manipulation. Destructive native pulmonary valve endocarditis with pericardial effusion was described in a 2-year-old French Guyanese infant in which the organism was detected by sequencing of 16S rRNA from the resected valve; the *tox* gene sequence was absent.[986] The patient initially presented with a febrile prodromal illness and may have had exudative pharyngitis. Echocardiography usually demonstrates large valvular vegetations that have a propensity to embolize to the small blood vessels in the brain, spleen, and kidneys. Mycotic aneurysms and septic arthritis may develop in the course of heart valve and bloodstream infection. Surgical intervention and valve placement or replacement may be required to effect cure in some cases. An association has also been made between the use of IV drugs and *C. diphtheriae* endocarditis.[448] In these cases, cutaneous colonization or infection with nontoxigenic *C. diphtheriae* may serve as the nidus for introduction in the bloodstream. The pathogenesis of severe infections due to nontoxigenic *C. diphtheriae* is not understood at the present time. The organism is able to actively invade tissue, causing a fulminant disease involving the cardiac, endothelial, and synovial tissues.

The capacity of *C. diphtheriae* to infect a wide variety of cell types may be responsible for some of the unusual presentations of diphtheria. Havaldar and Shanthala reported a case in an 11-year-old girl who presented with abdominal pain of 2 weeks duration.[479] The child subsequently developed bilateral, conjunctival pseudomembranes that were culture-positive for *C. diphtheriae*. Intestinal involvement became apparent with the passage of bloody stools and casts of sloughed intestinal tissue. Rare infections with nontoxigenic *C. diphtheriae* have included purulent pericarditis and percutaneous nephrostomy catheter insertion site infection in an elderly patient with bladder cancer.[415,617] In the latter

case, the isolate was demonstrated to form adherent biofilms on polystyrene, polyurethane, and glass surfaces. Other infections associated with *C. diphtheriae* (e.g., septic arthritis, osteomyelitis) have been reported but are rare.[69,877]

Treatment of Diphtheria. Treatment of diphtheria involves the administration of equine antitoxin to neutralize toxin not already bound to target cells. Antitoxin should be administered as soon as a presumptive diagnosis of diphtheria is made, since the antitoxin only inactivates extracellular toxin. The amount of intravenous antitoxin administered depends on the duration and extent of the disease. Hypersensitivity to horse protein must be evaluated by skin testing and, if an immediate type hypersensitivity reaction develops, desensitization with progressively higher antitoxin

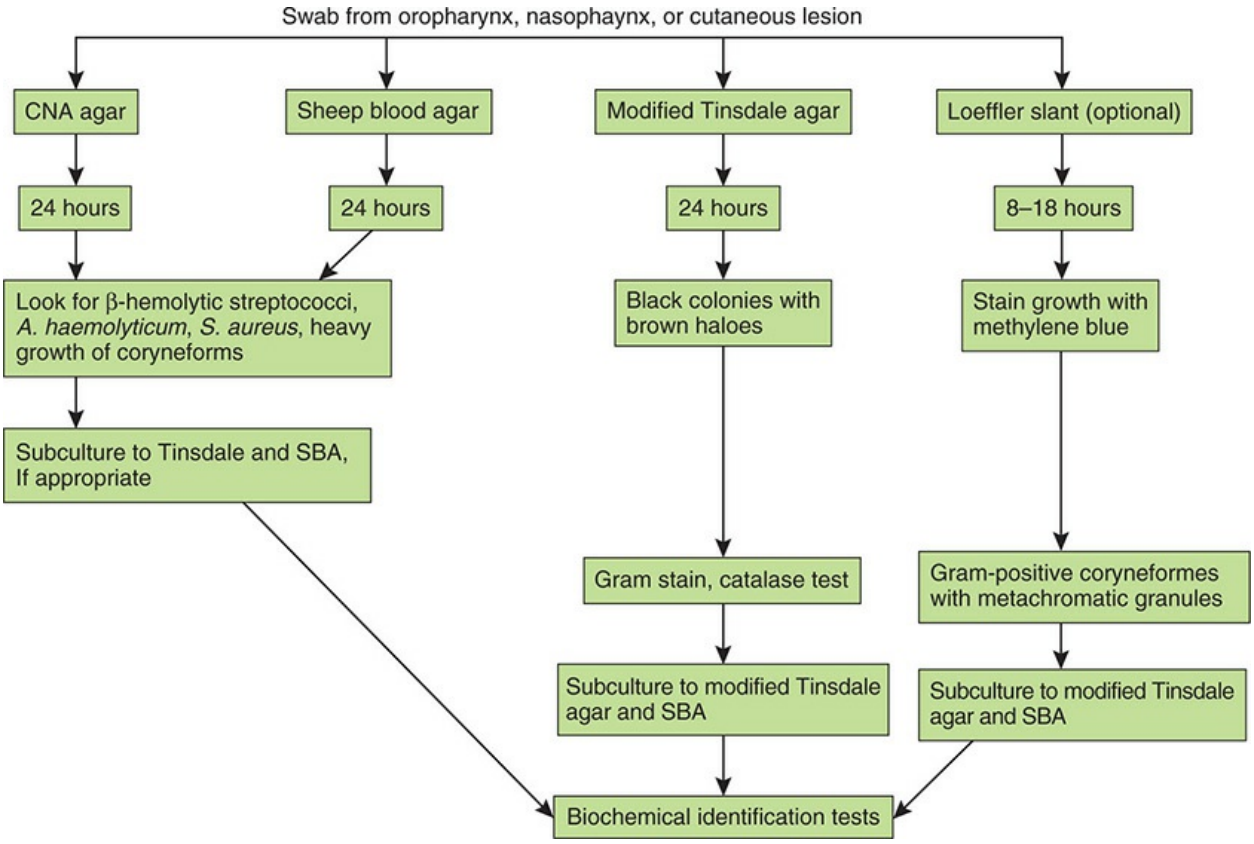
dose administration is warranted. Since diphtheria antitoxin is no longer licensed for production in the United States, the CDC must be notified to acquire the foreign product. No effective therapy is available for reversal of cardiac or neurologic involvement that might have occurred prior to specific diagnosis. Supportive care (i.e., tracheostomy or intubation, airway clearance, monitoring of cardiac function) is necessary and of extreme importance. Penicillin or erythromycin may also be administered to speed the eradication of the organisms from the respiratory tract of the patient, to decrease the toxin burden in the patient, and to prevent spread of the organism to others.[606] Procaine penicillin G (<20 lbs, 300,000 units; >600,000 units) is given intramuscularly every 12 hours, until the patient is able to swallow. Treatment is then switched to oral penicillin or erythromycin to complete a 14-day course. Both rifampin and erythromycin have been used to eradicate carriage of *C. diphtheriae* in exposed individuals.

Isolation and Identification of *Corynebacterium diphtheriae*. For the isolation of *C. diphtheriae*, swab specimens are obtained from the oropharynx, the nasopharynx, or from cutaneous lesions. Swabs should be made of Dacron and should be used to sample multiple, inflamed areas of the nasopharynx. If a pseudomembrane is present, swab specimens from beneath the membrane should be collected. Swab specimens may be sent to the laboratory in a routine semisolid transport medium such as Amies. Specimens are inoculated onto SBA and/or a Columbia CNA plate, and an agar medium containing cystine and potassium tellurite. For the cystine-tellurite medium, laboratories should use cystine-tellurite–blood agar or modified Tinsdale agar for the recovery of *C. diphtheriae* (Image Plate 14-3G).[1041] Although not routinely done, some laboratories will also inoculate a slant of Loeffler medium, which contains coagulated beef serum and egg. Methylene-blue staining of smears prepared from 8- to 18-hour growth on Loeffler medium may provide a preliminary diagnosis of diphtheria, since these organisms grow rapidly on this medium and produce distinctive, club-shaped, diphtheroidal cells containing metachromatic granules (Image Plate 14-3H) (Charts 14-1 and 14-2). However, since other coryneforms may also have this appearance on Loeffler medium, results should only be reported as presumptive and must be correlated with the clinical presentation of the patient. SBA and CNA are used to screen for β-hemolytic streptococci, *Staphylococcus aureus* and *Arcanobacterium haemolyticum*. *S. aureus* may be co-isolated with *C. diphtheriae* and will also grow as black colonies on Tinsdale medium, so the blood agar plate or CNA plate will help to assess the extent of staphylococcal growth on the tellurite-containing medium (Chart 14-3). Since some laboratories may not stock tellurite-containing medium because of infrequent requests, the inoculation of SBA and/or CNA agar will still enable the recovery of *C. diphtheriae* and other pathogenic organisms, enabling suspected coryneform isolates to be subcultured and examined further should the clinical

presentation and history of the patient (e.g., presence of a characteristic pseudomembranous oropharyngeal lesion, lack of immunizations, outbreak settings) strongly suggest *C. diphtheriae* infection.

C. diphtheriae, *C. ulcerans*, and *C. pseudotuberculosis* produce black colonies that are surrounded by brown halos on modified Tinsdale medium (Image Plate 14-3G). Black coloration of the colonies results from tellurite reductase activity, resulting in the reduction of tellurium, while the brown halos indicate cystinase activity. Other diphtheroids, staphylococci, and some streptococci may also reduce tellurite, although halos around the colonies are not generally present, so Gram's stains and catalase tests should be performed on all suspicious colonies. *C. diphtheriae* does not produce urease, so this test may be used to distinguish this organism from *C. ulcerans* and *C. pseudotuberculosis* (both urease-positive). The latter two organisms can be separated from one another by the production of acid from starch or glycogen. Deamidation of pyrazinamide to pyrazinoic acid by pyrazinamidase, a test used in mycobacteriology, is also a helpful test for distinguishing the "*C. diphtheriae*" organisms (pyrazinamidase-negative) from other *Corynebacterium* species (mostly pyrazinamidase-positive). A flow chart for detecting and presumptively identifying isolates recovered on Tinsdale medium is presented in Figure 14-1 and Figure 14-2.

C. diphtheriae may appear as four distinct colony types (biotypes) designated *gravis*, *mitis*, *intermedius*, and *belfanti*. These biotypes also differ slightly in Gram's stain morphology, certain biochemical reactions, and historically, in the severity of the disease processes they produce. *C. diphtheriae* biotype *gravis* and biotype *mitis* strains produce fairly large (1 to 2 mm in diameter at 24 hours), convex colonies with entire edges, while biotype *intermedius* strains produce small (less than 1 mm in diameter at 24 hours), blacker, dense colonies on tellurite-containing agar. Most *mitis* and some *gravis* strains are weakly β-hemolytic on SBA. All four biotypes grow as black colonies surrounded by brown halos on modified Tinsdale medium, all form gray-white, smooth, nonhemolytic colonies on SBA, and all are catalase-positive (Image Plate 14-4A). None of the biotypes have pyrazinamidase activity or produce alkaline phosphatase, and they do not hydrolyze esculin or urea. Acid is produced from glucose, maltose, fructose, galactose, mannose, and ribose, but not from sucrose, lactose, mannitol, xylose, raffinose, trehalose, glycerol, or glycogen; sucrose-fermenting strains have been reported (Table 14-6).[262] *C. diphtheriae* is included in the data bases of many manual and automated identification kits, including the API Coryne, the RapID CB-Plus, and the Vitek 2 *Anaerobe/Corynebacterium* card.[869,915] The API Coryne identification panel has been used as the primary phenotypic identification system for *C. diphtheriae* in many



■ **FIGURE 14-1** Flowchart for identification of *Corynebacterium diphtheriae*.

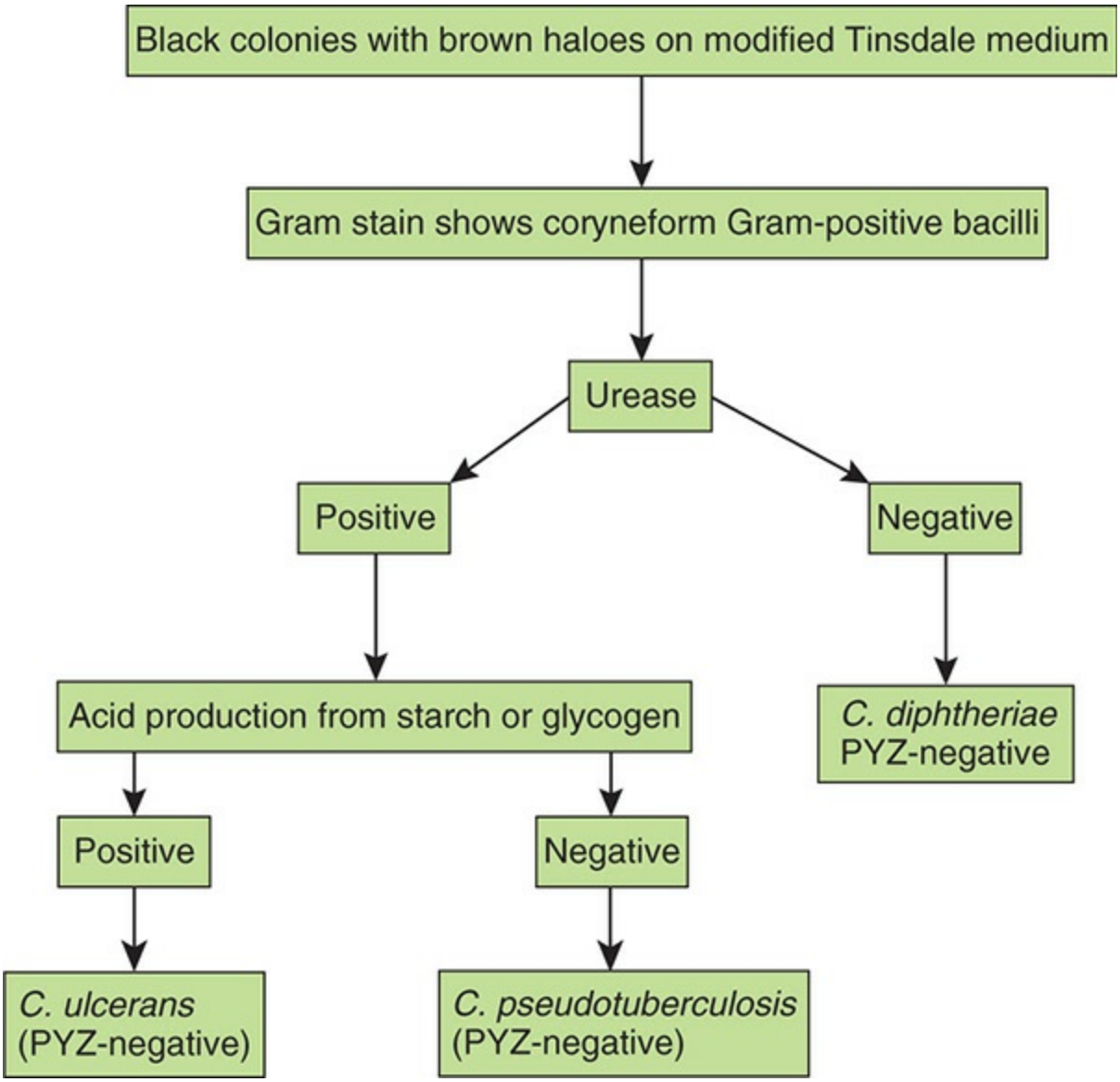
publications (see Image Plate 14-4B and 14-4C). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was also able to rapidly identify all 90 strains of *C. diphtheriae* tested, and these investigators proposed a testing algorithm using MALDI-TOF MS for rapid identification, followed by real-time PCR for *tox* gene detection, and the Elek test for detecting production of diphtheria toxin.[616]

Since isolation of *C. diphtheriae* is a rare event in developed countries, particularly in the United States, many laboratories do not keep the ingredients for modified Tinsdale medium or other cystine-tellurite-containing media on hand. Therefore, it is helpful to have a method to screen diphtheroidal colonies isolated on blood and chocolate agar for possible *C. diphtheriae,* particularly for those patients with compatible clinical presentations (e.g., severe pharyngitis or skin lesions) when immunization histories are not available. Pimenta and colleagues[869] evaluated the DNase test as an approach for screening. Of 91 *C. diphtheriae* isolates (37 toxigenic and 54 nontoxigenic), DNase activity was detected in 100% of the *C. diphtheriae* isolates regardless of biotype status (*mitis*, *interemdius*, *belfanti*, or *gravis*) and ability or inability to ferment sucrose. The DNase test was negative for 93.9% of 565 non-

diphtherial *Corynebacterium* species.

Once an organism is biochemically identified as a possible *C. diphtheriae*, the isolate must be tested for the ability to produce diphtheria toxin. The "gold standard" for detection of diphtheria toxin production is a Vero cell cytotoxicity assay.[301] Many laboratories that perform toxigenicity tests use the modified Elek immunoprecipitation procedure, which is an immunodiffusion method that is similar to the classical Ochterlony test. The test employs KL Virulence agar (Difco), a proteose peptone-based agar, supplemented with sterile rabbit serum or with KL Virulence enrichment (a non-serous enrichment designed for use with KL Virulence agar by Difco) and with 0.03% potassium tellurite (supplied as Bacto-Chapman tellurite solution, 1%; Difco) as the basal medium. This medium is poured into a sterile 150 mm petri plate. Before the medium solidifies, a 1 × 8 cm strip of filter paper that is saturated with diphtheria antitoxin (Bacto-KL antitoxin strips, Difco) is submerged in the medium and oriented across the diameter of the plate. After cooling, the isolate to be tested for toxin production is streaked perpendicular to the submerged antitoxin strip. Equidistant from either side of this streak, a known toxigenic *C. diphtheriae* strain and a known nontoxigenic *C. diphtheriae* strain are also streaked for control of the test. The plate is then incubated at 35°C and examined every 24 hours for 3 days. If the unknown strain is toxigenic, precipitin lines will form at 45° angles between the inoculum and the antitoxin strip. These lines will show identity with the toxigenic control *C. diphtheriae* strain. A modified Elek immunodiffusion procedures uses a centrally placed disk containing antitoxin with the test organisms being spot inoculated 7 to 9 mm away from the central antitoxin disk.[311] Such modifications enable simultaneous testing of multiple isolates and are adaptable to outbreak situations. High-quality antitoxin can be obtained from several sources (Pasteur-Merieux, Lyon, France; CNG, Peyrm, Russia; Wyeth Laboratories, Marietta, PA; Connaught Laboratories, Swiftwater, PA).

Several other methods, including enzyme immunoassays and reverse passive latex agglutination tests have also been



■ **FIGURE 14-2** Flowchart for isolation of *C. diphtheriae*.

developed for the detection of toxin production by *C. diphtheriae*.[309,310,311,1110] The rapid EIA developed by Engler and Efstratiou used equine polyclonal antitoxin as the capture antibody, and an alkaline phosphatase-labeled monoclonal antibody directed against fragment A of diphtheria toxin as the detection antibody. This method detected 87 toxigenic stains among 245 *C. diphtheriae* isolates and was completely concordant with the results from the modified Elek method.[309] In 2002, an immunochromatographic strip (ICS) assay was developed and marketed by the World Health Organization and the Program for Appropriate Technology in Health (Seattle, WA).[310] For this test, a suspension of the organism is prepared in an enrichment broth

and incubated for 3 hours. An ICS strip is placed in the tube. After 10 minutes, the strip is examined for the presence of a band that is parallel to an internal positive control present on the strip. This assay can detect as little as 0.5 ng/mL of diphtheria toxin and is 10 times more sensitive than EIA methods and 20 times more sensitive than agglutination assays.[310]

Molecular methods have also been developed for identification of *C. diphtheriae* and for the detection of the *tox* gene. For identification purposes, the most robust target is the *dtxR* gene, which is present in all toxigenic and nontoxigenic *C. diphtheriae* strains.[273,796,1117] The *dtxR* gene encodes a global regulator of metabolism in *C. diphtheriae*, and functions in the regulation of *tox* gene expression.[273,300,868] Pimenta and colleagues described a PCR assay for detection of *dtxR* in 91 *C. diphtheriae* isolates (54 nontoxigenic and 37 toxigenic) and 111 isolates of other *Corynebacterium* species. This assay was positive for all *C. diphtheriae*, and none of the 111 corynebacterial isolates, including one *C. ulcerans* and 2 *C. pseudotuberculosis* isolates that were *dtxR*-positive.[867] Most PCR assays for detection of *tox* gene sequences in *C. diphtheriae* use primers that flank sequences corresponding to the biologically active toxin fragment A.[754,795] These assays have been used for toxin detection in pure cultures of *C. diphtheriae* and directly in clinical specimens. Some studies have noted a 100% correlation between molecular-based techniques and phenotypic methods for detection of toxin production (e.g., the Elek test).[754]However, PCR methods may also detect toxin gene sequences in isolates that do not produce biologically active toxin. In one study, 6 of 55 *C. diphtheriae* isolates did not produce toxin but possessed the *tox* gene, in whole or in part.[300] Therefore, negative PCR tests on suspected isolates are helpful for excluding the diagnosis of diphtheria but may not predict phenotypic expression of toxin production. Pimenta et al.[867] designed a multiplex PCR assay that used three primer pairs for detection of the *dtxR* gene, toxin fragment A, and toxin fragment B, respectively. This approach correctly identified all 33 toxigenic and 51 nontoxigenic *C. diphtheriae*, and none of the other *Corynebacterium* isolates tested were positive for *dtxR* or tox A/tox B.[867] Complete correlation was also noted for positive PCR results for *toxA* and the Vero cell cytotoxicity assay, which is considered the "gold standard" in vitro assay for diphtheria toxin detection. In 2011, Sing et al.[1023] described a real-time PCR assay that detected the *tox* gene of toxigenic *C. diphtheriae* and the *tox* gene found in some *C. ulcerans* strains. This assay amplified the *tox* genes present in both species, and identification of the individual isolates was achieved by melting curve analysis.[1023] The CDC performs both the *dtxR* and *tox* gene PCR assays through consultation with state public health laboratories.

Many approaches have been used to develop a typing scheme for *C. diphtheriae*. Any typing scheme must be discriminatory, reproducible, and stable. Ribotyping has

been considered as the "gold standard" for molecular characterization of *C. diphtheriae*.[272,440] Ribotyping for *C. diphtheriae* was standardized in 1997 and, in 2004, ribotyping nomenclature was revised to construct a database with internationally recognized ribotype designations.[440,905] In 2008, a study was performed comparing ribotyping with three other methods for typing *C. diphtheriae* typing in order to address the global epidemiology of this important pathogen.[274] The study compared ribotyping with PFGE, random amplified polymorphic DNA (RAPD), and AFLP for their ability to discriminate between and among strains. This study found that ribotyping was still highly discriminatory and reproducible, followed by RAPD. AFLP and PFGE were less discriminatory than ribotyping and RAPD, even though the former tests were more rapid and less difficult to perform. However, a 2009 study examined 20 *C. diphtheriae* isolates from Belarus and found that all isolates belonged a single ribotype.[768,769] Application of a reverse-hybridization microassay that detected polymorphisms in two gene "spacer" sequences designated as DRA and DRB were able to subdivide these twenty isolates into three distinct clusters that were able to resolve links in organism transmission that were not explained by the ribotyping data.[769]In a 2010 study of 19 nontoxigenic isolates from Poland, ribotyping failed to show any differences among the isolates, whereas ERIC-PCR and PFGE were able to discriminate strains within different ribotypes.[1243] As more information becomes available on the molecular evolution of this bacterial agent, other typing methods or modifications to older methods are likely to be developed for global epidemiologic purposes.

C. ulcerans may cause diphtheria-like respiratory tract or cutaneous infections and, as mentioned above, can also

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produce diphtheria toxin.[1172] Depending on the molecular test used, toxigenic *C. ulcerans* may be detected in *tox* gene PCR assays for *C. diphtheriae*. The *tox* genes from the two organisms differ in their nucleotide sequences and, consequently, the amino acid sequences of A and B toxin subunits.[1024,1025] In one study, a previously described real-time PCR assay for the A and B subunits of the *tox* gene was compared with the conventional *tox* PCR assay for the same gene.[169] These tests differed in the sets of primers and probes used in the real-time versus the conventional *tox* assay.[784,796] Elek tests were also performed with each isolate and amplification products for both PCR assays were sequenced. Of the 20 *C. ulcerans* strains examined, four were positive for both toxin subunits by both PCR assays and all four were Elek test-positive. Another eight strains were negative in all PCR assays and were Elek test-negative. Seven isolates were positive for both *tox* gene subunits by the

conventional PCR assay, but the *tox* sequences for the B subunit were not detected and results for toxin A sequence detection were atypical in the real-time PCR assay. Sequencing of the *tox* gene from the seven isolates revealed several nucleotide differences in the regions where the primers and probes annealed in the real-time PCR assay. For the real-time PCR assay for subunit A, three to four mismatches were noted, while eight mismatches were found for subunit B. Corresponding mismatch values for the conventional PCR assay were three to four for subunit A and no mismatches for subunit B.[169] Other real-time *tox* gene PCR assays have been described that are based on published *tox* gene sequences from *C. diphtheriae* and *C. ulcerans* and are sensitive and specific enough to differentiate the two organisms.[991] Because of the results generated by toxigenic strains of *C. ulcerans* in molecular tests for the *C. diphtheriae tox* gene, negative or atypical real-time PCR results should be confirmed with conventional *C. diphtheriae* molecular assays and toxin tests (i.e., the Vero cell cytotoxicity assay, Elek test) should be performed.

Antimicrobial Susceptibility of *Corynebacterium diphtheriae*. Most isolates of *C. diphtheriae*, the primary pathogen in the genus, remain susceptible to penicillin, ampicillin, and erythromycin, the drugs used for treatment of diphtheria. These same agents are also used for treatment of infections caused by nontoxigenic *C. diphtheriae* strains. Most isolates are also susceptible to ceftriaxone, clindamycin, gentamicin, vancomycin, daptomycin, and linezolid.[606,715] Isolates that are resistant to β-lactam agents and erythromycin have been described, as has multidrug-resistant *C. diphtheriae*.[606,847,861] Multidrug-resistant strains have been isolated from individuals returning from regions of endemicity. For example, a wound isolate from an unvaccinated male patient was resistant to clindamycin, erythromycin, tetracycline, and trimethoprim–sulfamethoxazole, and intermediate in susceptibility to ceftriaxone and cefotaxime.[760] The isolate remained susceptible to gentamicin, ciprofloxacin, daptomycin, linezolid, meropenem, quinupristin-dalfopristin, and vancomycin. In 2012, this organism was the cause of a bloodstream infection in a patient with AML and neutropenic fever; the organism was susceptible to penicillin, clindamycin, erythromycin, gentamicin, ciprofloxacin, trimethoprim–sulfamethoxazole, imipenem, meropenem, daptomycin, and vancomycin, but was resistant to ceftriaxone (MIC 4 μg/mL).[1201] In a study of 19 nontoxigenic isolates from nasopharyngeal, bloodstream, and wound infections in Poland, all isolates were susceptible to every agent tested, except that a single strain was intermediate for ciprofloxacin, and 47% were intermediate for cefotaxime.[1243] A study performed in Italy also noted elevated MICs for cefotaxime for all *C. diphtheriae* isolates tested.[1167]

Reporting of *Corynebacterium diphtheriae*. Epidemiologic investigations of possible

diphtheria cases require cooperation between public health and the laboratory. A confirmed case of diphtheria must have the compatible upper respiratory tract illness and (a) isolation of *C. diphtheriae* from the nose, throat, or a sampling of the pseudomembrane, (b) histopathologic evidence of diphtheria, or (c) epidemiologic linkage to a laboratory-confirmed case. Positive PCR assays are considered as supportive evidence, and cases that have positive *dtxR* and *tox* gene PCR results without organism isolation, histopathologic evidence, or linkage to a confirmed case are considered as probable cases. Attempts must be made to recover the organism from properly collected nose and throat specimens of suspect cases and their close contacts as soon as diphtheria is suspected, and the laboratory must be informed so that the appropriate culture media can be obtained or prepared. Once isolated and identified, tests must be performed to determine the biotype and the Elek test should be performed to detect toxin production. Isolates of *C. diphtheriae* should be sent to the CDC Diphtheria Laboratory for reference testing through arrangement with the state public health laboratory. In the event that other possible toxin-producing isolates are obtained (e.g., *C. ulcerans*, *C. pseudotuberculosis*), these should be forwarded to the CDC as well. Healthcare providers must notify both the state health department and the CDC regarding probable or confirmed cases of diphtheria as soon as possible so that investigations can be promptly initiated.

Corynebacterium *Species Associated With Animals and the Environment*

Corynebacterium species have also been isolated from a variety of animal species, and some of these are regarded as pathogens in their respective animal hosts. A few animal isolates (e.g., *C. falseni*) have also been isolated from humans. Box 14-5 lists these *Corynebacterium* species and the animals associated with them. Several *Corynebacterium* species have also been isolated from various food and environmental sources, and none of these are implicated in human infections.

Other Coryneform Bacteria

Other coryneform bacteria that have been isolated from human clinical specimens include pleomorphic gram-positive bacilli that form characteristic Chinese letters, V-forms and occasional coccoid forms on Gram's stain. These species differ from recognized *Corynebacterium* species in several

BOX 14-5

Corynebacterium Species Isolated From Animals

Species	Source of Isolation—Infection
C. aquilae[331]	Spanish Imperial Eagles (*Aquila adalberti*)—mouth/tracheal isolates
C. auriscanis[224]	Canines—otitis externa
C. bovis	Cattle, others (humans)
C. camporealensis[330]	Sheep—mastitis
C. canis[363]	Dog—bite wound isolate
C. capitovis[221]	Sheep—head wound isolate
C. caspium[220]	Caspian seals—penile wound isolate
C. ciconiae[332]	Black storks—trachel culture isolates
C. cystiditis[1222]	Cows—genitourinary tract isolate
C. epidermidicanis[355]	Dogs—skin isolate from dog with pruritis
C. falsenii[331]	Eagles—mouth/tracheal isolates
C. felinum[222]	Wild cat—respiratory tract isolate
C. kutscheri[144]	Rats, mice
C. mastiditis[329]	Sheep—subclinical mastitis
C. mustelae[366]	Ferret—lung, liver, kidney tissue, lethal sepsis
C. phocae[845]	Common seals
C. pilosum[1222]	Cows—genitourinary tract isolate
C. pseudotuberculosis	Sheep (humans)
C. sphenisci[426]	Wild penguins—cloacal isolates from healthy animals
C. spheniscorum[427]	Wild penguins—cloacal isolates from healthy animals

C. suicordis[1153]	Pigs—respiratory tract isolates, pericardial isolates
C. testudinoris[222]	Tortoise—necrotic oral lesion isolate
C. ulceribovis[1225]	Cows—udder ulceration
C. vitarumen	Cow rumen isolate

respects. Most of them lack *meso*-DAP as the di-amino acid comprising the cross-linkages in the peptidoglycan structure, but rather have other amino acids (e.g., L-lysine, L-ornithine) present (Table 14-5). Other components normally found in the corynebacterial cell wall, such as arabinose, galactose, and short chain mycolic acids may also be absent. The menaquinones present as part of the electron transport system also vary from group to group. In addition, some of the coryneform organisms are oxidative rather than fermentative with respect to carbohydrate metabolism. These bacteria have been recovered from several types of human clinical specimens, including blood, cerebrospinal fluid, urine, and other normally sterile body sites and fluids.

While some of the former CDC coryneform groups have been formally reclassified in the genus *Corynebacterium,* others have been found to belong to genera not previously associated with human pathology. Several research groups, particularly in Europe, are working to unravel the complex taxonomic relationships among these disparate organisms using both genetic (e.g., DNA–DNA hybridization, 16S and 5S ribosomal RNA [rRNA] sequencing) and chemotaxonomic (e.g., cell wall, cellular fatty acid, cellular menaquinone analyses) approaches. These methods have established that reliance on phenotypic characteristics alone may not be sufficient for identification of these organisms, and that molecular and chemotaxonomic analyses of subcellular components may be necessary to differentiate among organisms that have identical or similar phenotypic characteristics. Although these organisms are relatively uncommon isolates in the clinical laboratory, they are increasingly being recovered as nosocomial colonizers and occasional pathogens, particularly in immunocompromised patients.

Actinotignum *and* Actinobaculum *Species*

Intensive genotypic examination of other "*Actinomyces*-like" strains from human clinical specimens has resulted in descriptions of totally new genera and reclassification of existing *Actinomyces* species. Lawson and associates[642] examined five strains of an unknown *Actinomyces*-like organism that were recovered from human specimens (including blood) and found that they were distinct from previously described *Actinomyces* species. The most closely related organism to these unknown strains was

the obligately anaerobic species

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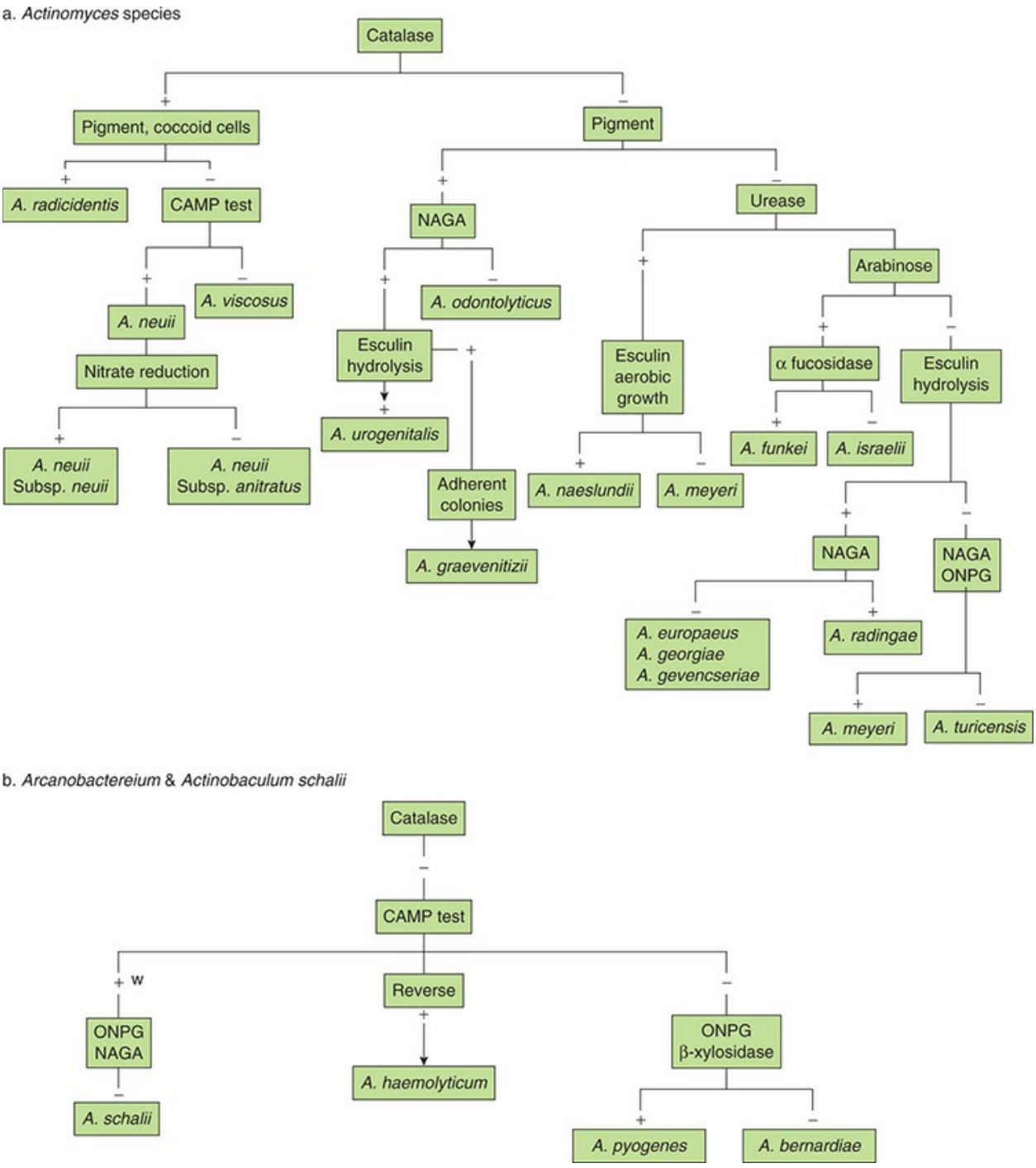

■ **FIGURE 14-3** Flowchart for identification of *Actinomyces, Actinobaculum,* and *Arcanobacterium* species from human clinical specimens.

Actinomyces suis, and even this organism was phylogenetically divergent from other *Actinomyces* species that were examined. Based on genetic, chemotaxonomic, and biochemical data, a new genus, *Actinobaculum*, and species,*Actinobaculum schaalii*, was proposed for the five previously undescribed human isolates from blood and urinary tract specimens and *Actinomyces suis* was also reclassified as *Actinobaculum suis*.[642] Since the description of *A. schaalii* and the reclassification of *A. suis*, two additional *Actinobaculum* species—*A. massiliae* and *A. urinale*—have been isolated from urine specimens from patients with chronic/recurrent urinary tract infections.[437,459] *A. massiliae* has also been isolated from superficial cutaneous infections.[1170] Figure 14-3 provides a flow chart for identification of the more common *Actinomyces* (Figure 14-3a), *Actinotignum, Actinobaculum,* and *Arcanobacterium* species (Figure 14-3b).

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Recent taxonomic dissection of *Actinobaculum* species using chemotaxonomic and molecular approaches has resulted in the reclassification of *A. schaalii* and *A. urinale* to the new genus *Actinotignum* as *Actinotignum schaalii* and *Actinotignum urinale*, respectively. These workers also found that the type strain of *A. massiliense* was actually a strain of *A. schallii*.[1228a]

Since its initial description, *A schaalii* has emerged as a significant urinary tract pathogen, causing urosepsis, acute and chronic cystitis, asymptomatic bacteriuria, and sepsis.[172,490,801] Reinhard et al.[909] reported on 10 patients in Denmark with urosepsis due to *A. schaalii*. Nine of the patients were elderly and had predisposing conditions, including prostatic hyperplasia, diabetes mellitus, rheumatoid arthritis, dementia, colon carcinoma, kidney stones, paraplegia, and pyelonephritis, and all patients had *A. schaalii* isolated from urine, blood, or both. A 2010 retrospective study of all cases identified in Switzerland since 2004 yielded 21 *A. schaalii* isolates from 19 patients.[84] Ten of the isolates were cultured from urine, six were from blood, three were from pus, and one was from both blood and urine cultures. Thirteen (76%) of the 17 patients with positive blood or urine cultures had underlying urinary tract disease, and, when urine cultures were positive, all these patients had pyuria and negative nitrite tests. Urosepsis with *A. schaalii* along with other uropathogens, including *Aerococcus urinae* and *Actinobaculum urinale*, have also been reported.[328,1064] Pediatric infection with *A. schaalii* was documented in 2003, with a case of pyelonephritis that occurred in a 5-year-old boy with hemiplegia secondary to a hemispheric tissue lesion and pyeloureteral obstruction.[836] Bank and colleagues[67] developed a real-time quantitative PCR assay for *A. schaalii* and tested urine specimens from 177 hospitalized patients and 75 out-patients in Denmark. PCR detected *A. schaalii* in

22% of specimens from individuals greater than 60 years old. In 9 of 10 PCR-positive specimens, other common urinary tract pathogens were also present, suggesting that *A. schaalii* may be present as an undetected urinary co-pathogen in many urinary tract infections. A subsequent study by the same group used quantitative PCR to screen urine samples from 76 patients with kidney stones, 29 childen, and 37 patients with indwelling catheters. Urine specimens from 7 (29%) of patients with kidney stones, 5 (14%) of 14 children less than 3 years old, none of 15 children 3 to 15 years old, and 8 (22%) of the catheterized patients were positive for *A. schaalii*.[66] Andersen and colleagues[34] documented *A. schaalii* in quantities of $>10^4$ to 10^5 cfu/mL by real-time PCR in 5 of 29 screened urine specimens from children less than 4 years of age. Two other children had urine specimens with colony counts of $\geq 10^6$ cfu/mL that were positive by both PCR and culture.

Other infections associated with *A. schaalii* and other members of the genus are rare. Gomez and coworkers[417] described 12 patients with *Actinobaculum* bacteremia. Blood cultures turned positive in from 1 to 3 days and only the anaerobic bottle was positive. *A. schaalii* was recovered from 10 patients, with *A. urinale* and *A. massiliense* being isolated from two patients, respectively. All patients were older than 65 years, 66% were male, and 10 of the 12 had underlying urogenital pathology (e.g., benign prostatic hypertrophy, prostate cancer, urinary retention, urethral stricture, prior urological instrumentation). Interestingly only 40% of the positive blood cultures were considered as significant by the physicians Vertebral osteomyelitis of the lumbar spine due was reported in a 71-year-old Swiss man with a past history significant for hepatitis B and tuberculosis requiring lung and rib resection.[466] In 2010, the first case of *A. schaalii* aortic prosthetic valve endocarditis was reported in a 52-year-old man in Austria.[505] This patient had no symptoms referrable to the urinary tract and urine cultures incubated both aerobically and anaerobically were negative after incubation for 5 days. *A. schaalii* was also isolated in pure culture from necrotic tissues of a 33-year-old obese male diagnosed with Fournier's gangrene, a type of necrotizing fasciitis affecting the genital, perineal, and perianal body sites.[1141] *A. schaalii* infection in North America was first reported in 2010 in a case report describing a chronic urinary tract infection in a 76-year-old Canadian woman.[636]

Actinobaculum species are anaerobic or facultatively anaerobic, catalase-negative, nonmotile gram-positive bacilli. *Actinobaculum* species may grow well or poorly at 37°C in a CO_2-enriched atmosphere and grow poorly at 37°C in air. Individual cells may be slightly curved or may exhibit branching. The principal product of glucose or maltose fermentation are lactic and acetic acids; *A. schaalii* produces succinate as well and *A. suis* produces acetic and formic acid plus some ethanol from maltose fermentation (glucose is not fermented). All members of the genus hydrolyze hippurate, but do not reduce nitrate to nitrite and esculin and gelatin are not

hydrolyzed. *A. suis* strains may ferment maltose but do not ferment sucrose. On the API Coryne system, *A. schaalii* is pyrazinamidase-negative, pyrrolidonyl arylamidase (PYR)-positive, alkaline phosphatase-variable, and α-glucosidase-positive, while *A. massiliae* is pyrazinamidase-positive, PYR-negative, alkaline phosphate-negative, and α-glucosidase-positive. *A. urinale* is pyrazinamidase, PYR and α-glucosidasenegative, but β-glucuronidasepositive.[909] Additional phenotypic characteristics for identification of *Actinobaculum* species are included in Table 14-7. *A. schaalii* isolates are susceptible to amoxicillin, mecillinam, ceftriaxone, gentamicin, vancomycin, linezolid, and nitrofurantoin and resistant to ciprofloxacin, metronidazole, and trimethoprim-sulfamethoxazole.[35,173] Isolates from patients with bacteremia reported by Gomez et al.[417] were all susceptible to penicillin (MIC, <0.5 μg/mL), resistant to metronidazole (MIC, >256 μg/mL), 10 were susceptible to clindamycin (MIC, <0.5 μg/mL), whereas two were clindamycin-resistant (MIC, >256 μg/mL). More than 90% of strains may be susceptible to other fluoroquinolones such as levofloxacin and moxifloxacin, and, in isolates that were ciprofloxacin- or levofloxacin-resistant, a mutation was noted in the *gyrA* but not in the *parC* gene, which are the quinolone-resistance-determining regions.[173]

Actinomyces *Species*

The genus *Actinomyces* is composed of a heterogeneous group of gram-positive, facultatively anaerobic or microaerophilic, non–spore-forming, non–acid-fast nonmotile bacilli, some of which display rudimentary branching. Many *Actinomyces* species are part of the oral microflora of humans and various animals.[992] *A. israelii*, *A gerencseriae*, *A. georgiae*, *A. odontolyticus*, and *A. naeslundii* are prevalent in both supra- and sub-gingival plaque specimens in patients with adult periodontitis and gingivitis.*Actinomyces* species may cause classical actinomycosis, wound infections, abscesses, genital tract infections, and urinary tract

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TABLE 14-7 Phenotypic Characteristics for Identification of *Actinotignum* and *Actinobaculum* Species

Test/Characteristic	*A. schaalii*	*A. suis*	*A. massiliae*	*A. urinale*
Strict anaerobe	–	+	–	–
CAT	–	–	–	–
Colony morphology/ pigmentation	Small, gray-white	White, granular, entire/ irregular edges	Small, gray, entire	Small, gray-white, convex, entire, smooth
HEM SBA	–	–	–	β
NO_3	–	–	–	–
PYZ	V	–	–	–
PAL	V	+	–	–
ESC	–	–	–	–
URE	–	+	–	V+
ADH	–	NA	–	–
MOT	–	–	–	–
HIPP	+	NA	+	+
VP	–	–	NA	–
GEL	–	–	–	–
LAP	NA	NA	–	–
CAMP	+w	NA	NA	NA
PYR	+	V	–	–
α-GAL	–	NA	–	–
β-GAL	–	–	–	–
α-GLU	+	+	+	–
β-GLU	–	NA	NA	–
β-GUR	–	–	–	+
NAGA	–	NA	–	–
Acid produced from:				
GLU	V+	–	+	+
MAL	+	V	+	+
SUC	V	–	–	+
MNTL	–	–	–	–
XYL	V	–	+	–
LAC	–	–	–	–
MANN	V	–	–	–
ARAB	V	–	NA	–
SBTL	–	–	–	–
RAFF	–	–	+w	–
RIB	+	–	+	+
TRE	V	–	+	–
GLYG	–	+	+	–
Products, glucose fermentation	A, S	A, f, EtOH (from maltose)	NA	L, a

+, positive reaction; −, negative reaction; V, variable reaction; +sl, slow positive reaction; V+, variable reaction, most strains positive; V−, variable reaction, most strains −; V+w, variable reaction, most strains weakly positive; NA, data not available; CAT, Catalase; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; ADH, Arginine dihydrolase; MOT, motility; HIPP; hippurate hydrolysis; VP, acetoin production (Voges–Proskauer); GEL, gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; GLU, Glucose; MAL, Maltose; SUC, Sucrose; MNTL, Mannitol; XYL, Xylose; LAC, Lactose; MANN, Mannose; ARAB, Arabinose; SBTL, Sorbitol; RAFF, Raffinose; RIB, Ribose; TRE, Trehalose; GLYG, Glycogen A, major amounts of acetic acid produced from glucose; S, major amounts of succinic acid produced from glucose; L, major amounts of lactic acid produced from glucose; f, minor amounts of formic acid produced from glucose; EtOh, ethanol.

infections.[362,890,1043] Rarely, an *Actinomyces* species has been reported to cause sepsis.[997] Genus *Actinomyces* is classified in the phylum *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, family *Actinomycetaceae.* Other genera in the family are *Actinobaculum, Arcanobacterium, Trueperella*, and *Mobiluncus.* (Box 14-1).

The genus *Actinomyces* has undergone rapid expansion over the last several years, with the description of many new species from both humans and animals. The plethora of new agents has created problems for the clinical laboratory as far as accurate identification of these organisms is concerned. Years ago, microbiology laboratories relied on conventional anaerobic bacteriology methods for phenotypic identification. Reference identifications were determined with anaerobic bacteriology techniques promulgated in the *Virginia Polytechnic Institute (VPI) Anaerobe Manual* and the *Wadsworth Anaerobic Bacteriology Manual.*[510,560,976] Unfortunately, the *VPI Anaerobe Manual* is no longer available and the *Wadsworth Anaerobic Bacteriology Manual* has not been updated since 2002. In the VPI approach to the identification of anaerobes, gas-liquid chromatography (GLC) for detection of volatile and nonvolatile products of glucose fermentation was very helpful for identification, since gram-positive, non–spore-forming bacilli that produce major amounts of succinic acid could be placed in the genus *Actinomyces* without further testing. Since new species are now validated by genotypic, molecular approaches, GLC data as well as phenotypic data on new species are often limited or unavailable. Several commercial vendors have marketed rapid enzymatic kits for identification of anaerobic bacteria, but these systems do not perform well for identifying *Actinomyces* species.[758] Santala and colleagues[975]evaluated the RapID ANA II (Remel), the RapID CB-Plus (Remel), the BBL Crystal ANR ID (BD Microbiology Systems) and the RapID 32A (bioMérieux, Inc.) for their ability to identify "classical" and newly described *Actinomyces* species. These kits correctly identified only 26% to 65% of the "classical" *Actinomyces* strains to the species level, and only 13% to 49% of the newer species to the genus level. A 2005 study evaluated the RapID ANA II, RapID32, RapID CB-Plus and BBL Crystal ANR ID systems for the identification of *Actinomyces* species.[586] Of 54 isolates tested, 46% were identified to the genus/group level by BBL Crystal, 30% by the RapID32A, 20% by the RapID CB-Plus, and 13% by the RapID ANA II. One of the newer systems for identification of anaerobic bacteria is the Vitek 2 Anaerobe/*Corynebacterium* (ANC) card. In the multicenter evaluation of the ANC card, two of two *A. meyeri* strains and one of two *A. isrealii* strains were correctly identified, and, in another study of the ANC card, along with RapID ANA II, and BBL Crystal ANR, only two *Actinomyces* isolates were included, so the performance of

these kits for *Actinomyces* identification was difficult to assess.[119,915] In another evaluation of the Vitek 2 ANC card, five *Actinomyces* isolates were tested; three were identified to the species level, while one isolate each was misidentified or gave no identification.[651] In another evaluation of the ANC card by Mory and colleagues,[780] six of six *Actinomyces* species were correctly identified to the species level, suggesting that this kit may have great potential for assisting in phenotypic identification of *Actinomyces* species. The phenotypic characteristics of new *Actinomyces* species as determined by these kits are often included as part of the original species descriptions. Because of the difficulties with phenotypic identification, genus- and species-specific DNA probes have been developed for investigating the role of *Actinomyces* species in periodontal disease and in the initiation of dental caries.[116,1091,1217] *Actinomyces* species can also be identified by molecular methods such as 16S rDNA sequencing and restriction fragment-length polymorphism analysis.[465,978] None of these techniques are available to the routine clinical laboratory. Box 14-6 includes descriptions of *Actinomyces* species from humans and animals. Phenotypic data presented in Table 14-8, which begins on page 908, includes both human and animal isolates and represents a synthesis of phenotypic characteristics and descriptions from several sources.[510,560]

Actinomycosis is a chronic infection that is characterized by localized swelling with suppuration, abscess and granuloma formation, draining sinus tracts, and tissue fibrosis and is caused primarily by members of the genus *Actinomyces*.[140] *A. israelii* is the most common cause of actinomycosis in humans, although other species (i.e., *A. meyeri*, *A. naeslundii*, *A. odontolyticus*, *A. gerencseriae*, and *A. viscosus*) may also occasionally cause this infection. Although the classic association of *Actinomyces* with intrauterine devices is well known, *Actinomyces* species may be associated with infections of other prosthetic material.[145] Because of their chronic nature, actinomycotic infections frequently mimic carcinomas. The clinical course of the infections are also affected by the immune competence of the host and other predisposing, comorbid conditions, such as alcoholism, radiation, debilitation, diabetes, malignancies, immunosuppression, and HIV infection. Actinomycosis can be divided into several clinical forms, including cervicofacial actinomycosis, primary cutaneous actinomycosis, thoracic actinomycosis, abdominal/pelvic actinomycosis, and CNS actinomycosis. Most actinomycotic infections are polymicrobial, and other facultative (e.g.,*Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, streptococci, staphylococci, *Enterobacteriaceae*) and anaerobic bacteria (e.g., *Bacteroides* species, *Fusobacterium* species) are involved in pathogenesis and disease.[140]

Cervicofacial actinomycosis is the most common clinical presentations of actinomycosis and a rare complication of dental manipulations, oral surgery, maxillofacial trauma, and other head and neck infections (e.g., otitis,

mastoiditis).[18,413,825] At least nine different *Actinomyces* species can cause this infection, although *A. israelii* and *A. gerencseriae* predominate.[886] In most cases, endogenous oral *Actinomyces* are introduced by trauma and begin to grow in the head and neck tissues to form an acute, pyogenic abscess that may evolve into an indurated submandibular mass.[632] Spontaneous sinus tracts discharging purulent material may be seen in up to 40% of cases.[413] Lymph node involvement is often present and the patient often present with lymphadenopathy. Co-infecting organisms present in the tissues along with the actinomycete help determine the clinical evolution of the infection as subacute, acute, or chronic. Microcolonies of the organisms called "sulfur granules' may be discharged from draining sinus tracts in some cases. These sulfur granules are matted collections of the organism and show basophilic organism masses with distinct eosinophilic clubs radiating from the border of the granule. These granules provide excellent material for culture and organism detection. Cervicofacial actinomycosis may involve the submandibular space, parotid glands, teeth, tongue, nasal cavity, epiglottal area, and pharynx. Osteomyelitis may occur due to extension of infection

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BOX 14-6

Members of the Genus *Actinomyces*

Species	Clinical Significance and Identification
Actinomyces bovis	*A. bovis* causes actinomycosis in cattle and other animals.[714] This organism was confirmed with phenotypic, chemotaxonomic, and genotypic approaches. On the API Coryne, this isolate produced biocode number 0101104, indicating positive reactions for alkaline phosphatase, urease, glucose and catalase.
Actinomyces bowdenii	This species was originally isolated from clinical specimens obtained from dogs and cats and has not yet been recovered from clinical specimens obtained from human sources.[846]
Actinomyces canis	This species, described in 2000, is a catalase-positive species isolated from dogs.[519] *A. canis* is closely related to *A. hyovaginalis*, *A. georgiae*, *A. meyeri*, *A. odontolyticus*, *A. radingae,* and *A. turicensis*.
Actinomyces cardiffensis	*A. cardiffensis* has been isolated from human pleural fluid, a paracolonic abscess, a jaw abscess, a post-mastoidectomy ear abscess, a sinus washout, and intrauterine devices.[458] On Gram's stain, the organisms appear as slender, slightly curved rods with beaded, branching filaments. After 48 hours incubation, colonies are small, convex, entire, and may be creamy to pinkish. *A. cardiffensis* is a facultative anaerobe that is catalase-negative and produces major amounts of succinic and lactic acids and small amounts of acetic acid from glucose fermentation. Esculin, urea, and gelatin are not hydrolyzed.
Actinomyces catulii	*A. catuli* has been isolated from dogs.[522]
Actinomyces coleocanis	*A. coleocanis* was isolated from the vagina of a dog.[518]
Actinomyces dentalis	*A. dentalis* was described in 2005, when it was isolated from pus aspirated from a dental abscess.[464] This organism is obligately anaerobic and forms filamentous, beaded, branching gram-positive rods. It is catalase-negative and the major end product of glucose metabolism is lactic acid, with minor amounts of acetic acid; it does not form succinic acid from glucose.
Actinomyces denticolens	This species was originally isolated from dental plaque specimens from cattle.[268]
Actinomyces europaeus	This species was described in 1997, when it was isolated as the sole organism or as the only aerobic organism recovered from human abscess material.[358] This organism has also been recovered from skin-related infections (e.g., pilonidal sinus infection, perianal abscesses, decubitus ulcers) and urinary tract infections.[959] *A. europaeus* was co-isolated with *A. turicensis* from a subcutaneous fistula associated with severe congenital hypoplasia of the right leg in a 23-year-old male and from a breast abscess.[1018,1245] On Gram-stained smears, this organism appears as short, gram-positive rods arranged in small clusters with no discernable branching. Colonies on SBA are small (less than 0.5 mm after 48 hours incubation in CO2), gray, and translucent. This organism is catalase-negative, nonmotile, and ferments glucose and maltose, but not mannitol or xylose. Some strains of this organism also hydrolyze esculin and gelatin, but these reactions are delayed. Nitrate is not reduced to nitrite and urease is not produced. As with other *Actinomyces* species, succinic acid is produced as the major product of glucose fermentation.
Actinomcyes funkei	This recently described species was originally recovered from blood cultures, a sternal wound culture, n abdominal incision, and was reported as a cause of native tricuspid valve endocarditis.[644,1195] These isolates were orginally identified as strains of *A. turicensis*, but certain phenotypic traits and 16S rRNA sequencing showed that this organism was closely related to but not identical with *A. turicensis*. Cells of *A. funkei* are gram-positive, slender, slightly curved, and exhibit some branching. Growth occurs under both aerobic and anaerobic conditions. On SBA, colonies are small, nonhemolytic, and gray after 24 hours incubation. Acid is produced from glucose, sucrose, and xylose, but not from arabinose, mannitol, glycogen, raffinose, sorbitol or trehalose. Hippurate is hydrolyzed, but esculin and gelatin are not. On the API Coryne system, the original isolated produced biocode numbers 0130761 or 3530761, indicating the variability of the pyrazinamidase, nitrate reductase, and β-galatosidase reactions on the test strip.
Actinomyces georgiae	*A. georgiae* was described in 1990 and is found as a part of the normal microbiota of the gingival crevices of adults and children. In 2008, this organism was isolated from blood cultures of a patient with endocarditis in Thailand.[549] Strains of this species are facultatively anaerobic. On Gram's stain, this species appears as rods that occurring in pairs or short chains; cells may also have swellings along their length and branching is seldom seen.[553] In older broth cultures, cells may have a coccoid appearance. After 48 hours of anaerobic incubation, colonies are 1 mm in diameter, circular, entire, shiny, and smooth with entire adges. Colonies may be white, tan, or beige. The organism is catalase-negative, and succinic, acetic, formic, and small amounts of lactic acid are produced from glucose fermentation. Acid is produced from a variety of carbohydrates, including glucose, maltose, sucrose, xylose, trehalose and glycogen. The majority of strains hydrolyze esculin, but do not reduce nitrate to nitrite.

Actinomyces gerencseriae	This species was described in 1990 and was formerly called *A. israelii* serotype II.[553] However, it is genetically unrelated to the type strains of *A. israelii*. *A. gerencseriae* is found in the human oral cavity and in the gingival crevices. About 12% of isolates are obligately anaerobic, while the remainder produce scant to moderate growth on blood agar incubated in 5%–7% CO_2. On Gram's stain, the organisms appear as filamentous cells with occasional swellings and branching. After 48 hours incubation under anaerobic conditions, colonies are about 0.2 mm in diameter, white, opaque, nonhemolytic, and may have a "lumpy" topography. This species is catalase-negative, esculin hydrolysis-positive, gelatin-hydrolysis-negative, and produces acid from a variety of carbohydrates, including glucose, maltose, mannose, sucrose and trehalose. Lactic, succinic, formic, and acetic acids are produced by glucose fermentation. In 2010, *A. gerencseriae* was isolated from a case of thoracic actinomycosis presenting as empyema necessitatis, which is an extension of purulent pleural fluid through the tissues forming an abscess on the thoracic wall.[681]
Actinomcyes graevenitzii	The original report of this new species described four human isolates, three of which were from respiratory tract specimens, while the fourth was isolated from the jaw of a patient with osteitis.[895] These isolates were gram-positive, nonmotile, slightly curved rods with some branching and occasional swollen ends. Colonies on SBA are very small (0.2 mm in diameter after 24 hours' incubation) and are adherent to the agar, particular on primary isolation. This species is catalase-negative, does not hydrolyze esculin, urea or hippurate, and does not reduce nitrate to nitrite. This species is also strongly *N*-acetyl-β-d-glucosaminidase positive on the API Coryne strip, which helps to differentiate it from the other closely related *Actinomyces* species. Succinic and lactic acids constitute the end-products of glucose fermentation. Since its initial description, *A. graevenitzii* has been isolated from a patient with disseminated co-infection with this organism and *M. tuberculosis*, and from a patient with bacteremia and alcoholic liver cirrhosis.[533,1108]
Actinomyces hominis	*A. hominis* was described in 2010, when a wound swab collected from a 89-year-old German women grew a pure culture of a catalase-positive coryneform bacterium after 72 hours of aerobic incubation.[363] The isolate was confirmed as an *Actinomyces* species by 16S rRNA gene sequencing and was closely related to *A. europaeus*. *A. hominis* is catalase-positive (one of the 10 *Actinomyces* species that is catalase-positive), CAMP test-positive, produces acid from raffinose and is *N*-acetyl-β-d-glucosaminidase positive.
Actinomyces hongkongensis	This novel species, described in 2003, was isolated from purulent material of a patient with pelvic actinomycosis.[1208] This species is strictly anaerobic, grows on SBA as pinpoint, nonhemolytic colonies and is catalase-negative. By 16S rRNA gene sequencing, this species is most closely related to *A. maramammalium*, which has been isolated from seals and porpoises.
Actinomyces hordeovulneris	*A. hordeovulneris*, along with *A. viscosus* and *A. bovis*, are the three species that cause actinomycosis in dogs.[147,857] *A. hordeovulneris* has been isolated from patients with pleuritis, septic arthritis, peritonitis and abscesses.
Actinomyces houstonensis	This human species originated with three isolates from serious subcutaneous abscesses that required drainage.[212] *A. houstonensis* is a facultative anaerobe, producing α-hemolytic, gray colonies that are about 0.2 mm in diameter after 48 hours of incubation at 35°C–37°C. The cells are pleomorphic gram-positive rods that tend to form half-circles. The organism is catalase-negative and does not hydrolyze esculin, urea or gelatin. Nitrate is reduced to nitrite and acid is produced from glucose and sucrose, but not from xylose.
Actinomyces howellii	*A. howellii* is found in dental plaque of dairy cattle.[269] This organism is antigenically related to *A. naeslundii* and *A. viscosus*.[888]
Actinomyces hyovaginalis	Described in 1993, *A. hyovaginalis* was originally isolated from purulent vaginal lesions of pigs.[228] Subsequent genotypic studies of isolates from porcine necropsy specimens identified a new biotype of *A hyovaginalis* from kidney, lung, brain, bladder and joint tissue that was termed the "general" biotype to distinguish these from the "vaginal" biotype described previously.[1061] Vaginal biotypes are associated with porcine abortion.[509] *A. hyovaginalis* is also a cause of lymphadenitis in goats.[974] More recent sequencing and expanded phenotypic studies have documented that certain strains of *A. hyovaginalis* cause disease in sheep similar to *C. pseudotuberculosis*, *A. pluranimalium* and *T. pyogenes*.[351]
Actinomyces israelii	*A. israelii* is the agent most frequently associated with the various presentations of actinomycosis, including cervicofacial, thoracic, pulmonary, and pelvic actinomycosis (often associated with the presence of intrauterine devices) (see text).[515,882] *A. israelii* has been recovered from cases of head and neck actinomycosis with CNS, spinal cord, vertebral, ocular, and periocular involvement, including endophthalmitis and osteomyelitis.[392,511,759,807,1239] *A. israelii* has also been isolated from rare cases of indolent breast

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	abscess, primary sternal actinomycosis and endocarditis.[6,653,870,882,971] Pulmonary actinomycosis may manifest due to *A. israelii* may present as pleural, chest wall and splenic abscesses with empyema.[451] Abdominal actinomycosis involving *A. israelii* include abdominal wall masses, and intrabdominal, liver and pancreatic abscesses, intestinal actinomycosis with bowel obstruction and retroperitoneal involvement with obstructive uropathy.[5,401,653,1129,1135,1219] Infections of prosthetic joints following bacteremia with *A. israelii* have also been reported.[1241] This organism is primarily anaerobic, with facultative growth in a CO_2-enriched atmosphere only occurring after repeated subcultures. Colonies of *A. israelii* may resemble a molar tooth, are nonhemolytic on SBA and are not pigmented. This species is catalase-negative, esculin hydrolysis-positive and most strains reduce nitrate to nitrite. Acid is produced from glucose, maltose, sucrose, raffinose, xylose, lactose and trehalose.
Actinomyces johnsonii	During an examination of 115 type/reference strains, clinical isolates, and oral isolates of *A. naeslundii*, the partial gene sequences of several housekeeping genes were determined. These sequences identified three distinct genospecies. *A naeslundii* genospecies 1 and genospecies 2 formed two distinct clusters that were well separated from the third cluster, designated genospecies WVA 963. Genospecies 1 clustered with the type strains of *A. naeslundii sensu stricto*, so this genospecies 1 strains remained as *A. naeslundii*. The name *Actinomyces oris* was proposed for the *A. naeslundii* genospecies 2 isolates, while the name *Actinomyces johnsonii* was proposed for *A. naeslundii* genospecies WVA 963.[486]
Actinomyces marimammalium	*A. marimammalium* was originally isolated from multiple internal organs of a dead hooded seal, lung tissues of a dead harbor porpoise, and small intestinal tissue of a dead gray seal.[523]
Actinomyces massiliensis	This species was isolated in 2009 from a blood culture of a 38-year-old man with pleuropneumonia.[918] This obligately anaerobic organism is catalase-negative, oxidase-negative, and produces acid from glucose, fructose, and lactose, but not from mannitol, xylose, raffinose or amygdalin. This species is most closely related to *A. gerencseriae*, *A. israelii*, *A. oricola*, *A. ruminicola* and *A. dentalis*.
Actinomyces meyeri	*A. meyeri* is an obligately anaerobic species that has been associated with a variety of human infections, including cervicofacial, thoracic and pulmonary actinomycosis.[240,325,674,1136] Primary cutaneous actinomycosis due to *A. meyeri* has been reported in persons with minor cutaneous trauma, and usually presents as indolent ulcerated lesions draining purulent material.[416,488] In one instance, this infection was the first manifestaion of HIV infection in a 23 year-old man.[416] This organism is an uncommon cause of postoperative endophthalmitis, brain abscess, cutaneous and intra-abdominal abscesses, endocarditis and osteomyelitis.[526,688,859,1148] Intra-abdominal *A. meyeri* infections have included splenic abscesses.[399] *A. meyeri* has a propensity to disseminate from localized infection, and, in one patient, the infection was mistaken for lung cancer with metastases to the brain.[230,1148] *A. meyeri* has also been isolated from synovial fluid from a patient with metastatic squamous cell carcinoma.[190] Colonies on blood agar are white and non-pigmented. Strains of *A. meyeri* are catalase-negative, do not hydrolyze esculin and do not reduce nitrate. The CAMP test is positive, and some strains also produce urease. Acid is produced from glucose, maltose, sucrose, and xylose, and some strains will also ferment glycerol and glycogen. Mannitol, trehalose, raffinose, and mannose are not fermented.
Actinomyces naeslundii	*A. naeslundii* is found in the human oropharnyx and is a rare cause of cervicofacial actinomycosis, IUD-associated endocervical actinomycosis, septic arthritis, and periodontal disease.[465,667,1216] *A. naeslundii* may cause bacteremia following dental procedures in patients with periodontal disease.[104] *A. naeslundii* strains were divided into genospecies 1 and 2; genospecies 1 is now recognized a *A. naeslundii* sensu stricto, and genospecies 2 has been reclassified as *Actinomyces oris*.
Actinomyces nasicola	This species was originally isolated from pus that was aspirated from the antrum of a patient undergoing a routine nasal polypectomy.[463] On Gram's stain, this organism appears as coryneform gram-positive bacilli with some branching and coccoid forms being evident as well. The organism is facultatively anaerobic and catalase-negative; acetic, lactic and succinic acid are produced as products of glucose fermentation. Esculin, gelatin and starch are not hydrolyzed and urease is not produced. Acid is produced from fructose and cellobiose, but not from glucose, maltose, sucrose, ribose, xylose, lactose, mannitol or amygdalin.
Actinomyces neuii subsp. *neuii and Actinomyces neuii* subsp. *anitratus*	These *Actinomyces* species were described in 1994 and encompass former members of CDC coryneform group 1 and "group 1-like" strains that were recovered from a variety of clinical specimens, including cerebrospinal shunt fluid, ear cultures, vitreous fluids, mammary gland abscesses, furuncles and blood.[376] Group 1 strains were α-hemolytic on human blood agar, reduced nitrate to nitrite, were unable to ferment adonitol, and were alkaline phosphatase-negative, while the "group 1-like" strains were nonhemolytic, failed to reduce nitrate to nitrite, fermented adonitol and were alkaline phosphatase-positive. Both groups of organisms grew under both aerobic and strictly anaerobic conditions.

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	Cell wall analysis showed that these isolates lacked *meso*-DAP and mycolic acids in their cell walls. Analysis of volatile and nonvolatile fatty acids showed that succinic acid was the major end product of glucose fermentation for both groups of organisms, and examination of fatty acid methyl esters revealed that the cell walls contained C16:0, C18:1*cis*-9, and C18:0 fatty acid methyl esters as their major cell wall constituents. These data suggested a phylogenetic relationship between CDC group 1 and "group 1-like" strains and *Actinomyces* species.[376] Subsequently, 16S rRNA gene sequences of these organisms were determined and found to support the inclusion of CDC group 1 and "group 1-like" isolates in the genus *Actinomyces*. In 1994, CDC group 1 and "group 1-like" strains were formally proposed as *Actinomyces neuii* subsp. *neuii* and *Actinomyces neuii* subsp. *anitratus*, respectively.[388] Since the deposition of this coryneform in the genus *Actinomyces*, this species has been associated with abscesses, infected skin lesions, bacteremia and both native valve and prosthetic valve endocarditis.[215,387,419,444,623,713,1165] Bacteremias have been associated with urinary tract infections, joint infections, and chorioamnionitis.[388,470,713] Other infections have included pericarditis, osteomyelitis, ventriculo-peritoneal shunt infections, endophthalmitis and infection of a mammary and a total hip prostheses.[145,400,430,862,894,925,1138,1165,1180] Both *A. neuii* subsp. *neuii* and *A. neuii* subsp. *nitratus* are catalase-positive, nonhemolytic gram-positive bacilli. Colonies are circular, smooth, convex, and white with entire edges; subsp. *neuii* is α-hemolytic on SBA, while subsp. *anitratus* is nonhemolytic. These organisms are very active fermenters and produce acid from glucose, maltose, sucrose, mannitol, lactose, mannose, trehalose and xylose. Both species produce leucine arylamidase, α-galactosidase, β-galactosidase, and α-glucosidase and both are CAMP test-positive. *A. neuii* subsp. *neuii* reduces nitrate to nitrite, while subsp. *anitratus* is negative for this characteristic. Rare strains of *A. neuii* may be catalase-negative.[144] *A. neuii* isolates are susceptible to penicillin, ampicillin, cephalosporins, imipenem, erythromycin, clindamycin and vancomycin. Suceptibility to the aminoglycosides and fluoroquinolones is variable.[388]
Actinomyces odontolyticus	*A. odontolyticus* is found in the human oropharnyx in association with the gingival crevices and deep carious lesions. This species, along with *A. naeslundii* and *A. viscosus*, has been documented as a cause of bacteremia after dental procedures.[104] This organism is also a rare cause of bacteremia, thoracopulmonary infections, empyema thoracis, lung and liver abscesses, soft tissue infections and IUD-associated pelvic actinomycosis.[72,73,193,196,234,679,767,1084] *A. odontolyticus* has also been isolated from an abscess adjacent to the exit site of a Tenckhoff catheter for chronic ambulatory peritoneal dialysis.[1029] This species is catalase-negative, produces pinkish colonies on SBA, and forms red colonies on brain heart infusion agar. Nitrate is reduced to nitrite and urease is not produced. Acid is produced from glucose, maltose, and sucrose and some strains also ferment glycerol, glycogen, and xylose.
Actinomyces oricola	*A. oricola*, like *A. radicidentis* (see below), was isolated originally from a dental abscess.[462] This species includes gram-positive rods that display both branching and filament formation. The organism is facultatively anaerobic, catalase-negative and produces pinpoint colonies on SBA after 48 hours incubation. GLC analysis of glucose broth cultures reveals the formation of acetic, lactic, and succinic acids. On the API Coryne strip, acid is produced from glucose, maltose and sucrose only. Other positive tests (i.e., pyrazinamidase, alkaline phosphatase, esculin hydrolysis, α-glucosidase, and β-galactosidase) produces a biocode of 2550121.
Actinomyces oris	During an examination of 115 type/reference strains, clinical isolates, and oral isolates of *A. naeslundii*, the partial gene sequences of several housekeeping genes were determined. These sequences identified three distinct genospecies. *A. naeslundii* genospecies 1 and genospecies 2 formed two distinct clusters that were well separated from the third cluster, designated genospecies WVA 963. Genospecies 1 clustered with the type strains of *A. naeslundii sensu stricto*, so this genospecies 1 strains remained as *A. naeslundii*. The name *Actinomyces oris* was proposed for the *A. naeslundii* genospecies 2 isolates, while the name *Actinomyces johnsonii* was proposed for *A. naeslundii* genospecies WVA 963.[486]
Actinomcyes radicidentis	*A. radicidentis* has been isolated in pure cultures from infected root canals in humans.[223,568] These isolates were gram-positive, catalase-positive, coccoid to coccobacillary in morphology, and grew under both aerobic and anaerobic conditions. Colonies on SBA may produce a pink pigment. This species is fermentatively active, producing acid from glucose, maltose, mannitol, lactose, ribose, and sucrose on the API Coryne. The organism hydrolysisof esculin, reduction of nitrate and production of urease are variable characteristics. A PCR assay for *A. radicidentis* was developed and applied to dental root canal specimens obtained from patients with primary (root canal infections) and persisting (i.e., posttreatment) endodontic infections.[1026] *A. radicidentis* was detected in 2 (4%) of 50 samples collected from primary infections and in 1 (8%) of 12 root canal samples from patients who failed therapy. This organism is probably a previously unrecognized member of the normal indigenous oral microbiota of humans.

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Actinomyces radingae	CDC coryneform group E organisms were originally described as "*A. pyogenes*-like" bacteria that were recovered from several clinical sources. These organisms were believed to be aerotolerant strains of the anaerobe *Bifidobacterium adolescentis* that differed in the volatile and nonvolatile end-products produced from glucose fermentation.[449] Using genetic and chemotaxonomic approaches, Wust and colleagues[1214,1215] found that these strains were closely related to *Actinomyces* species and proposed two new species: *Actinomyces radingae* and *Actinomyces turicensis*. VanDamme and associates subsequently used whole-cell protein electrophoresis, fatty acid analysis, phenotypic tests (i.e., API Coryne and the IDS Rapid ANA panel) and species-specific oligonucleotide probes to characterize a group of unidentified, catalase-negative coryneform bacteria.[1215] These workers found that the organisms formed two clusters that corresponded to *A. turicensis* and *A. radingae*, respectively. These analyses enabled the publication of emended descriptions of these "*Actinomyces pyogenes*"-like isolates from human clinical specimens.[1140] Using species-specific oligonucleotide probes for *A. turicensis* and *A. radingae*, Sabbe and colleagues examined 294 "*Actinomyces*-like" isolates from human clinical specimens collected over a 7-year period and found that *A. radingae* is primarily associated with skin-related infections (e.g., skin abscesses, pilonidal cysts, perianal abscesses).[52,959] Isolates of *A. radingae* are facultative, catalase-negative, gram-positive bacilli. After 48 hours incubation, colonies on SBA are small, gray, convex and circular with a butyrous consistency. Most strains are α-hemolytic. Esculin is hydrolyzed, but gelatin and urea are not. Nitrate is not reduced to nitrite and the CAMP test is negative. Acid is produced from glucose, maltose, sucrose, lactose, xylose and ribose, but not from mannitol or glycogen. With enzymatic tests on the API Coryne, *A. radingae* produces α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and *N*-acetyl-β-d-glucosaminidase, but does not produce leucine arylamidase, pyrrolidonyl arylamidase, alkaline phosphatase or β-glucuronidase. *A. radingae* isolates are susceptible to penicillin, cephalosporins, erythromycin, and tetracyclines.[1140]
Actinomyces ruminicola	*A. ruminicola* is an obligately anaerobic species that has been isolated from the bovine rumen.[33]
Actinomyces slackii	*A. slackii* is found in the dental plaque of dairy cattle.[270]
Actinomyces suimastidis	This species was isolated from a sow with chronic granulomatous mastitis.[521]
Actinomyces timonensis	*A. timonensis* was described in 2010, when an unusual isolate was recovered from an osteo-articular specimen obtained from the sacral area of a 13-year-old girl with chronic lower back pain. This organism is most closely related to the type strain of *A. denticolens*.[919] This organism is an obligately anaerobic catalase-negative, gram-positive rod.
Actinomyces turicensis	*A. turicensis* is found mainly in genital tract infections, skin-related infections, urinary tract infections and some isolates have been recovered from appendiceal tissue, hepatic abscess, head/neck infections and blood.[940,959] Since its initial description, *A. turicensis* has been isolated from pilonidal cysts, cutaneous and peri-rectal abscesses, gas gangrene-associated scrotal exudates, liver abscess, an ovarian cyst infection, subcutaneous fistulae, furuncles, and carbuncles.[205,824,940,1245] *A. turicensis* has also been isolated as a cause of mitral valve endocarditis in a Labrador Retriever.[565] On Gram's stain, cells of *A. turicensis* are straight, slightly curved, or club-shaped. Colonies on SBA are small, gray, convex, and entire after 48 hours' incubation in a CO_2 atmosphere. The organism is catalase-negative, does not hydrolyze esculin, urea or gelatin, does not reduce nitrate to nitrite and is CAMP test-negative. Acid is produced from glucose, maltose, sucrose, xylose, ribose and trehalose, but lactose and mannitol are not fermented. On the API Coryne, α-glucosidase is detected, but the other enzymatic tests are negative. *A. turicensis* is broadly susceptible to a variety of agents, including pencillin, cephalosporins, erythromycin and doxycycline.[1140]
Actinomcyes urogenitalis	Isolates of this species were originally recovered from urine, vaginal discharge and urethral cultures.[803] In 2006, a previously healthy 38-year-old woman, with IUD-associated pelvic actinomycosis due to *A. urogenitalis* was reported.[305] She presented with a 2-week history of fever, a heavy malodorous vaginal discharge, and lower back pain that appeared 8 months after insertion of the IUD. These organisms are facultative, regular gram-positive, catalase-negative rods. This species produces acid from glucose, maltose, sucrose, lactose, xylose, and some strains also ferment mannitol and ribose. Nitrate is reduced to nitrite and esculin is hydrolyzed, but gelatin, urea and hippurate are not.
Actinomyces vaccimaxillae	This isolate was recovered from pus exuding from a jaw lesion of an adult cow.[460]
Actinomyces viscosus	*A. viscosus* is found in the oral cavity of human and animals. This species, along with *A. naeslundii* and *A. viscosus*, has been documented as a cause of bacteremia after dental procedures.[104] In 2005, *A. viscosus* was recovered as a cause of endocarditis that required aortic valve replacement.[562] This organism was isolated from multiple draining sinus tracts on the back of a patient with an unusual presentation of chronic cutaneous actinomycosis and from a loculated pleural effusion in a patient with acute

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	lymphoblastic leukemia.[290,750] *A. viscosus* was also co-isolated with *Streptococcus constellatus* from a subdural empyema in a previously healthy 8-year-old girl, which unfortunately was fatal.[132] This species is a catalase-positive, gram-positive rod. Colonies on SBA are small and nonhemolytic after growth for 48 hours. Esculin and urea are hydrolyzed and nitrate is reduced to nitrite. Acid is produced from glucose and sucrose and some strains produce acid from xylose.
Actinomyces weissii	This species is based on two isolates from the mouths of two dogs.[493] The first was isolated from an oral wound infection caused by a foreign body in a 4-year-old male Labrador Retriever, while the second was isolated from a gingival specimen from a 13-year-old West Highland White Terrier. Both were recovered in mixed culture with other flora. This organism was noted to be reverse CAMP test-positive.

from perimandibular areas of infection.[1004] Crossman and Herold reported a case of actinomycosis of the maxillary and nasal bones requiring debridement in an 85-year-old male.[244] CT scans initially were suggestive of a diffuse malignant process, but histological staining made the diagnosis. In some cases, other maxillofacial dysplasia or neoplasia may mimic actinomycotic infection or may co-exist in the same infected site.[1038] Mucocutaneous actinomycosis involving the mucosa of the lips and mouth has also been reported.[657]

Thoracic actinomycosis occurs as an extension of cervicofacial infection into the chest and mediastinal region, as a result of aspiration of oropharyngeal contents, or as an extenision of abdominal/pelvic infection that has spread retroperitoneally or transdiaphragmatically.[140] Infection may also result from contiguous extension into the mediastinum from the neck, or may reach the lung parenchyma via the bloodstream.[700] This infection presents initially as an aspiration pneumonitis with the eventual development of lung mass or cavitations. Chest roentgenogram (i.e., X-ray) may reveal masses, fibronodular lesions, or cavitary lesions with accompanying pleural effusions and/or empyema.[761] Pulmonary infection may also extend to involve the bones (i.e., ribs, sternum, thoracic vertebrae, shoulder girdle), chest wall muscles, and soft tissues, including the pericardium and myocardium, and mediastinum.[73] Symptoms include cough, hemoptysis, chest pain, and low-grade fever, and weight loss. The differential diagnosis of thoracic and thoracopulmonary actinomycosis in broad and includes tuberculosis, fungal infection (e.g., blastomycosis, cryptococcosis), nocardiosis, and carcinoma.[761] Up to 25% of cases of thoracic actinomycosis may be initially thought to represent a malignancy.[700]

Abdominal actinomycosis develops when the gastrointestinal mucosa is compromised by surgical procedures, lower gastrointestinal tract trauma, immunosuppression, presence of a foreign body, or as a complication of colonic diverticulitis.[140,1069]Postsurgical actinomycosis develops sometime months to years

after procedures such as appendectomy, with extension from the intestine and involvement of adjacent tissues and organs. Usually, the ileocecal region of the colon is involved, and the infection may remain localized or can spread hematogenously or by direct extension to the liver, abdominal wall, spleen, stomach, gallbladder, pancreas, kidneys, and retroperitoneal tissue.[5,280,1103] The colon may be involved at multiple discrete sites, and infection of the sigmoid colon and rectum has also been described.[883,1040] As with craniofacial infection, mass lesions that develop in the abdomen may be misdiagnosed as solid tumors.[5,887] Mass abdominal lesions may cause large bowel obstruction and can result in intestinal perforation as well.[808,883] Radiological methods are not very helpful for diagnosing abdominal actinomycosis, but Computerized tomography (i.e., CT) can delineate the site and size of intra-abdominal masses and the relation of the mass to adjacent tissues and organs.[525] Anorectal actinomycosis may originate in an infected anal crypt, fissure, or abscess or by direct extension from an intra-abdominal location. Omental actinomycosis that presented as an inflammatory tumor with fistula formation between the transverse colon and the jejunal region of the small intestine has also been described.[675] Isolated hepatic and hepatosplenic actinomycosis are rare clinical entities that may present as a palpable tender mass in the left mid-abdomen as with constitutional symptoms such as fever, night sweats, bloating, anorexia, and weight loss.[629,1178] Abdominal actinomycosis may also develop as an extension and complication of pelvic actinomycosis associated with intrauterine contraceptive devices.[159] Pusiol and colleagues[887] described a patient with abdominal-pelvic actinomycosis that consisted of a retroperitoneal mass lesion extending from the lower pole of the right kidney to the lower pelvis. This mass involved the ascending colon, cecum, the distal ileum, the right Fallopian tube and ovary, ureter, and the psoas muscle. The patient had an intrauterine contraceptive device (IUD) in place for the prior 3 years. Symptoms of abdominal actinomycosis include fever, weight loss, nausea, vomiting, abdominal pain, and changes in bowel function and habits.[1169] Abdominal actinomycosis may mimic colon cancer, particularly in immunocompromised patients (e.g., liver transplant recipients).[628] A diagnosis can be made if sulfur granules are present and microscopic examination reveals, branching gram-positive bacilli. Both surgical and medical interventions may be necessary to diagnose and treat these infections.[1069] Surgery enables the removal of necrotic tissue and persistent sinus tracts and may shorten the time required for parenteral therapy so a switch to oral agents can be made more quickly.

Pelvic actinomycosis may result from contiguous spread of abdominal actinomycosis or may originate in the pelvis itself. This infection is often associated with IUDs that become colonized with *Actinomyces* organisms.[140,1208] Pelvic actinomycosis is more common in women who have had an IUD in place for more

than 4 years. The clinical presentation ranges from asymptomatic endocervical colonization to tubo-ovarian infection. Pelvic actinomycotic infection may cause endometritis, salpingitis, salpingo-oophoritis, and tubo-ovarian abscess. Adnexal masses may become large enough to palpate and suggest the presence of a pelvic neoplasm.[1022] Pelvic actinomycosis may extend into adjacent

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TABLE 14-8 Phenotypic Characteristics for Identification of *Actinomyces* Species

Species	CAT	HEM SBA	NO_3	PYZ	AP	ESC	URE	ADH	MOT	GEL	HIPP	VP	LAP	PYR	CAMP	α-GAL	β-GAL	α-GLU	β-GLU	β-GUR	NAGA
A. bovis	–	–/β	–	NA	–	V+	–	NA	–	–	–	–	+	NA	NA	–	–	–	–	–	+
A. bowdenii	+	–	+	+	V	+	–	–	–	–	–	–	+	NA	NA	+	+	V	+	–	–
A. canis	+	–	–	+w	–	–	–	–	–	–	–	–	+	–	NA	+	+	+	–	–	+
A. cardiffensis	–	–	V	–	–	–	–	–	–	NA	–	–	+	–	NA	–	–	+	–	–	–
A. catuli	V	–	+	+	–	+	–	V	–	–	–	–	+	+	NA	V	+	+	+	+	V
A. coleocanis	–	–	–	+w	–	–	–	–	–	–	–	–	NA	–	NA	–	+	+	–	–	–
A. dentalis	–	–	–	–	–	+	–	–	–	–	–	–	+	–	NA	+	V	+	+	–	–
A. denticolens	–	–	+	NA	–	+	NA	NA	–	NA	–	NA	+	NA	NA	V	+	+	V	–	–
A. europaeus	–	–/βw	–	–	–	V	–	NA	–	+sl	NA	NA	+	–	–	+	+	+	–	–	–
A. funkei	–	–	V	V	+	–	–	–	–	–	+	–	+	–	+	–	V	+	–	–	V
A. georgiae	–	–	V–	NA	NA	V+	–	NA	–	V	NA	NA	NA	NA	–	NA	+	+	NA	NA	–
A. gerencseriae	–	V	V–	NA	NA	+	–	NA	–	V–	NA	NA	NA	NA	–	NA	+	+	NA	NA	–
A. graevenitzii	–	–	–	NA	NA	–	–	NA	–	–	–	NA	+	–	–	–	+	–	–	–	+
A. hominis	+	–	+	+	+	–	–	NA	–	–	NA	NA	+	–	+	+	+	+	–	–	+
A. hongkongensis	–	–	–	NA	+	–	–	+	–	–	NA	NA	+	NA	NA	–	–	–	–	–	–
A. hordeovulneris	+w	–	–	V	–	+	–	NA	–	+w	NA	NA	NA	V	NA	+	NA	+	V	–	+
A.houstonensis	–	–	+	NA	–	–	–	NA	–	–	NA	NA	NA	–	NA	NA	NA	+	NA	NA	NA
A. howellii	+	NA	–	+	+	+	–	+	–	–	NA	NA	NA	NA	NA	NA	NA	NA	+	–	+
A. hyovaginalis	–	–	V	–	+	+	–	–	–	–	–	–	+	–	NA	V	+	+	+	–	NA
A. israelii	–	–	V+	NA	NA	+	–	NA	–	–	NA	NA	NA	NA	–	NA	+	+	NA	NA	–
A. johnsonii	V	–	+	NA	V	V+	V	–	–	–	NA	NA	NA	NA	NA	+	+	+	+	–	–
A. marimammalium	–	–	–	–	V	–	–	–	–	–	–	–	+	–	NA	–	+	–	–	–	+
A. massiliensis	–	–	+	NA	–	–	–	–	–	NA	NA	NA	+	NA	NA	–	+	+	–	–	–
A. meyeri	–	–	V–	NA	–	–	V	NA	–	–	–	–	+	NA	+	–	V	+	–	–	V
A. naeslundii genospecies 1	V–	–	V+	+	–	+	+	–	–	–	–	–	+	NA	–	V	+	+	+	–	–

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A. naeslundii genospecies 2	V+	–	+	+	+w	+	+	–	–	–	NA	NA	+	NA	+	V	V	+	+	–	–
A. nasicola	–	–	–	NA	–	V	–	V	–	–	NA	–	+	–	NA	–	+	+	V	–	+
A. neuii subsp. *neuii*	+	α	+	+	–	–	–	NA	–	–	NA	NA	+	NA	+	+	+	+	–	–	–
A. neuii subsp. *anitratus*	+	–	–	+	V	–	–	NA	–	–	NA	NA	+	NA	+	+	+	+	–	–	–
A. odontolyticus	–	–	+	NA	–	V	–	–	–	–	–	–	V	NA	–	–	+	V	V	–	–
A. oricola	–	–	–	+	V	+	–	–	–	–	–	–	–	NA	NA	+	V	+	+	–	–
A. oris	V+	–	V+	NA	NA	V	–	–	–	NA	NA	NA	+	NA	NA	V+	V+	+	V+	–	–
A. radicidentis	+	–	V	+	–	+w	V	–	–	+w	–	+w	+	NA	–	+	+	+	+	–	–
A. radingae	–	α/βw	–	V	–	+	–	NA	–	V	NA	NA	–	–	–	+	+	+	+	–	+
A. ruminicola	–	–	+	NA	NA	V	–	NA	–	NA	–	–	NA	NA	NA	NA	NA	NA	NA	NA	NA
A. slackii	+	–	+	V	–	+	V–	–	–	V	NA	NA	NA	+	NA	+	NA	+	+	–	–
A. suimastiditis	–	–	–	+w	V	+	–	–	–	–	–	+w	NA	NA	NA	+	+	+	+	–	V
A. timonensis	–	–	–	NA	–	NA	–	–	–	NA	NA	NA	+	NA	NA	–	–	V	+	–	–
A. turicensis	–	βw	–	–	–	–	–	NA	–	–	NA	NA	+	–	–	–	–	+	–	–	–
A. urogenitalis	–	–	+	–	V	+	–	–	–	–	NA	V	+	+	NA	+	+	+	+	–	+
A. vaccimaxillae	–	–	–	+	+	+	–	–	–	–	–	–	+	–	NA	–	–	+	–	–	–
A. viscosus serotype 1	+	–	V	–	–	+	+	–	–	–	–	–	+	NA	NA	–	V	V	+	–	–
A. viscosus serotype 2	+	–	V	+	–	V	V	V	–	–	–	–	+	NA	NA	–	V	V	+	–	–
A. weissii	+	βw	–	–	+	+	–	NA	–	–	NA	–	NA	–	+	NA	+	+	+	–	+

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Species	Relation to O_2	Products of GLU Fermentation	Acid Produced From:																Common API CORYN Biocode (If Available)
			GLU	MAL	SUC	MNTL	XYL	LAC	MANN	ARAB	SBTL	RAFF	RIB	TRE	GLYCG	AMYG	MELB	MELZ	
A. bovis	A/F	S, A, L, F	+	+	+	–	–	+	V	–	–	–	–	–	+	–	V	–	0101104
A. bowdenii	F	S, L, A	+	+	+	–	–	+	NA	–	–	+	+	+	NA	NA	+	+	3410365, 3400365, 3510365, 3500365
A. canis	F	NA	+	+	V	–	+	+	NA	+	–	V	+	–	+	NA	–	–	2430766, 2430767
A. cardiffensis	F	L, S, a	+	V	V	–	–	–	V	–	–	V	V	–	–	–	–	–	0010121, 0010321, 1010121, 1010321
A. catuli	F	S, L, a	+	+	+	–	+	+	NA	V	–	+	+	+	–	NA	V	–	7650761, 7650765, 7670761, 7670765
A. coleocanis	F	S, L	+	+	–	–	–	+	NA	–	–	–	–	–	+	NA	–	–	2410162
A. dentalis	A	L, a	+	+	+	–	–	+w	+	–	–	+	–	+	–	–	+	NA	0450121
A. denticolens	F	S, A, L	+	+	+	V	–	+	V	–	–	+	V	–	NA	–	NA	–	NA
A. europaeus	F	S	+	+	V	–	–	–	V	–	–	–	V	V	V	–	V	V	04(1/5)0(1,3) 2[0/1/2/3]; GEL+ after 5 days
A. funkei	F	NA	+	V	+	–	+	V	NA	–	–	–	V	–	–	NA	–	–	0130761, 3530761
A. georgiae	F	S, I, a, f	+	+	+	V–	+	V+	V–	V–	V–	V–	–	+	+	V–	–	V–	NA
A. gerencseriae	A/F	S, L, a, f	+	+	+	V	+	+	+	–	V	+	V+	+	V–	V+	V	V	NA
A. graevenitzii	F	L, S	+	+	+	–	–	+	NA	–	–	–	+	–	–	–	–	–	(0,2)(0,4)20361
A. hominis	F	NA	+	+	+	–	–	–	+	+	–	+	+	NA	+	–	–	+	NA
A. hongkongensis	A	NA	–	NA	NA	NA	–	NA	–	–	NA	–	NA	–	NA	NA	NA	NA	NA
A. hordeovulneris	A/F	L, S	+	+	+	–	+	+	+w	V	NA	+w	V	V	V	NA	–	–	NA
A. houstonensis	F	NA	+	NA	+	NA	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
A. howellii	A/F	NA	+	+	+	–	+	V	V	–	NA	+w	–	V+	–	–	V	V	NA
A. hyovaginalis	F	NA	+	+	+	–	+	V	+	+	–	–	V	–	–	+	–	–	0570721, 0170721
A. israelii	A/F	S, A, L, f	+	+	+	V+	+	+	+	V+	V–	+	V+	+	–	V+	V+	V	NA
A. johnsonii	F	NA	+	V+	V+	–	–	V+	V	–	V–	+	V	V	V	NA	+	–	NA
A. marimammalium	F	S, A, L	+	+	–	–	–	+	NA	–	–	–	–	–	V	NA	–	–	0520162, 0420160, 0520160
A. massiliensis	A/F	NA	+	+	+	–	+	+	+w	–	–	–	+w	+	–	–	–	+	NA
A. meyeri	A/F	S, a, l, f	+	+	+	–	+	V+	–	V–	–	–	+	–	V	V+	–	–	NA
A. naeslundii genospecies 1	F	L, s, a, f	+	+	V+	–	V–	V	+	–	–	+	V	V+	–	V	V	–	NA
A. naeslundii genospecies 2	F	L, s, a, f	+	+	V+	+	V+	V	+	–	V	+	V	V+	V	V	V	V	NA

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A. nasicola	F	S, a, l	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NA
A. neuii subsp. *neuii*	F	S, l	+	+	+	V	+	+	+	V+	NA	+	+	+	V+	NA	V+	+	Included in API Coryne data base
A. neuii subsp. *anitratus*	F	S, l	+	+	+	V+	+	+	+	–	NA	+	+	+	V	NA	+	+	Included in API Coryne data base
A. odontolyticus	A/F	S, A, f	+	V+	+	–	V	V	–	V	–	–	V	V	V	–	–	–	NA
A. oricola	F	S, A, l	+	V+	+	–	–	–	NA	–	–	+	V	+	NA	+w	–	–	NA
A. oris	F	NA	+	+	+	–	V–	V+	V	NA	V–	+	V	V	V	NA	V+	V	NA
A. radicidentis	F	NA	+	+	+	+	–	+	NA	–	–	+	+	+	–	NA	+	+	NA
A. radingae	F	NA	+	+	+	–	+	V+	NA	NA	NA	V	+	NA	_	NA	NA	NA	Included in API Coryne data base
A. ruminicola	A	F, a, l	+	+	+	V	+	+	V	+	–	NA	+	+	V	NA	+	+	NA
A. slackii	F	NA	+	NA	V	–	+	NA	NA	–	NA	NA	–	+	–	NA	+	NA	NA
A. suimastiditis	F	NA	+	V	+	–	+	–	NA	+w	–	+	+w	–	NA	NA	+	–	NA
A. timonensis	F	NA	+	+	+	+	NA	+	V	–	NA	+	V	+	V	–	+	NA	NA
A. turicensis	F	NA	+	+	+	–	+	–	NA	NA	NA	–	+	+	–	NA	NA	NA	0010721, 0010701,. 0010723, 1010701
A. urogenitalis	F	NA	+	+	+	V	+	+	NA	V	–	+	V	+	–	NA	+	+	NA
A. vaccimaxillae	F	S, L, a	+	–	V	V	+	+	NA	+	–	–	+	+	+	–	–	–	NA
A. viscosus sero-type 1	F	S, A, l, f	+	V	+	–	V–	V+	V	–	–	+	–	V–	V	–	–	–	NA
A. viscosus sero-type 2	F	S, A, l, f	+	V	+	–	–	V+	V	–	–	+	+	V	V	–	V	–	NA
A. weissii	F	NA	+	+	+	–	–	+	NA	NA	NA	NA	–	NA	+	NA	NA	NA	NA

+, Positive; –, Negative; V, Variable reaction; +w, Weak positive reaction; V+, Variable reaction, most strains positive; V–, Variable reaction, most strains negative; NA, Data not available; CAT, Catalase; HEM SBA, Hemolysis on SBA; NO_3, Reduction of nitrate; PYZ, Pyrazinamidase; AP, Alkaline phosphatase; ESC, Esculin hydrolysis; URE, Urease; ADH, Arginine dihydrolase; MOT, Motility; GEL, Gelatin hydrolysis; HIPP; Hippurate hydrolysis; VP, Acetoin production (Voges–Proskauer); LAP, Leucine arylamidase; PYR, Pyrrolidonyl arylamidase; CAMP, CAMP test; α-GAL, α-Galactosidase; β-GAL, β-Galactosidase; α-GLU, α-Glucosidase; β-GLU, β-Glucosidase; β-GUR, β-Glucuronidase; NAGA, *N*-acetyl-β-d-Glucosaminidase.
A, Anaerobic; F, Facultative; A/a, Major/minor acetic acid; L/l, Major/minor lactic acid; S/s, Major/mino succinic acid; F/f, Major/minor formic acid; GLU, Glucose; MAL, Maltose; SUC, Sucrose; MNTL, Mannitol; XYL, Xylose; LAC, Lactose; MANN, Mannose; ARAB, Arabinose; SBTL, Sorbitol; RAFF, Raffinose; RIB, Ribose; TRE, Trehalose; GLYG, Glycogen; AMYG, Amygdalin; MELB, Melibiose; MELZ, Melizitose.

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organs, including the intestine, bladder, liver, and peritoneum.[129,593] Imaging studies, particular CT scans, may be particular useful for defining size of pelvic lesions and the extent of involvement of adjacent organs.[57] Symptoms can include fever, chronic vaginal discharge, abnormal vaginal bleeding, pelvic pain, and weight loss. Kim and colleagues[593] described a patient with multiple, large ovarian and hepatic masses, plus peritoneal metastases for which the working diagnosis was metastatic ovarian cancer. Pathology established that the cause was actinomycosis. Pelvic actinomycosis mimicking ovarian malignancy may also occur in female patients without a history of IUD placement or use.[786]

CNS actinomycosis occurs as a result of hematogenous dissemination or by direct extension from actinomycotic infections in the cervicofacial or neck areas.[456,1034] Patients usually present with a chronic meningitis or meningoencephalitis complicated by the development of subdural empyema, epidural and spinal abscesses, and cranial osteomyelitis. In 75% of cases, CNS actinomycosis presents with single or multiple encapsulated brain abscesses, usually in the temporal or frontal lobes.[1035] Patients may have the signs and symptoms of a chronic meningitis that resembles tuberculous

meningitis or another chronic CNS infections. CSF usually shows a mononuclear pleocytosis, elevated protein, and normal or low glucose concentrations. On CT scans, the lesions are ring enhancing and resemble the CNS lesions of nocardiosis. Interestingly, headache and focal neurological signs are usually present but fever is found in only about half of patients with CNS actinomycosis.

Actinomycosis may have unusual presentations because of the propensity of the organism to spread contiguously. Acevedo and colleagues[3] described a 37-year-old man with a previous history of pectoral actinomycosis who presented with lower back pain, multiple nodules with purulent discharge on the lumbar region, and genital and abdominal wall edema resulting in swelling of his legs. CT scans showed retroperitoneal and paravertebral masses compressing the inferior cava and ureters, hydronephrosis, and osteolytic lesions in the lumbar vertebrae. Another patient, a 41-year-old woman, had actinomycotic cholecystitis involving the gallbladder with adhesions and extension to the stomach and right colon.[3] Other unusual clinical presentations have included actinomycotic pericarditis with tamponade.[3] Primary cutaneous actinomycosis is rare. In one instance, an 18-year-old male with common variable immunodeficiency developed large, tumor-like masses on two of his fingers several days after receiving a lamb bite.[714] *A. bovis* was cultured from purulent material oozing from the lesions. Finally, a 35-year-old woman presented with a 7-year history of back wounds that discharged purulent material on occasion.[750] Gram's stain of the sinus tract discharge showed branching, filamentous gram-positive bacilli and *A. viscosus* grew from the cultured specimen.

Arcanobacterium *and* Trueperella *Species*

Arcanobacterium species are facultative, gram-positive, nonmotile bacilli that may be slightly curved and show rudimentary branching along with "Chinese letters" and V-forms. Unlike *Corynebacterium* species, these organisms are mostly catalase-negative, do not possess cell wall mycolic acids, and contain L-lysine and L-ornithine rather than *meso*-DAP as the peptidoglycan di-amino acid. The genus *Arcanobacterium* formerly included nine species: *A. haemolyticum*, *A. pyogenes*, *A. bernardiae*, *A. phocae*, *A. pluranimalium*, *A. hippocoleae*, *A. bialowiezense*, *A. bonsai* and *A. abortisuis*.[56,226,520,643,661,896] *A. haemolyticum*, *A. pyogenes*, and *A. bernardiae* were formerly members of the genus *Corynebacterium* and subsequently the genus *Actinomyces* before 16S rRNA sequence studies established the genus *Arcanobacterium*.[226,896,903] *A. phocae* was isolated from seals and other marine animals, while *A. pluranimalium* was recovered from a harbor porpoise, and ovine material.[350,643,896] *A. hippocoleae* has been isolated from vaginal secretions of a horse and from cases of equine placentitis and stillbirth, and *A. bialowiezense* and *A. bonasi*

were isolated from prepuce cultures from bison bulls.[475,520,661] *A. abortisuis* is an obligately anaerobic species that was isolated from the placenta of a sow who had experienced an abortion, and, subsequently, isolates of this species have been obtained from vaginal, urine, kidney, and cervical cultures from pigs.[56,87,1130] In 2006, Lehnen and coworkers[661] examined the menaquinones of *A. pyogenes* and found they were similar to those found in *A. bialowiezense* and *A. bonasi*, but divergent from those of *A. haemolyticum*. The suggestion was made that genus *Arcanobacterium* should be restricted to *A. haemolyticum*, *A. phocae*, *A. pluranimalium*, and *A. hippocoleae*, whereas *A. pyogenes*,*A. bernardiae*, *A. bialowiezense*, and *A. bonasi* should be reclassified in a new genus.[661] Yassin and colleagues then undertook an polyphasic investigation of the structure of the genus *Arcanobacterium*, examining 16S rRNA gene sequences, peptidoglycan structure, fatty acid and whole cell wall carbohydrate analysis, and polar lipid and menaquinone composition. On the basis of these studies, it was formally proposed that the genus *Arcanobacterium* be restricted to *A. haemolyticum*, *A. hippocoleae*, *A. phocae*, and *A. pluranimalium*, and that *A. abortisuis*, *A. bernardiae*, *A. bialowiezense*, *A. bonasi*, and *A. pyogenes* be reclassified in the new genus *Trueperella* as *Trueperella abortisuis*, *Trueperella, bernardiae*, *Trueperella bialowiezense*, *Trueperella bonasi*, and *Trueperella pyogenes,* respectively.[1227] In 2012, the new *Arcanobacterium* species, *A. canis,* was described. This species was isolated from an otitis externa of a 7-year-old English Bulldog.[496]

A. haemolyticum is associated with acute pharyngitis in humans, with the highest incidence in individuals between 15 and 18 years old.[701] Cervical lymphadenopathy, tonsillitis, and rash frequently may accompany the infection. Because of the rash, patients may be thought to have scarlet fever. *A. haemolyticum* may also cause other types of head and neck infections, including sinusitis and peri-orbital cellulitis.[676,1163] *A. haemolyticum* has also been recovered from chronic skin ulcers, soft tissue cellulitis, postoperative wound infections, and deep-tissue abscesses, including brain, peritonsillar, paravertebral, pelvic, and intra-abdominal abscesses.[709,1032,1147,1149] *A. haemolyticum* may occasionally cause bacteremia and sepsis in both immunocompetent and immunocompromised hosts. Bacteremia usually results from spread of the organism from local sites, including abscesses, pneumonia, and cellulitis.[1088,1104] *A. haemolyticum* has also been associated on three two occasions with Lemierre's disease, which is characterized by peritonsillar infection followed by unilateral swelling and tenderness along the sternocleidomastoid muscle due to

sole isolate from blood cultures, while, in the two other cases, the organism was co-isolated with *F. necrophorum*, the typical agent involved in Lemierre's disease.[339,698,1238] *A. haemolyticum* is a rare cause of endocarditis, osteoarticular infections, thyroid abscess, pelvic abscess, septic arthritis, chorioamnionitis, and urinary tract infections.[28,208,428,981,982,1988,1104,1147] *A. haemolyticum* may also cause infections in horses and rabbits.[476] *A. haemolyticum* is usually susceptible to all classes of antimicrobial agents, including penicillins, cephalosporins, macrolides, and vancomycin, with resistance to trimethoprim-sulfamethoxazole being a consistent finding.[163,164] Occasional isolates may be resistant to tetracyclines, the macrolides, clindamycin, and ciprofloxacin.[164]

Trueperella bernardiae includes isolates that were formerly called CDC coryneform group 2 and, subsequently *Actinomyces bernardiae*.[383] Sequence analysis of the 16S rRNA of these isolates resulted in the transfer of this species to the genus *Arcanobacterium* as *Arcanobacterium bernardiae* and recent polyphasic taxonomy studies resulted in transfer of this species to the new genus *Trueperella* as *Trueperella bernardiae*.[896,1227] *T. bernardiae* was isolated from a blood cultures of a 72-year-old woman who had large sacral decubiti; the organism was not recovered from the ulcers, but was isolated from the blood.[1190] Two patients with complicated urinary tract infections by this organisms have been described in individuals with chronic bladder dysfunction and structural urinary tract abnormalities.[535,664] One of these patients developed bacteremia and perirenal abscesses.[535] *T. bernardiae* has also been associated with osteoarticular infections, including septic arthritis, osteitis of the knee, and infection of a left total hip prosthesis.[9,86,686,891] *T. bernardiae* has also been isolated from an anal swab from a piglet.[495] *T. bernardiae* is susceptible to amoxicillin, cephalothin, cefotaxime, imipenem, aminoglycosides, erythromycin, clindamycin, linezolid, levofloxacin, and vancomycin.[86,686]

T. pyogenes is an inhabitant of the upper respiratory, gastrointestinal, and urogenital tracts of many domesticated animals and is also recognized as a veterinary pathogen, causing infections in pigs, cattle, sheep, goats, and domesticated companion animals (i.e., dogs and cats).[109,497] In this regard, the organism and the infections is causes have significant economic importance. It may also be recovered from milk and other dairy products produced from infected animals. While human infections caused by this organism have been reported since 1940, the validity of these reports is questionable due to lack of microbiological data ensuring a definite distinction of this agent from closely related bacteria like *A. haemolyticum*. Bacteriologically confirmed infections have occurred mostly in persons living in rural areas who have close contact with animals and have included bacteremia in a patient with colon cancer, five patients with intra-abdominal infections (including one patient with cervical cancer), two patients

with otitis media, one with mastoiditis, two with cystitis (including one with prostatic carcinoma), two patients with skin abscesses, and one diabetic patient with a mixed wound infection and positive blood cultures containing *T. pyogenes*.[70,295,391,699] In 2007, Plamondon et al.[875] reported the first instance of ultimately fatal *T. pyogenes* endocarditis in a 57-year-old Canadian man. This patient had several underlying conditions, including alcoholic cirrhosis, poorly controlled type II diabetes, peripheral neuropathy, and chronic leg ulcers.[875] In 2009, *T. pyogenes* sepsis was reported in a immunocompetent farmer from Brazil, and in 2010, three patients with skin and soft tissue infections were reported from India.[573,671] These patients had diabetes, Hansen's disease (leprosy), or scabies as underlying conditions that were likely involved in the pathogenesis of the infections. *T. pyogenes* is susceptible to ampicillin, third- and fourth-generation cephalosporins, aminoglycosides, macrolides, linezolid, rifampin, and vancomycin; some strains are resistant to tetracyclines and trimethoprim-sulfamethoxazole.[1116,1237]

A. haemolyticum, *T. bernardiae*, and *T. pyogenes* are the species that may be encountered in clinical specimens from humans. On SBA, *A. haemolyticum* grows as small, dull, matte colonies that are β-hemolytic (Image Plate 14-6E). Hemolysis may first be noted in areas of confluent growth after 24 hours incubation; isolated colonies will be β-hemolytic after 48 hours incubation. Use of horse blood (5%) instead of sheep blood allows recognition of β-hemolysis surrounding isolated colonies up to 24 hours earlier than sheep blood.[396] All three species are negative for arginine dihydrolase, β-glucosidase, reduction of nitrate, and urease, and none of them produce acid from arabinose, raffinose, sorbitol, or sucrose. *A. haemolyticum* is catalase-negative and does not hydrolyze esculin or urea. Most strains are pyrazinamidase, alkaline phosphatase, β-galactosidase, α-glucosidase and *N*-acetyl-β-D-glucosaminidase positive, but gelatin hydrolysis and β-glucuronidase negative. Most isolates ferment glucose, maltose, lactose, and ribose, and do not ferment mannitol, xylose, glycerol, or glycogen (Image Plate 14-6F). *A. haemolyticum* is reverse CAMP test-positive (or CAMP test inhibition-positive). In this test, the effect of the β-hemolysin of *S. aureus* on sheep red blood cells is inhibited by the phospholipase of *A. haemolyticum*. This appears on the SBA plate as a dark "arrowhead-shaped" non-hemolyzed area between *S. aureus* and the *A. haemolyticum* that has been streaked at right angles to the streak of *S. aureus*. *T. bernardiae* forms small, smooth, glassy colonies on SBA that may or may not be β-hemolytic on SBA. This organism is catalase-negative, and produces acid from glucose, maltose, ribose, and glycogen, but not from sucrose, mannitol, xylose, and lactose. Unlike *A. haemolyticum*, glucose is more readily fermented than maltose. *T. pyogenes*, the most infrequently isolated species from animals, is also β-hemolytic and catalase-negative. This species is easily differentiated from *A. haemolyticum* and *T. bernardiae* because it is obviously β-hemolytic at 24 hours, is

gelatin hydrolysis-positive, ferments xylose, and is β-glucuronidase (β-GUR)-positive. All three species are readily identified with the API Coryne. In a study of 56 *A. haemolyticum* isolates, 7 API Coryne profiles were obtained. The profiles and the percentage of strains with the indicated profile were 6530361 (12.5%), 6730161 (3.6%), 6530360 (7.2%), 6730360 (39.2%), 2730160 (17.8%), 2530360 (7.2%), and 2730260 (12.5%).[397] MALDI-TOF has also been shown to be a reliable and rapid tool for identifying *Arcanobacterium* and *Trueperella* species.[494,1157] A method for typing of *A. haemolyticum* isolates using PFGE has been developed.[348] The phenotypic characteristics of *Arcanobacterium* and *Trueperella* species are found in Table 14-9.

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TABLE 14-9 Phenotypic Characteristics for Identification of *Arcanobacterium* and *Trueperella* Species

Test	*A. canis*	*A. haemolyticum*	*A. hippocoleae*	*A. phocae*	*A. pluranimalium*	*T. abortisuis*	*T. bernardiae*	*T. bialowienzense*	*T. bonasi*	*T. pyogenes*
CAT	–	–	–	–	+	V	–	–	–	–
HEM SBA	βw	β	β	β	α	NA	V	β	β	β
NO_3	–	–	–	–	–	+	–			V
PYZ	–	+	–	–	–	–	+	+	+	–
PAL	+	+	+	+	–	–	+	–	–	–
ESC	–	–	+w	–	+w	–	–	NA	NA	–
URE		–	–	–	–	NA	–	NA	NA	–
ACET	–	–	–	–	–	+	–	–	–	–
HIPP HYDR		–	+	–	+	+	–	+	+	+
MOT	–	–	–	–	–	–	–	–	–	–
GEL HYDR		–	–	–	+	–	–	–	–	+
CAMP	+	Rev+	+	Rev+	+	–	–	–	–	–
LAP	NA	–	+	+	+	+	+	–	–	+
PYR	–	–/+w	–	–	+	–	–	+	+	–
α-GAL	–	–	–	+	–	+	–	–	–	–
β-GAL	+	+	–	+	–	+	–	–	–	+
α-GLU	+	+	+	+	–	+	+	+	–	+
β-GLU	–	–	–	+w	+w	–	–	–	–	–
β-GUR	+	–	+	–	+	+	–	+	+	+
NAGA	+	+	+	–	–	–	+	–	–	–

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Acid produced from:										
GLU	+	+	+	+	+	+	+	+w	+w	+
MAL	+	+	+	+	V	+	+	–	+w	+
SUC	+	+w	–	–	–	+	–	–	–	+w
LAC	+	+	+	+	–	+w	–	–	+w	+
MNTL	–	–	–	+	–	+	–	–	–	–
XYL	–	–	–	–	–	–	–	–	–	+
TRE	NA	–	–	–	–	–	–	–	–	+
RIB	+	+	–	+	+	–	+	–	–	+
GLY	NA	+	–	+	–	+	+	–	–	–
GLYG	+	–	–	–	–	+	+	–	–	+

+, positive reaction, −, negative reaction; V, variable reaction; +w,weak or slow positive reaction; V+, variable reaction, most strains positive; V−, variable reaction, most strains −; V+w, variable reaction, most strains weakly positive; Rev+, reverse CAMP test positive; NA, data not available; LIP, lipid requirement; CAT, catalase; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; ACET, Acetoin (VP); HIPP HYDR, Hippurate hydrolysis; MOT, motility; GEL HYDR, Gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; GLU, Glucose; MAL, Maltose; SUC, Sucrose; LAC, Lactose; MNTL, Mannitol; SBTL, Sorbitol; XYL, Xylose; TRE, Trehalose; RAFF, Raffinose; RIB, Ribose; GLY, Glycerol; GLYG, Glycogen.

Arthrobacter *and Related Species*

Members of the genus *Arthrobacter* are gram-positive cornyneform bacilli that occupy several ecologic niches in the environment. For example, some species are psychrophilic and have been isolated from extreme environments (e.g., Antarctic snow) and some species have been isolated from veterinary specimens. One species described in 2004 was isolated from filtered air samples from the Mir Russian Space Laboratory![673,1226] These organisms have a marked rod-to-coccus cycle, where bacilli predominate in younger cultures and cells become more coccobacillary and coccoid with age. The cellular fatty acid content is consistent among all *Arthrobacter* species as well. In the cell wall, *meso*-DAP is absent, lysine is the predominant amino acid, and alanine and glutamic acid comprise the interpeptide (A3α-type) linkages. During their examination of various CDC group B-1 and B-3 strains, Funke and colleagues[370] encountered 11 human clinical isolates (from urine, skin infection, blood cultures, endophthalmitis) that had characteristics consistent with genus *Arthrobacter*. Newer *Arthrobacter* species—*A. cumminsii* and *A. woluwensis*—were proposed for these CDC group B-1 and B-3 strains. A 1998 analysis of 15 clinical isolates of *A. cumminsii* from the urinary tract, middle ear, amniotic fluid, vagina, calcaneal osteomyelitis, deep-tissue cellulitis, cervix, blood, and wounds revealed unique cellular fatty acid patterns, with the consistent presence of C14:0i and C14:0, and unusually high amounts of C16:0 and C16:0i fatty acids, which are not found in other *Arthrobacter* species.[378] *Arthrobacter* species were also reported in a patient with endophthalmitis following intraocular lens implantation and from a neutropenic patient with acute lymphoblastic lymphoma who developed septicemia.[313,524]

Since 1998, several new *Arthrobacter* species associated with serious infections have been isolated and characterized phenotypically, chemotaxonomically, and

genetically. Clinically relevant species and their sources of isolation include *A. creatinolyticus* (from urine specimens), *A. oxydans*, *A. luteolus* (from surgical wounds), *A. albus* (from multiple blood culture from patient with severe phlebitis), and *A. scleromae* (exudates from multiple dorsal dermal scleromata).[517,527,1183] In 2004, *A. woluwensis* was reported as the cause of endocarditis in an injection drug user.[96] Mages and colleagues performed an exhaustive examination of 50 *Arthrobacter* strains from several types of clinical specimens, including 12 strains each from blood and wounds, 8 from urine samples, 5 from sterile sites, and the remainder from miscellaneous sites. Thirty-eight strains were true *Arthobacter* species, and more than half of these were either *A. cumminsii* (14 isolates) or *A. oxydans* (11 isolates). The third most common isolate was *A. aurescens*, which had never before been isolated from human specimens. This work also revealed a new species, *A. sanguinis*, from blood cultures.[703] As had been reported earlier for *A. woluwensis* and *A. cumminsii*, all 38 bona fide *Arthrobacter* species were susceptible to β-lactams, doxycycline, gentamicin, linezolid, rifampin, and vancomycin. A 2012 report documented another new species, *A. mysorens*, from a soil-contaminated erythrasma-like skin lesion; the same organism was isolated from the soil.[536] Molecular techniques were used to detect an *Arthrobacter* species in blood and placental tissue associated with fetal death and maternal disseminated intravascular coagulation.[1009] These organisms have also been isolated from semen specimens from patients with inflammatory prostatitis.[1126]

Arthrobacter species demonstrate a rod-to-coccus cycle, with cocci predominating in older cultures. Colonies may be creamy white, gray/white, or yellow, and are smooth, glistening, and circular with entire edges. Some environmental *Arthrobacter* species may have red to blue/green colonies. Nitrate reduction and hydrolysis of urea and esculin are variable characteristics. Acid is produced oxidatively from a variety of carbohydrates, as determined by assimilation. In most reports on these organisms, the API CHO (bioMérieux, Inc.), a carbohydrate assimilation, not fermentation, panel was used for characterization, but this system is not usually used in clinical laboratories. *A. cumminsii,* the most frequently isolated *Arthrobacter* species from human clinical specimens, is LAP-positive, and negative for both nitrate reductase and esculin hydrolysis. Some strains produce urease, and both DNase and gelatinase activity are produced by most isolates. While most strains produce acid from ribose, acid production from glucose is variable, and no acid is produced from maltose, sucrose, mannitol, xylose, lactose, or glycogen. *A. woluwensis* hydrolyzes esculin, urea, gelatin and DNA, and, unlike *A. cumminsii*, produces several enzymatic activities, including β-galactosidase (β-GAL), α-glucosidase (α-GLU), β-glucosidase (β-GLU), and *N*-acetyl-β-D-glucosaminidase (NAGA). *Arthrobacter* species are included in the API Coryne database; however, the carbohydrate fermentation cupules will usually be negative after 24 hours. Prolonged incubation of the identification panels (up to 5 to 7

days) may result in slow oxidative utilization of some carbohydrates. When *A. cumminsii* isolates were tested on the API Coryne, numerous different biotype numbers were generated after 24 hours, 48 hours, and 7 days incubation.[370,378] Molecular methods (e.g., sequencing studies) and chemotaxonomic methods are necessary for definitive identification of *Arthrobacter* species. Phenotypic and biochemical characteristics of *Arthrobacter* species isolated from clinical species are presented in Table 14-10.

The antimicrobial susceptibility of *Arthrobacter* species may be quite variable. In a study of 10 *A. cumminsii* strains, the β-lactams, rifampin, and vancomycin showed good activity against most isolates.[378] All strains were susceptible to the macrolides, and most were tetracycline susceptible. MICs for the aminoglycosides were surprisingly high, with the MIC_{50} being 8 μg/mL for gentamicin and 64 μg/mL for tobramycin. Similarly, the fluoroquinolones showed elevated MICs for most strains tested. The *A. woluwensis* endocarditis isolate reported by Bernasconi et al.[96] was resistant to penicillin and ciprofloxacin, and susceptible to tetracycline and vancomycin by disk diffusion.[96] *Arthrobacter* strains examined by others have exhibited susceptibility to theβ-lactams, doxycycline, gentamicin, rifampin, linezolid, and vancomycin, with less susceptibility seen for ciprofloxacin.[370,378,703] These studies also noted the multiresistant nature of *A. woluwensis*. The single *A. scleromae* isolate reported by Huang and colleagues[527] was susceptible to ceftriaxone, rifampin, and tetracycline, intermediate in susceptibility to cefazolin, cefotaxime, doxycycline, erythromycin, and vancomycin, and resistant to penicillin, ampicillin, oxacillin, and all aminoglycosides (gentamicin, tobramycin, amikacin, streptomycin, kanamycin) by the CLSI criteria established for

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TABLE 14-10 Phenotypic Characteristics for Identification of *Arthrobacter* Species

Characteristic	*A. albus*	*A. citreus*	*A. creatinolyticus*	*A. cumminsii*	*A. luteolus*	*A. oxydans*	*A. sanguinis*	*A. scleromae*	*A. woluwensis*
CAT	+	+	+	+	+	+	+	+	+
HEM SBA	–	NA	–	–	–	–	–	–	–
Growth, 20°C	–	+	NA	V	+	+	NA	+	+
Growth, 42°C	+	–	NA	+	+	V	NA	–	+
NO_3	–	+	+	–	+	+	–	+w	–
PYZ	+	NA	NA	V	+	NA	+	–	NA
PAL	+	NA	NA	V	NA	NA	+	NA	+
ESC	–	NA	NA	–	–	–	–	+	+3 days
URE	–	NA	NA	V	–	–	–	–	+3 days
MOT	–	+sl	–	–	+	–	–	NA	–
GEL HYDR	+sl	+	+	+10 days	+	+	+	+	+24 hours
DNase	–	+	NA	+10 days	+	+	NA	NA	+24 hours
LAP	+	NA	NA	+	+	NA	+	NA	+
PYR	+	–	NA	V	–	–	NA	–	+
α-GAL	–	NA	NA	–	–	NA	+	NA	–
β-GAL	–	+	NA	–	–	+	+	–	+
α-GLU	–	+	NA	–	+	+	+	–	+
β-GLU	–	NA	NA	–	–	NA	–	+	+
β-GUR	–	NA	NA	–	–	NA	–	–	–
NAGA	–	+	NA	–	–	–	+	–	+
Acid oxidatively from:									
GLU	–	+	–	V	+	+	+	–	+
MAL	–	NA	NA	–	+	–	+	–	NA
SUC	–	NA	NA	–	+	+	+	–	NA
MNTL	–	+	NA	–	–	+	–	+	+
XYL	–	NA	–	–	+	–	–	–	NA
LAC	NA	NA	NA	–	–	NA	–	–	NA
Common API Coryne biocodes	6102004, 6100004	NA	NA	Varies (depends on length of incubation)	3110004	3750004	NA	1042014	NA

+, positive reaction; −, negative reaction; V, variable reaction; +sl, slow positive reaction; NA, data not available; +w, weak or slow positive reaction; V+, variable reaction, most strains positive; V−, variable reaction, most strains negative; V+w, variable reaction, most strains weakly positive; NA, data not available; LIP, lipid requirement; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; ACET, Acetoin (VP); GEL, gelatin hydrolysis; HIPP HYDR, Hippurate hydrolysis; MOT, motility; GEL HYDR, Gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-d-glucosaminidase; GLU, Glucose; MAL, Maltose; SUC, Sucrose; LAC, Lactose; MNTL; Mannitol; XYL, Xylose; TRE, Trehalose.

staphylococci. Reports of infections with uncharacterized *Arthrobacter* species have also noted isolates with elevated MIC against β-lactams, macrolides, and tetracyclines, and susceptibility to vancomycin.[517]

During their examination of the 50 *Arthrobacter* isolates, 7 isolates were found to be other species. While three belonged to genus *Brevibacterium*, and two were *Microbacterium* species, single isolates belonged to the genera *Pseudoclavibacter*,*Leucobacter*, or *Brachybacterium*.[703] All of these genera include various environmental organisms. The *Pseudoclavibacter* species in this study was isolated from an aortic valve of a 74-year-old male. Lemaitre et al.[662] isolated a gram-positive rod from a chronic subcutaneous infection of the right leg. The isolate was sequenced and determined to have 99% sequence similarity with genus *Pseudoclavibacter*.[662] *Brachybacterium* species have been recovered from clinical material before, but *Leucobacter* had never been previously reported in human clinical samples.[1044]

Brevibacterium *Species*

Brevibacterium species are irregular, slender, rod-shaped bacteria that also display a marked rod-to-coccus cycle. Fresh subcultures (<3 days) stain as bacilli, but as the culture ages (4 to 7 days), the cells may appear coccal in morphology. Cells are gram-positive, but may be easily decolorized. The rod-shaped cells show diphtheroidal morphology and are non–acid-fast. *Brevibacterium* species are obligately aerobic and are oxidative in their metabolism. They are nonmotile, salt-tolerant (>6.5% NaCl), produce catalase and proteinases, do not possess urease, and do not produce acid from glucose or other carbohydrates in peptone media. Brevibacteria also characteristically produce methanethiol (CH_3SH) from L-methionine. The peptidoglycan contains *meso*-DAP as the principal di-amino acid, but does not contain arabinose, lysine, or mycolic acids (galactose may or may not be present).[150]

Genus *Brevibacterium* contains more than 45 species, most of which are environmental bacteria. Colonies are grayish white, yellowish white, yellow, or tan and have an intense "cheesy" odor. The habitat of the many brevibacteria is primarily milk products, where the organisms contribute to the aroma (e.g., Limberger cheese) and the color (e.g., orange-pigmented *B. linens*) of surface-ripened cheeses. They are also found on human skin surfaces; under moist, macerating conditions (e.g., between the toes and other intertriginous areas), these organisms are believed to contribute to body odor. Brevibacteria are rare causes of human infections and examination of strains previously classified as CDC groups B-1 and B-3 established that some were *Brevibacterium* species. Gruner, Pfyffer, and von Graevenitz isolated nine *Brevibacterium* strains from dialysate fluids, blood cultures, sputum, CSF, pleural fluid, and a miscellaneous body fluid.[445] Comparison of their biochemical characteristics with reference *Brevibacterium* strains indicated that these organisms were either *B. casei* or *B. epidermidis*. These researchers suggested that aerobic, gram-positive and catalase-positive bacilli that are nonmotile and fail to ferment sugars may be identified as presumptive *Brevibacterium* species based on colony pigmentation and morphology (gray-white to yellow, opaque, convex and smooth) and an intensive "cheesy" or "stinky feet" odor. A subsequent examination of 41 clinical strains of CDC groups B-1 and B-3 found that 22 were identical to *B. casei*, with 5 additional isolates forming another phenotypic cluster within the genus *Brevibacterium*.[446] Funke and Carlotti also examined 43 *Brevibacterium* strains recovered from clinical specimens over the last 20 years and compared them to reference *Brevibacterium* strains.[360] Using chemotaxonomic analysis and carbohydrate assimilation tests with the API 50CH assimilation panel, 41 were identified as *B. casei* and 2 were identified as *B. epidermidis*.

Brevibacterium species have been isolated from several human infections since their initial characterization, and 10 species have been isolated from human clinical

specimens: *B. casei*, *B. epidermidis*, *B. iodinum*, *B. mcbrellneri*, *B. otitidis*, *B. lutescens*, *B. paucivorans*, *B. sanguinis*, *B. massiliensis,* and *B. ravenspurgense*. *B. casei* has been isolated from blood cultures as a cause of catheter-related bacteremia in AIDS patients, bacteremia in a patient with choriocarcinoma, central venous catheter infections, and as a cause of brain abscess, corneal ulcers, and pericardial infection.[38,135,156,404,544,908,1132,1186] *B. sanguinis* has been isolated from skin and from dialysate fluids and is phenotypically similar to, but genotypically distinct from *B. casei*.[1186] *B. epidermidis* has caused central venous line infection and native valve endocarditis in an immunocompetent 52-year-old man.[711,734] *B. otitidis* has been isolated from ear drainage of patients with bilateral otorhea, from blood cultures as a cause of prosthetic valve endocarditis in an elderly woman, and from peritoneal fluid of a patient on CAPD.[257,844,1187] *B. lutescens* is a *B. otitidis*-like species that was also isolated from human peritoneal dialysis fluid and infected ear drainage in 2003.[1182] *B. iodinum* peritonitis in a CAPD patient was reported in Greece in 1997.[40] *B. mcbrellneri* was originally isolated from human genital hairs that were co-infected with *Trichosporon beigelii* and *B. paucivorans*, which was described in 2001, has been isolated from CSF, abscesses, wounds, blood, ear discharge, and intravascular catheters.[733,1184] *B. massiliensis* was isolated from discharge from an ankle wound resulting from a car accident.[951] *B. avium* has only been isolated from granulomatous lesions of poultry.[843] In their survey of *Arthrobacter* species, Mages et al.[703] identified a new *Brevibacterium* species, *B. ravenspurgense*, that was isolated from a wound.

All brevibacteria are aerobic, gram-positive, non–spore-forming bacilli that are catalase-positive. They are nonhemolytic on SBA, nonmotile, and asaccharolytic. Colonies of *B. casei* are usually whitish-gray, while *B. epidermidis* strains develop yellowish colonies with time (Image Plate 14-6G). Detection of methanthiol and cellular fatty acid analysis can be used to confirm the assignment of the isolate to the genus *Brevibacterium*, and carbohydrate assimilations can also be useful for identification of these organisms.[360] Some *Brevibacterium* species are included in the data bases of the API Coryne and the RapID CB-Plus (Image Plate 14-6H). Molecular characterization most accurately identifies these organisms. Biochemical characteristics of *Brevibacterium* species are shown in Table 14-11.

The antimicrobial susceptibility of *Brevibacterium* species may be quite variable. Funke, Punter, and von Graevenitz examined 50 *B. casei* strains, which are the the most frequently isolated species, and found that all isolates showed decreased susceptibility to all β-lactam agents, with half of

TABLE 14-11 Phenotypic Characteristics for Identification of *Brevibacterium* Species

Characteristic	*B. avium*	*B. casei*	*B. epidermidis*	*B. iodinum*	*B. linens*	*B. lutescens*	*B. massiliensis*	*B. mcbrellneri*	*B. otitidis*	*B. paucivorans*	*B. ravensburgense*	*B. sanguinis*
CAT	+	+	+	+	+	+	+	+	+	+	+	+
HEM SBA	–	–	–	–	–	–	–	–	–	–	–	–
NO_3	+	V–	V+	+	NA	–	–	–	–	–	–	–/+w
PYZ	NA	+	+	+	+	+	+w	–	+	–	+	+
PAL	+	+	NA	NA	NA	V	–	NA	+	–	V	+
ESC	–	–	NA	NA	NA	–	–	NA	–	–	–	–
URE	–	–	NA	NA	NA	–	–	NA	–	–	–	–
Growth, 6.5% NaCl	NA	+	+	NA	NA	+	+	NA	NA	NA	NA	NA
GEL	+	+	+	+	+	+	–	+sl	+	–	–	+
DNase	+	+	NA	NA	NA	NA	NA	NA	+	NA	NA	–
LAP	+	+	NA	NA	NA	+	+	NA	+	+	+	+
PYR	NA	V+	–	–	–	+	–	–	+	–	–	V
α-GAL	–	–	NA	NA	NA	–	–	NA	–	–	–	NA
β-GAL	–	–	NA	NA	NA	–	–	NA	–	–	–	NA
α-GLU	–	V+	–	–	–	–	–	–	–	–	–	+
β-GLU	–	–	NA	NA	NA	–	–	NA	–	–	–	NA
β-GUR	–	–	NA	NA	NA	–	–	NA	–	–	–	NA
NAGA	–	–	+	–	–	+	–	+	–	V–	–	–
Acid from GLU and other CHOs	–	–	–	–	–	–	–	–	–	–	–	NA
Hydrolysis of:												
CAS	+	+	+	+	+	+	NA	+	+	–	NA	+
XAN	+	+	+	+	+	–	NA	V	–	–	NA	+
Common API Coryne Biocodes	NA	6112004, 4112004	NA	NA	NA	6002004, 6102004	NA	NA	6002004	0000004	NA	6112004

+, positive reaction; –, negative reaction; V, variable reaction; +w, weak or slow positive reaction; V+, variable reaction, most strains positive; V–, variable reaction, most strains negative; V+w, variable reaction, most strains weakly positive; NA, data not available; LIP, lipid requirement; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; GEL, gelatin hydrolysis; GEL HYDR, Gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; GLU, Glucose; CHOs, Carbohydrates; CAS, casein; XAN, xanthine.

the 50 isolates tested having β-lactam MICs of >1 μg/mL.[380] Troxler and colleagues[1119] also found that *B. casei* strains tended to have higher β-lactam MICs, with some isolates having cephalosporin MIC's of >8 μg/mL. Both research groups found that *B. casei* displayed elevated MICs to other drug classes, including the macrolides, lincosamides, and fluoroquinolones. Others have found that *B. casei* strains are susceptible to β-lactams, aminoglycosides, tetracyclines, rifampin, linezolid, vancomycin, teicoplanin, imipenem, meropenem, and linezolid.[38] The clinically significant isolates of *B. otitidis* (from blood and peritoneal fluid) were susceptible to all agents tested, including penicillin, ampicillin, cephalosporins, fluoroquinolones, macrolides, gentamicin, ciprofloxacin, linezolid, and vancomycin.[257,1187] The *B. iodinum* isolate reported by Antoniou et al.[40] was resistant to ampicillin, the cephalosporins, and vancomycin, but susceptible to fluoroquinolones and amikacin.

Cellulomonas, Cellulosimicrobium, *and* Oerskovia *Species*

Cellulomonas species are principally environmental organisms that appear as small, slender, irregular coryneform rods that become more coccoid in older cultures. They are gram-positive (although easily decolorized), non–acid-fast, and may be nonmotile or motile due to single polar or sparse lateral flagella. Colonies of these organisms are usually opaque, convex, and yellow-pigmented. The cell wall peptidoglycan contains L-ornithine and does not contain *meso*-DAP, glycine, lysine, or homoserine. These organisms are either oxidative or fermentative in their metabolism; most strains produce acid from glucose both aerobically and anaerobically. *Cellulomonas* species are catalase-positive, hydrolyze cellulose, starch, and gelatin (weak reaction), reduce nitrate to nitrite, and produce DNase. The genus *Cellulomonas* includes several valid species that are isolated from various environments (e.g., soil, compost, water).[381] The genus *Cellulomonas* (and *Cellulosimicrobium*) are in the phylum *Actinobacteria*, class *Actinobacteria*, in the *Micrococciniae* suborder of the order *Actinomycetales*, family *Cellulomonadaceae.*

Some strains of former CDC coryneform groups A-3 and A-4 have been reclassified as *Cellulomonas* species that have been isolated from human clinical specimens.[381,389,478] Funke, Ramos, and Collins examined clinical isolates from CSF (two isolates) and blood (two isolates); both of the CSF isolates had been phenotypically identified as CDC group A-3, while the blood culture isolates were identified as CDC group A-4.[381] These isolates were compared to type strains of the characterized environmental *Cellulomonas* species using peptidoglycan and cellular fatty acid analysis in addition to the phenotypic tests used for characterization of coryneforms. Phenotypic test results for these isolates did not correspond to any of the extant *Cellulomonas* species. The four clinical isolates contained L-ornithine as the peptidoglycan di-amino acid, thereby reinforcing affinity with *Cellulomonas* species. Partial sequencing of the 16S rRNAs of these organisms further validated the similarities with *Cellulomonas* species, and Funke and coworkers[389] formally proposed that CDC group A-3 and some group A-4 strains be assigned to a new genus, *Cellulomonas hominis*. These organisms have been isolated from vitreous humor specimens following penetrating injuries or intraocular lens transplants, blood cultures associated with catheter-related sepsis, native valve endocarditis, and cerebrospinal fluid.[115,389,478] In 2005, the remaining six coryneform A-3 isolates strains (i.e., two blood isolates, one CSF isolate, one each from a homograft valve, a pilonidal cyst, and a lip wound) were placed in the new species *Cellulomonas denverensis.*[142] *C. denverensis* has also been isolated from the blood and bile cultures of an 82-year-old woman with acute cholecystitis.[813] In 2009, an uncharacterized *Cellulomonas* species was isolated from several sets of blood cultures from a 78-year-old female with endocarditis complicated by osteomyelitis of the lumbar spine.[626]

C. hominis and *C. denverensis* are both small, thin, motile gram-positive rods. Colonies of both organisms on SBA are smooth, circular, convex, and white; a pale yellow to yellow pigmentation develops after 2 to 3 days. Both species are catalase-positive, hydrolyze esculin, reduce nitrate to nitrite, and are urease-negative. Both species produce acid from several carbohydrates including, glucose, maltose, sucrose, xylose, and lactose. Mannitol is not fermented by either species. The two species can be differentiated by sorbitol fermentation; *C. denverensis* produces acid from sorbitol, while *C. hominis* does not.[142,381] Unlike environmental *Cellulomonas* species, *C. hominis* does not hydrolyze cellulose. *Cellulomonas* species are included in the API Coryne database, but these organisms will usually give a cross-call with *Microbacterium* species.

The genus *Cellulosimicrobium* includes the organisms formerly included in the genus *Oerskovia* (*O. xanthineolytica* and *O. turbata*) that were subsequently transferred to the genus *Cellulomonas* as *C. cellulans* and *C. turbata,* respectively. Analysis of 16S rDNA dendrograms provided evidence that "*C. cellulans"* branched outside of the phylogenetic confines of the genus *Cellulomonas* and was a phylogenetic "nearest neighbor" of the genus *Promicromonospora*. In addition, chemotaxonomic analysis of the cell wall of "*C. cellulans*" showed that the type strain and other strains of this species possess a "type A4α" peptidoglycan, a characteristic not found among members of the genus *Cellulomonas*, including "*C. turbata*". L-lysine was found to be the principal cell wall di-amino acid of "*C. cellulans*", while other *Cellulomonas* species contain L-ornithine. The agreement between the phylogenetically distinct position of this organism in the 16S rDNA dendrogram, and the different chemotaxonomic properties exhibited by this organism justified the assigment of "*C. cellulans*" to the new genus *Cellulosimicrobium,* with the type species being *Cellulosimicrobium cellulans* ("*Oerskovia xanthineolytica*").[993] In 2006, Brown and colleagues[143] proposed that some clinical isolates of "*C. turbata*" be included in the genus *Cellulsosimicrobium* based on 16S rRNA sequence and chemotaxonomic data; this species is named *Cellulosimicrobium funkei*. In 2007, a third *Cellulosimicrobium* species, *Cellulosimicrobium terreum*, was isolated from soil samples from Dokdo, South Korea.[1233] This latter species description is based on a single environmental isolate, and this species does not grow at or above 35°C. In 2002, another *Cellulosimicrobium* species, *C. variabile*, was isolated from the hindgut of a termite, but this species was reclassified in as *Isoptericola variabilis* in 2004.[62,1055]

C. cellulans is found in the environment (soil, water, grass cuttings), as a cause of infection in animals (e.g., horses)

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and as a rare cause of opportunistic infections primarily in in immunocompromised hosts, with *C. cellulans* being isolated much more frequently than *C. funkei*.[41,124] *C. cellulans* has been reported as a rare cause of keratitis, endophthalmitis, pneumonia, prosthetic joint-related infection, catheter-related bacteremia, ventriculoperitoneal shunt-associated meningitis, and peritonitis in patients receiving chronic ambulatory peritoneal dialysis.[474,567,696,735,800,1000] *C. cellulans* bacteremia has also been documented in bone marrow transplant recipients, in AIDS patients, and in patients with underlying liver disease.[304,705] These organisms may contaminate total parenteral nutrition solutions; this was only discovered after a patient developed signs and symptoms of bacteremia immediately following parenteral infusion, with recovery of the organisms from blood cultures, from the associated central venous catheter, and from a sampling of the nutritional supplement.[453] More recently. *C. cellulans* has been isolated as the cause of neonatal sepsis with negative CSF and urine cultures and as the agent of central venous catheter-related bacteremia in a 13-year-old male with midgut volvulus and short bowel syndrome.[167,952] Pyogenic flexor tenosynovitis of the right middle finger and septic arthritis of the knee due to *C. cellulans* were described in two patients, both of whom had had penetrating injuries to the involved site with retained foreign bodies serving as the nidus of infection.[704,1122] Molecular methods using universal primers based on conserved sequences of the *rrn* gene (encoding 16S rRNA) were used to amplify a 479-base-pair fragment from a biopsy specimen of a tongue lesion from a patient with AIDS. On sequencing, the fragment was identical to the 16S rRNA gene of the *C. cellulans* type strain.[491] *C. cellulans* was also identified as the causative agent of postoperative endophthalmis following cataract surgery in three patients.[19]

C. funkei is isolated less frequently than *C. cellulans* and has been documented as the cause of Broviac catheter-related bacteremia in a 3-year-old male and as a cause of endocarditis in a 68-year-old man whose aortic valve had been replaced with a homograft heart valve because of severe aortic insufficiency related to ankylosing spondylitis.[666,910] In 1996, this organism was reported as a cause of bacteremia (along with *Comomonas acidovorans*) in a patient with AIDS.[627] More recently, *C. funkei* bacteremia and possibly prosthetic valve endocarditis was reported in an 81-year-old male who had received a tissue aortic valve replacement 7 months previously due to aortic stenosis and from a patient with peritonitis.[100,865]

C. cellulans produces smooth, glistening, entire colonies that are strongly yellow-pigmented (Image Plate 14-7C). The organism's actinomycetous affinities are manifested by the production of a substrate "mycelium" that fragments into irregular, curved and club-shaped rods arranged in V-forms and "Chinese letters." After exhaustion of fermentable carbohydrate from broth media, these rod-shaped organisms are transformed into even shorter rods or coccal forms. These organisms

are biochemically active and are included in the databases of both the API Coryne and the IDS RapID CB-plus systems for the identification of coryneform bacteria. The common API Coryne biocodes for *C. cellulans* are 3552727, 7552727, 3572727, or 7572727 (Image Plate 14-7D).[952] Both *C. cellulans* and *C. funkei* hydrolyze casein, but only *C. cellulans* hydrolyzes xanthine. In addition, *C. cellulans* grows at 42°C, while *C. funkei* does not. MALDI-TOF MS has been used successfully for identification of *C. cellulans*.[704]

C. cellulans and *C. funkei* are variable in their susceptibility to antimicrobial agents. While many isolates have been reported as susceptible to penicillin, ampicillin, cephalothin, tetracycline, clindamycin, erythromycin, gentamicin, trimethoprim-sulfamethoxazole, ciprofloxacin and vancomycin, strains that are resistant to these agents have also been reported.[735,1122] The isolate from the neonatal sepsis case reported in 2010 was resistant to penicillin, ampicillin, cefotaxime, clindamycin, ciprofloxacin, and erythromycin, and susceptible to linezolid, rifampin, sulfamethoxazole-trimethoprim, and vancomycin using the CLSI standards for *Corynebacterium* species.[167,214] In the species description of *C. funkei*, all 13 isolates evaluated were susceptible to ampicillin, amoxicillin clavulanate, ceftriaxone, clarithromycin, minocycline, and vancomycin.[143] The *C. funkei* blood isolate described by Petkar et al.[865] was resistant to penicillin, erythromycin, tetracycline ciprofloxacin, rifampin, sulfamethoxazole-trimethoprim, and imipenem, and susceptible to vancomycin and gentamicin. Linezolid and vancomycin may be considered the drugs of choice for *C. cellulans* and *C. funkei* infections.[704] High-level vancomycin and teicoplanin resistance has been reported in a clinical isolate of *C. funkei*.[881] This glycopeptide resistance was shown to be due to modified cell wall peptidoglycan structure related to the presence of *vanA* gene sequences similar to those found in vancomycin-resistant strains of *Enterococcus faecium*. Treatment of some *C. cellulans* infections required removal of foreign bodies in order to effect cure with the appropriate agents.[1122]

Members of the emended genus *Oerskovia* demonstrate branching vegetative hyphae that penetrate into the agar and fragment into motile rod-like elements.[1054] On smears, the organisms appear as coryneform bacilli. Organisms may or may not be motile. They are catalase- and oxidase-positive and facultative with respect to oxygen, although some strains may be strictly aerobic. Major fatty acids, isoprenoid quinone composition, and other characteristics of the genus are shown in Table 14-5. *O. enterophila* is recovered from insects and was formerly a *Promicromonospora* species, while *O. jenensis* and *O. paurometabola* are soil isolates.

Dermabacter *and* Helcobacillus *Species*

In 1988, Jones and Collins described four strains of coryneform bacteria that were isolated exclusively from human skin.[557] The cell walls of these isolates contained *meso*-DAP, alanine, and glutamic acid, but lacked cell wall mycolic acids.[150] These bacteria also contained predominantly branched-carbon cellular fatty acids, a characteristic found only in *Brevibacterium* species and the environmental organism *Brachybacterium faecium*. The organisms were fermentative (not oxidative like *Brevibacterium* and *Brachybacterium* species) and produced acid from several carbohydrates. The name *Dermabacter hominis* was proposed for these strains. Subsequent examination of CDC group 3 and group 5 isolates recovered human clinical established that these two CDC groups were identical to the type strain of *Dermabacter hominis* described by Jones and Collins.[94,386,557] *D. hominis* has been recovered from a variety of clinical specimens

including blood, lung tissue, abscesses, CSF, peritoneal fluid, conjunctivae, calcaneal osteomyelitis, and infected vascular grafts.[420,447,1138] In 1998, Bavbek and colleagues[76] described a renal transplant recipient that presented as a contrast-enhancing brain mass, which was found to be a cerebral abscess caused by *D. hominis*. *D. hominis* has also been recovered as a cause of peritonitis from a patient on CAPD and was co-isolated with *S. aureus* and *Finegoldia magna* from a recurrent abscess.[718,892] A *Dermabacter*-like organism was isolated by Renvoise and colleagues[917] from an erythrasma-like lesion from a 58-year-old man. The organisms showed 95.1% similarity with *D. hominis* and represented a new genus and species. This organism was named *Helcobacillus massiliensis*. Both of these species are classified in the family *Dermatobacteriaceae,* suborder *Micrococcineae*, order *Actinomycetales*, class *Actinobacteria*, in the phylum *Actinobacteria*.

D. hominis and *H. massiliensis* are catalase-positive, nonhemolytic, nonmotile coryneform rods. On Gram's stain, both organisms appear coccobacillary or coccoid. Colonies of *D. hominis* are usually white, convex, and have a creamy or slightly sticky consistency. After 48 hours of incubation, colonies are usually about 1.5 mm in diameter. *H. massiliensis* also forms shiny, white, smooth, nonhemolytic colonies on SBA. *D. hominis* does not reduce nitrate or produce pyrazinamidase and is PYR-positive, while *H. massiliensis* reduces nitrate to nitrite, is pyrazinamidase-positive, and is PYR-negative. These species also produce acid from different carbohydrates as well. *D. hominis* is included in the API Coryne database. Additional phenotypic characteristics of this organism and *H. massiliensis* are presented in Table 14-12.

Exiguobacterium *Species*

Exiguobacterium species are short, irregular coryneform bacilli that demonstrate a marked rod-coccus cycle in their growth. They characteristically produce yellow or pale orange colonies on agar media and are facultative and fermentative in their metabolism.[227] Mycolic acids are absent and L-lysine is the peptidoglycan di-amino acid. *Exiguobacterium* species are catalase-positive, motile, and resemble *Microbacterium* and *Oerskovia* species, although they differ in their cell wall fatty acids. More than fourteen species of *Exiguobacterium* have been described and they are all alkaliphilic, halotolerant, and found in environmental sources. *E. aurantiacum* and *E. acetylicum* are the two species that have been previously isolated from human clinical specimens (e.g., skin, wound, cerebrospinal fluid).[389] *E. aurantiacum* was isolated from blood cultures of six patients in London over a 10-year period; three of these patients had myeloma and one had endocarditis.[873] *E. acetylicum* was isolated from blood cultures as a cause of catheter-related bacteremia in an elderly woman.[588] In 2006, an unnamed *Exiguobacterium* species was isolated from a blood culture of a patient in Belfast. This isolate was misidentified as *C. cellulans* by the API Coryne, and confirmed as an *Exiguobacterium* species by 16S rRNA sequencing.[582] These bacteria produce light yellow, intense yellow, or orange colonies on SBA. Both organisms are catalase-positive and produce acid from variety of carbohydrates. Phenotypic properties of *E. acetylicum* and *E. aurantiacum* are shown in Table 14-13.

Leifsonia *Species*

During a taxonomic investigation of microbes isolated from grassroot galls induced by a free-living nematode, Evtushenko and colleagues[315] recognized a group of coryneform bacteria that were phenotypically similar to the motile coryneform that has historically been called "*C. aquaticum*." Although long considered a *Corynebacterium* species, certain properties (e.g., motility and oxidative carbohydrate metabolism) serve to exclude this organism from this genus. Strains previously called "*C. aquaticum*" also contained DL-2, 4-diaminobutyric acid in the cell wall, as did the grassroot gall isolates. This unusual di-amino acid is also found in the cell walls of *Clavibacter* species. Further analysis of the grassroot gall isolates, reference strains of "*C. aquaticum*," and existing *Clavibacter* species isolates established that these organisms were chemotaxonomically and phylogenetically related. The genus *Leifsonia* was created to accommodate previously described *Clavibacter* species, the isolate from grassroot galls, and "*C. aquaticum*." Therefore, the genus *Leifsonia* is comprised of *L. xyli* subsp. *xyli* and *L. xyli* subsp. *cynodontis* (former *Clavibacter* species), *L. poae* (the isolate from *Poa annua* root galls induced by the nematode *Subanguina radicola*), and *L. aquatica* ("*C. aquaticum*").[1071]

L. aquatica has been described as a rare cause of infections in humans, including

bacteremia, endocarditis, access device infection, meningitis, chronic ambulatory peritoneal dialysis-associated peritonitis, wound infections, and urinary tract infections.[80,168,346,638,772] Two patients with central venous catheter (CVC)-related bacteremias due to *L. aquatica* have been described in immunocompromised men.[878,1067] In Italy, *C. aquaticum* was isolated from blood cultures of 10 patients in a cluster of CVC-related bacteremias in a hemodialysis unit.[251] Colonies of *L. aquatica* are opaque, butyrous, and yellow-pigmented; pigmentation increases with the age of the culture. Some isolates may appear mucoid and, on Gram's stain, the organisms may appear as curved, gram-positive bacilli in tangled masses.[878] The cells are motile at both 35°C to 37°C and at room temperature via long, peritrichous flagella. The organism demonstrates a rod-coccus cycle, so in older cultures, coccoid cells predominate. *L. aquatica* is aerobic, catalase-positive and oxidase-positive (most other corynebacteria are oxidase-negative) (Table 14-13). Acid is produced oxidatively from glucose, sucrose, fructose, arabinose, galactose, and mannose. The organism is urease-negative and esculin is not hydrolyzed. In the past, some *Microbacterium* species recovered from human clinical specimens have been misidentified as "C. *aquaticum*."[390,443] *L. aquatiam* is in the database of the API Coryne, but some biocodes yield cross-calls with *Microbacterium* species (i.e., biocode 247004). In one report, the API Coryne identified this organism as "*Microbacterium/Leifsonia aquatica*, with 98.7% probability."[878] Because of their oxidative metabolism, carbohydrate acidification tests may be negative or uninterpretable after 24 hours' incubation. Hydrolysis of casein and gelatin are helpful for separating possible *Microbacterium s*pecies (gelatin hydrolysis- and casein hydrolysis-positive) from "*C. aquaticum*" (gelatin hydrolysis- and casein hydrolysis-negative). *L. aquatica* also produces strong DNase activity. Other *Leifsonia* species (i.e., *L. cynodontis*, *L. rubra*, *L. aurea*, *L. shinshuensis*, *L. naganoensis*) are environmental

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TABLE 14-12 Phenotypic Characteristics for Identification of *Cellulomonas* Species, *Cellulomonas hominis, Cellulosimicrobium* Species, *Dermabacter hominis* and *Helcobacillus massiliensis*

Characteristic	*Cellulomonas* spp.	*Cellulomonas hominis*	*Cellulomonas denverensis*	*Cellulosimicrobium cellulans*	*Cellulosimicrobium funkei*	*Dermabacter hominis*	*Helcobacillus massiliensis*
CAT	+	+	+	+	+	+	+
HEM SBA	–	–	–	–	–	–	–
NO_3 RED	+	+	+	+	+	–	+
PYZ	NA	NA	NA	+	–	–	+
PAL	V	–	NA	+	NA	+	–
ESC	+	+	+	+	+	+	–
URE	–	–	–	V–	+	–	–
MOT	V+	+	+	V	+	–	–
GEL HYDR	+	–	–	+	+	–	+
DNase	V	+	NA	+	+	+	NA
LAP	+	+	NA	NA	NA	+	NA
PYR	NA	NA	NA	V+	–	+	–
α-GAL	NA	NA	NA	NA	NA	V+	NA
β-GAL	NA	NA	NA	+	+	+	–
α-GLU	+	+	NA	+	+	+	+
β-GLU	NA	NA	NA	NA	+	+	–
β-GUR	NA	–	NA	–	–	–	–
NAGA	NA	NA	NA	V+	+	+	+
Acid from:	Ferm	Ferm	Ferm	Ferm	Ferm	Ferm	
GLU	+	+	+	+	+	+	+
MAL	+	+	+	+	+	+	+

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SUC	+	+	+	+	+	+	+
LAC	NA	+	+	–	+	+	–
MNTL	V	–	–	–	–	–	+
SBTL	V	–	+	NA	–		
XYL	+	+	+	+	+	–	+
RIB	NA	NA	NA	+	NA	+	–
GLYG	NA	NA	NA	V	NA	–	–
Hydrolysis of:							
CAS	NA	NA	–	+	+	NA	NA
XAN	NA	NA	NA	+	–	NA	NA
HYPX	NA	NA	NA	+	+		
TYR	NA	NA	NA	–	–	NA	NA
STA	NA	NA	NA	+	NA	+	–
Common API Coryne Biocodes	NA	NA	NA	3552727, 7552727, 3572727, 7572727	NA	4570365, 4570565, 4570765	3012535

+, positive reaction; −, negative reaction; V, variable reaction; +w, weak or slow positive reaction; V+, variable reaction, most strains positive; V−, variable reaction, most strains negative; V+w, variable reaction, most strains weakly positive; NA, data not available; LIP, lipid requirement; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; GEL, gelatin hydrolysis; HIPP HYDR, Hippurate hydrolysis; MOT, motility; GEL HYDR, Gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; GLU, Glucose; MAL, Maltose; SUC, Sucrose; LAC, Lactose; MNTL; Mannitol; SBTL, Sorbitol; XYL, Xylose; RIB, Ribose; GLYG, Glycogen; CAS, casein; XAN, xanthine; TYR, tyrosine; STA, starch.

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TABLE 14-13 Phenotypic Characteristics for Identification of *Exiguobacterium Species, Leifsonia aquatica, Microbacterium* Species, and *Turicella* Species

Characteristic	*Exiguobacterium acetylicum*	*Exiguobacterium aurantiacum*	*Leifsonia aquatica*	*Microbacterium* spp.	*Microbacterium arborescens*	*Microbacterium binotii*	*Microbacterium imperiale*	*Microbacterium paraoxydans*	*Turicella otitidis*
CAT	+	+	+	+	+	+	+	+	+
HEM SBA	–	–	–	–	–	–	–	–	–
NO_3 RED	V	V	–	V	–	–	–	–	–
PYZ	NA	+	+	V+	NA	NA	NA	NA	–
PAL	NA	V	–	V+	NA	NA	NA	NA	+
ESC	+	+	+	V+	+	+	+	+sl	–
URE	–	–	–	V–	–	–	–	–	–
MOT	+	+	+	V	+	–	+	+	–
GEL HYDR	+	V	–	V	+	–	+	+	–
DNase	+	+	–	NA	NA	NA	NA	+	+
LAP	NA	NA	NA	NA	NA	NA	NA	+	+
PYR	NA	–	–	V	NA	NA	NA	NA	NA
α-GAL	NA	NA	NA	NA	NA	+	NA	NA	–
β-GAL	NA	V	+	+	+	+	+	V	–
α-GLU	NA	+	+	NA	NA	+	NA	+	–
β-GLU	NA	+	NA	+	+	+	+	–	–
β-GUR	NA	–	–	NA	NA	–	NA	–	–
NAGA	NA	V	+	+	+	+	+	V	–
Acid from:	Ferm	Ferm	Oxid	Oxid	Ferm	Oxid	Ferm	Oxid	Asacch
GLU	+	+	+	+	+	+	+	+	–
MAL	+	+	NA	+	+	+	+	+	–

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SUC	+	+	+	V	+	+	+	+	–
MNTL	+	V	NA	V+	+	+	+	+	–
XYL	–	V	NA	V	+	+	+	NA	–
LAC	–	–	NA	V	NA	+	NA	NA	–
ARAB	–	NA	+	–	+	NA	+	NA	–
RAFF	–	NA	NA	–	–	NA	+	NA	–
Hydrolysis of:									
CAS	+	+	–	+	NA	–	NA	+	NA
XAN	NA	NA	–	NA	NA	NA	NA	NA	NA
TYR	NA	–	–	NA	NA	NA	NA	–	NA
STA	+	NA	+	NA	NA	+	NA	NA	NA
Common API Coryne Biocodes	NA	NA	2470004	2550004, 2570004	NA	NA	NA	NA	2100004

+, positive reaction; −, negative reaction; V, variable reaction; +w, weak or slow positive reaction; V+, variable reaction, most strains positive; V−, variable reaction, most strains negative; V+w, variable reaction, most strains weakly positive; NA, data not available; LIP, lipid requirement; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; GEL, gelatin hydrolysis; HIPP HYDR, Hippurate hydrolysis; MOT, motility; GEL HYDR, Gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; Ferm, fermentative acid production; Oxid, oxidative acid production; Asacch, asaccharolytic; GLU, Glucose; MAL, Maltose; SUC, Sucrose; LAC, Lactose; MNTL; Mannitol; XYL, Xylose; RAFF, Raffinose; CAS, casein; XAN, xanthine; TYR, tyrosine; STA, starch; ARAB, arabinose.

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isolates and have not been isolated from human clinical specimens.[904,1071] The blood culture isolate described by Porte et al.[878] was tested on the MALDI-TOF mass spectrometry instrument (Bruker Daltronics, Bremen, Germany) and gave an identification of *Leifsonia,* genus level only; retesting provided no identification. However, at the time of testing, the Bruker database contained only the type strain of

L. aquatica. Isolates of *L. aquatica* tend to have intermediate susceptibility to vancomycin.[389] Historically, this organism has had vancomycin MIC_{90}s of 8 μg/mL, and recent isolates have had MICs of 4 μg/mL.[878,1067] Resistance to penicillin and other β-lactams is also common.

Microbacterium *Species*

Microbacterium species are small, irregularly-shaped, gram-positive, catalase-positive obligately aerobic bacilli that grow with typical "diphtheroid" morphology. They may be nonmotile or motile via one to three flagella and are oxidative and aerobic in their metabolism, although some species may be fermentative. Unlike *Cellulomonas* species, *Microbacterium* do not hydrolyze cellulose. Instead of *meso*-DAP, these organisms contain D-ornithine or lysine as the di-amino acid in their cell walls and have "glycine-glycine-lysine" or "glycine-lysine" as the interpeptide cross-linkage in the cell wall peptidoglycan. In addition to D-ornithine and glycine, the cell wall also contains alanine, glutamic acid, and homoserine. Unlike *Corynebacterium* species, arabinose, galactose, and mycolic acids are not components of the cell wall. In 1998, Takeuchi and Hitano analyzed extant *Microbacterium* and *Aureobacterium* species using 16S rRNA sequencing and found that the species in these two genera formed a monophyletic intermixed association.[980] This and additional physiological and chemotaxonomic features resulted in the unification of these genera in a re-defined genus *Microbacterium.*[1083] All of the *Microbacterium* species (and former *Aureobacterium* species) are widely distributed in the environment (i.e., food, plants, soil, sewage, insects).

Microbacterium species have been isolated from human clinical specimens and some former CDC coryneform group A-4 and group A-5 isolates have been assigned to the genus *Microbacterium* based on phenotypic, chemotaxonomic, and genotypic analyses. Funke and colleagues[364] examined 22 strains of yellow- and orange-pigmented, gram-positive bacilli by a variety of chemotaxonomic and phenotypic techniques. Strains included *Microbacterium* type strains, environmental isolates, and 13 (10 from blood) All were *Microbacterium* species by cell wall analysis; three clinical isolates were phenotypically identical to *M. imperiale* and two others were identical to the *M. arborescens* type strain. The remaining clinical isolates did not phenotypically resemble any other recognized *Microbacterium* species, although they were *Microbacterium* species based on chemotaxonomic analysis. In 2010, an isolate with 96% rDNA sequence homology with *M. arborescens* was isolated from the peritoneal fluid of a 57-year-old woman with end-stage renal disease on continuously cycling peritoneal dialysis.[7] The isolate was resistant to clindamycin and erythromycin, and susceptible to β-lactams, ciprofloxacin, linezolid, rifampin, and vancomycin.

Microbacterium species have been associated with severe human infections,

including a fatal disseminated infection in a 75-year-old man and persistent bacteremia from cellulitis in a 39-year-old man with acute myelogenous leukemia and porphyria cutanea tarda.[806,989] In the latter instance, the isolate was vancomycin-resistant. These organisms have also been associated with nosocomial and catheter-related bacteremias in patients with leukemia, and with contamination of peripheral blood stem cell products infused into transplant recipients.[8,27,154,501,641] Laffineur et al.[624] described a yellow-pigmented coryneform isolate that was recovered from blood cultures of a child with leukemia. Genetic and chemotaxonomic comparisons with existing *Microbacterium* species resulted in the characterization of *Microbacterium paraoxydans*, another new *Microbacterium* species. *M. paraoxydans* has also been associated with central venous catheter-related bacteremia.[312] Funke and colleagues[368] in Switzerland and Germany reported a patient with endophthalmitis due to a *Microbacterium* species following a penetrating eye injury. Phylogenetically, the organism clustered with recognized *Microbacterium* species, suggesting that the isolate may actually represent yet another new *Microbacterium* species.[368] The isolate was susceptible to cefazolin, cefotetan, ciprofloxacin, clindamycin, imipenem, piperacillin, teicoplanin, and vancomycin and was resistant to gentamicin and tobramycin. In 2006, a novel *Microbacterium* species was isolated from the sputum of a heart transplant recipient and was thought to be the cause of insterstitial pulmonary inflammation and pleural effusion. This isolate showed more than 98% 16S rRNA sequence similarity with eight other *Microbacterium* species, including *M. oxydans* and *M. paraoxydans*.[406] Two novel gram-positive rods recovered from blood cultures in 1976, 1977, and 2007 were analyzed by genotypic and chemotaxonomic approaches and were determined to represent two new *Microbacterium* species; the 1976/1977 strains were named *Microbacterium binotii*, while the 2007 isolate was named *Microbacterium pyrexiae*.[213,610] In 2008, Gneiding, Frodl, and Funke characterized 50 *Microbacterium* species that had been recovered from a variety human clinical specimens over the previous 5-year period using molecular genetic and phenotypic methods.[411] In their study, the 50 isolates represented 18 different species, with 3 species (*M. oxydans*, *M. paraoxydans*, and *M. foliorum*) representing more than half of the isolates. Four isolates belonged to species never recovered from humans before, and two isolates represented undescribed species.

Microbacterium species form whitish-yellow, yellow- or orange-pigmented colonies on SBA (Image Plate 14-7E). All species are catalase-positive, and some species (*M. arborescens*, *M. imperiale*, *M paraoxydans*) are motile. Most species hydrolyze esculin and do not hydrolyze urea. Acid is produced oxidatively or fermentatively from a variety of carbohydrates. Original members of genus *Microbacterium* tend to be fermentative, while former *Aureobacterium* species are more oxidative in their utilization of carbohydrates. *Microbacterium* species are included in the API Coryne

database, but additional tests are required to separate them from *Cellulomonas* species and *L. aquatica.* Acidification of carbohydrates may not be apparent after 24 hours incubation. API Coryne biocodes (0470004, 0452004, 0472004, 0570004, 0572004) included in the database usually give cross-calls

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with "*L. aquaticum*" (Image Plate 14-7F). Incubation beyond 24 hours may be necessary to determine acidification of carbohydrates. Additional phenotypic data is found in Table 14-13. *Microbacterium* species may vary in their susceptibility to various antimicrobial agents. Among the 50 isolates examined by Gneiding and coworkers,[411] 100% were susceptible to meropenem and linezolid, 98% were susceptible to vancomycin and doxycyline found, and 56% were susceptible to ciprofloxacin. For the β-lactams, 78% were susceptible to penicillin and 72% were susceptible to cefotaxime. Individual clinical isolates from case reports have generally been pan-susceptible, although resistance to aminoglycosides, macrolides, and erythromycin has been encountered in several clinical isolates.[7,312,368,406]

Turicella *Species*

In 1993, Simonet and colleagues[1020] in France reported the recovery of a coryneform isolate in pure culture from middle ear fluids collected by tympanocentesis from children with acute otitis media. These isolates contained *meso*-DAP, arabinose, and galactose in their cell walls, but corynemycolic acids were absent. The isolate grew as convex, whitish, creamy, nonhemolytic colonies on blood agar that became yellowish with age. This colonial morphology was distinctly different from the flat, gray-white, nonhemolytic colonies produced by *C. afermentans* subspecies or the convex dry, adherent, slightly yellowish colonies of *C. auris*, coryneforms that have both been associated with acute and chronic otitis media.[373,931] On chemotaxonomic analysis, the cellular menaquinone patterns of this middle ear isolate were also different from the two *Corynebacterium* species. Even though these organisms displayed markedly different colony morphologies, the two *Corynebacterium* species and the third isolate had identical phenotypic characteristics. These middle ear isolates have now been assigned to the new genus *Turicella*, (referring to Turicum, the Latin name for Zurich, Switzerland, where the first isolates were collected) as *Turicella otitidis*.[385] Since its description, *T. otitidis* has been recovered from other head and neck infections (e.g., posterior auricular abscess) and from blood cultures as well.[252,687,921]

On Gram's stains, *T. otitidis* appear as long rods, and in culture, colonies of the organism are white-gray, are convex, and have entire edges. Colonies on SBA are 1.5 to 2 mm in diameter after 48 hours' incubation. *T. otitidis* does not have a lipid

requirement for growth. All *T. otitidis* isolates produce the biocode number 2100004 on the API Coryne system, indicating positive test results for pyrazinamidase, alkaline phosphatase, and catalase, and negative tests for glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, and glycogen fermentation, nitrate reductase, urease, esculin hydrolysis, β-glucuronidase, β-galactosidase, α-glucosidase, esculin hydrolysis, urease, and gelatin hydrolysis (Table 14-13). These phenotypic characteristics are similar to those of both *C. afermentans* subsp. *afermentans* and *C. auris*. *T. otitidis*, *C. afermentans* subsp. *afermentans* and *C. auris* can be differentiated most efficiently by genetic and chemotaxonomic analyses. However, Renaud et al.[913] have shown that colony morphology, the CAMP test, certain enzymatic activities (e.g., LAP, DNase), and carbon substrate assimilations are useful for separating these species. Colonies of *T. otitidis* are creamy and pale yellow, while colonies of *C. auris* and *C. afermentans* are slightly yellowish and adherent (*C. auris*) or flat, smooth, and grayish white (*C. afermentans*). *T. otitidis* is DNase-positive, while *C. afermentans* subsp. *afermentans* and *C. auris* are DNase-negative. Both *T. otitidis* and *C. auris* are LAP-positive, while *C. afermentans* is LAP-negative. Lastly, *T. otitidis* and *C. auris* are CAMP test-positive, while this characteristic is variable for *C. afermentans*. Isolates of *T. otitidis* are susceptible to ampicillin, cephalosporins, ciprofloxacin, gentamicin, rifampin, tetracyclines, vancomycin, and linezolid; some strains are resistant to clindamycin, erythromycin, and azithromycin.[380,418,934]

Rothia *Species*

Rothia species are members of the phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, order *Actinomycetale*s, suborder *Micrococcineae,* in the family *Micrococcaceae.* This family also includes the *Micrococcus*, *Arthrobacter*, *Kocuria*,*Stomatococcus*, and *Nesterenkonia* genera. Prior to 2000, the genus *Rothia* included a single species, *Rothia dentocariosa*. Based on 16S rDNA sequencing and chemotaxonomic data, *Stomatococcus mucilaginosus*, the catalase-variable gram-positive coccus found in the human oropharynx, was transferred to genus *Rothia* as *Rothia mucilaginosa*.[92,225] A third species in the genus called *Rothia nasimurium* was described at the same time and is found in the anterior nares of mice. In 2004, a novel gram-positive coccobacillary organism was isolated from air and condensation water samples from the Russian space station Mir and this organism was named *Rothia aeria*.[673] Two additional environmental species were characterized in 2002 and 2008, respectively.*Rothia amarae* was isolated from sewer sludge in China, and *Rothia terrae* was isolated from soil sample from Taiwan.[202,319] While *R. mucilaginosa, R. nasimurium, R. aeria* and *R. terrae* are gram-positive cocci, *R. dentocariosa* is a gram-positive coryneform bacillus that shows rudimentary branching, although, in older broth cultures, coccoid cells may predominate. *R. dentocariosa* does not

produce major amounts of succinic acid from glucose, a characteristic that distinguishes this organism from facultative *Actinomyces* species. Like the corynebacteria and aerobic actinomycetes, these organisms are catalase-positive and tend to grow better under aerobic conditions. However, unlike *Nocardia* species, these organisms are fermentative, which facilitates identification by phenotypic tests routinely used for *Corynebacterium* species and the coryneform groups described above.

Rothia dentocariosa is part of the normal human oropharyngeal and gingival flora and may be isolated from saliva and supragingival plaque. During the 1950s and 1960s, the Special Bacteriology Branch at the CDC collected several isolates, most of which were from abscesses, respiratory tract specimens, pilonidal cysts, cerebrospinal fluids, urine, and blood. *R. dentocariosa* has most frequently been associated with endocarditis involving both native and prosthetic valves.[111,131,615,1002] In several cases, this infection was associated with severe, life-threatening complications, including the multi-valve infections and development of aortic root and paravalvular abscesses.[340,615,1066] Valvular infections may result in the formation of large vegetations, which may lead to the development of pulmonary infarcts, intracranial

hemorrhages, and brain abscesses.[538,922,961] Bacteremia without endocarditis has also been reported in adults, children, and neonates.[965,1010,1223] Shin and colleagues[1010] reported a neonate with meconium aspiration syndrome who developed bacteremia with this organism in 2004; then, in 2007 this same species was isolated from a fetal blood culture that also involved meconium aspiration. Immunocompromised children and adults on cytotoxic chemotherapy are at increased risk for sepsis and bacteremia with this organism, particularly if there is mucositis or poor dentition.[1196] Septic arthritis due to *R. dentocariosa* was diagnosed in a patient with rheumatoid arthritis who was on etanercept, which is known to place patients at increased risk of infection.[324] Other case reports have documented *R. dentocariosa* as a cause of endophthalmitis, corneal ulcer, vertebral osteomyelitis with paraspinal abscess, and peritonitis associated with chronic ambulatory peritoneal dialysis.[580,702,776,778] Although rare, *R. dentocariosa* has also been recognized as a cause of pneumonia in patients with severe underlying diseases, including acute myelocytic leukemia and adenocarcinoma of the lung.[984,1174]

R mucilaginosa and *R. aeria* are also rare causes of infection. Surprisingly, *R. mucilaginosa* has been isolated from several cases of meningitis in patients with ALL, AML, multiple myeloma, and non-Hodgkin's lymphoma.[650] *R. mucilaginosa* was isolated as the cause of meningitis in two pediatric stem cell transplant patients and as

a cause of bacteremia associated with infectious granulomatous dermatitis in a man with acute myeloid leukemia.[650,774] Bacteremia with this organism was also documented in a child with Shwachman–Diamond syndrome, a rare autosomal-recessive multisystem disorder that causes bone marrow dysfunction, immune deficiency, and neutrophil deficiencies that lead to recurrent infections.[1134]Septic arthritis and late prosthetic hip arthroplasty infection due to *R. mucilaginosa* have been reported in two elderly patients with histories of diabetes mellitus and arterial hypertension.[566,751] This organism has also been isolated from peritoneal fluid of a patient on CAPD.[504] *R. mucilaginosa* are usually susceptible to most antimicrobial agents including vancomycin, rifampin, and linezolid, although occasional isolates may be resistant to penicillin or clindamycin. The first isolation of *R. aeria* from a human was from a blood culture of a neonate delivered to a mother who had a tooth extraction without antimicrobial prophylaxis 4 days prior to delivery.[770] *R. aeria* was subsequently isolated from a sputum specimen obtained from a diabetic patient with acute bronchitis who was being treated with etanercept for rheumatoid arthritis and from the joint fluid of an elderly female with abscessed teeth who was also being treated with methotrexate and prednisone for rheumatoid arthritis.[752,1156] All three isolates were susceptible to all agents tested including vancomycin and linezolid, except one isolate was resistant to ofloxacin and a second isolate was clindamycin-resistant.

CDC fermentative coryneform group 4 organisms are closely related to *R. dentocariosa*. These organisms have been recovered mostly from female genitourinary tract specimens, although no specific human infections with CDC group 4 isolates have been reported. Daneshvar and colleagues[253] at the CDC investigated several black colony-forming isolates that were provisionally categorized as group 4 bacteria. These organism segregated into two groups by molecular genetic analysis. One group (including only one genital tract isolate) was most closely related to *R. dentocariosa*, while the other group, which included isolates that were mainly recovered from female genital tract specimens, were most closely related to *C. aurimucosum*, a more recently described *Corynebacterium* species isolated predominantly from the female genital tract (Box 14-4). This work resulted in emendations of the species descriptions of both *R. dentocariosa* and *C. aurimucosum* to include variants that formed charcoal-black colonies on agar media. Consequently, the colony morphologies of *R. dentocariosa* include bone-white, off-white, or charcoal-black colonies that are dry, rough, and adherent, or smooth and entire. *C. aurimucosum* colonies are sticky, white-to-yellow, or charcoal black Table 14-14 presents the phenotypic characteristics of *R. dentocariosa* and *C. aurimucosum* for comparison to aid in the identification of coryneforms that produce charcoal-black pigmented colonies. "*C. nigricans*" is identical to *C. aurimucosum* and is a later synonym for the same organism.[253]

The similarities of *R. dentocariosa* with other aerobic actinomycetes are apparent upon examination of colony morphology. After 72 hours incubation at 35°C to 37°C in CO_2, colonies are usually dry, rough, and bone-white (Image Plate 14-7A). Rare isolates may be black-pigmented and may correspond to pigmented *R. dentocariosa* or *C. aurimucosum*. Pigmented *R. dentocariosa* strains are more likely to be isolated from blood cultures or the respiratory tract, and not the female genital tract, while *C. aurimucosum* is isolated from the latter site. The colonies often appear heaped and cerebriform, particularly in areas of confluent growth. Inspection of the edges of colonies reveals that the colonies tend to "dig into" the agar surface like those of *Nocardia* species. Strains of *R. dentocariosa* also have relatively uniform phenotypic characteristics and are quite biochemically active. *R. dentocariosa* is catalase-positive (although catalase-negative strains may be encountered), reduces nitrate to nitrite and produces pyrazinamidase and pyrrolidonyl arylamidase (Table 14-14). The organism hydrolyzes esculin but does not hydrolyze urea or gelatin, and acid is produced fermentatively from glucose, maltose and sucrose. *R. dentocariosa* is included in the data bases of both the API Coryne and the RapID CB-Plus. On the API Coryne, most strains produce the biocodes 7050125, 7052125, 7050165, or 7052165 (Image Plate 14-7B). While these organisms are usually uniformly susceptible to all classes of antimicrobial agents, strains that are resistant to β-lactams because of β-lactamase production have been isolated.[253] The latter strains contain plasmids bearing the β-lactamase structural genes.

Gardnerella vaginalis

Taxonomy and Cellular Morphology

Gardnerella vaginalis was first described in 1953 and has been known by various names, including *Haemophilus vaginalis* and *Corynebacterium vaginale*. In 1980, this organism was formally placed in the new genus *Gardnerella* by Greenwood, Pickett, and others based on electron microscopic, biochemical and chemotaxonomic data, and DNA–DNA

TABLE 14-14 Phenotypic Characteristics for the Identification of *Rothia* Species

Characteristic	*Rothia aeria*	*Rothia dentocariosa*	*Corynebacterium aurimucosum*	*Rothia mucilaginosa*
CAT	V	V+	+	V
HEM SBA	–	–	–	–
NO_3 RED	+	+	–	+
PYZ	+	+	+	+
PAL	–	V	+	–
ESC	+	+	V	+
URE	–	–	V–	–
MOT	–	–	–	–
GEL HYDR	–	V	V–	+
DNase	NA	NA	NA	+
LAP	NA	+	+	NA
PYR	NA	+	–	NA
α-GAL	NA	–	–	NA
β-GAL	–	–	–	–
α-GLU	+	+	–	+
β-GLU	+	+	–	+
β-GUR	–	–	–	–
NAGA	–	–	–	–
Acid from:	Ferm	Ferm	Ferm	Ferm
GLU	+	+	+	+
MAL	+	+	+	+
SUC	+	+	+	+
MNTL	–	–	V	–
XYL	–	–	–	–
LAC	–	V	–	–
RIB	–	–	–	–
GLYG	–	–	–	–
ARAB	NA	V	–	–
RAFF	NA	–	NA	–
Hydrolysis of:				
CAS	NA	–	NA	+
XAN	NA	NA	NA	NA
TYR	NA	NA	NA	NA
STA	NA	–	–	+
Common API Coryne Biocodes	7050125	7050165, 7050125, 7052165, 7052125	NA	NA

+, positive reaction; −, negative reaction; V, variable reaction; +w,weak or slow positive reaction; V+, variable reaction, most strains positive; V−, variable reaction, most strains −; V+w, variable reaction, most strains weakly positive; NA, data not available; LIP, lipid requirement; HEM SBA, hemolysis on sheep blood agar; NO_3, Reduction of nitrate; PYZ, pyrazinamidase; PAL, alkaline phosphatase; ESC, esculin hydrolysis; URE, urease; GEL HYDR, gelatin hydrolysis; HIPP HYDR, Hippurate hydrolysis; MOT, motility; GEL HYDR, Gelatin hydrolysis; LAP, leucine aminopeptidase; PYR, pyrrolidonyl arylamidase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GLU, β-glucosidase; β-GUR, β-glucuronidase; NAGA, *N*-acetyl-β-D-glucosaminidase; GLU, Glucose; MAL, Maltose; SUC, Sucrose; LAC, Lactose; MNTL; Mannitol; XYL, Xylose; RAFF, Raffinose; RIB, Ribose; GLYG, Glycogen; CAS, casein; XAN, xanthine; TYR, tyrosine; STA, starch; ARAB, arabinose.

hybridization studies.[435] Ultrastructural studies of *G. vaginalis* indicate that this organism has a gram-positive type of cell wall, but the peptidoglycan layer is much thinner than that found in the cell walls of *Corynebacterium*, *Lactobacillus*, or *Staphylococcus* species. The peptidoglycan content of the *G. vaginalis* cell wall constitutes about 20% of the total cell wall weight, which is similar to that found in *Enterobacteriaceae,* such as *Escherichia coli*, where the peptidoglycan constitutes about 23% of the cell wall weight. Consequently, different strains of *G. vaginalis* may appear predominantly gram-positive, gram-negative, or gram-variable. Cell wall extracts of *G. vaginalis* do not contain compounds normally present in gram-negative cell wall lipopolysaccharides (e.g., *meso*-DAP, 2-keto-3-deoxy-D-manno-2-octonoic acid, hydroxy fatty acids). The absence of *meso*-DAP, arabinogalactans, and mycolic acids confirms that the *G. vaginalis* cell wall is distinct from the gram-positive cell wall type found in *Corynebacterium* species.

Molecular studies on *G. vaginalis* have shown that this organism is closely related to the bifidobacteria. Miyake and colleagues[763] used 16s rRNA gene sequence analysis to examine the type strains of 21 *Bifidobacterium* species and showed that *G. vaginalis* clustered with 19 of the 21 *Bifidobacterium* species. Another study used phenotypic, chemotaxonomic (e.g., whole-cell protein and fatty acid analysis), and 16S rRNA sequence analysis to characterize *G. vaginalis* and "*G. vaginalis*-like" strains.[1146]Chemotaxonomic methods resulted in recognition of two "clusters" that were shown to represent different genera by sequencing, and these different genera could be distinguished by phenotypic methods. Cluster I strains represented *G. vaginalis*, with its closest relative being members of the genus *Bifidobacterium*. *G. vaginalis* strains demonstrated a level of similarity of 93.1% to *Bifidobacterium bifidum*.[1146] These investigators felt that the G+C contents of *G. vaginalis* and *Bifidobacterium* species were different enough that *G. vaginalis* could not be considered a member of the *Bifidobacterium*. Analysis of heat-shock protein gene sequences also suggested a phylogenetic relationship between *G. vaginalis* and bifidobacteria, but this relationship was more distant than suggested by 16S rRNA analysis and confirmed the genus status of *Gardnerella.*[547] Amplified ribosomal DNA restriction analysis has shown that *G. vaginalis* strains are fairly heterogeneous and may be divided into at least three or four genotypes depending upon the restriction enzymes used.[537] In culture, it may be difficult to differentiate some *G. vaginalis* strains from "look-alike" organisms, which are termed "unclassified, catalase-negative coryneform" bacteria. *G. vaginalis* is the only member of the genus *Gardnerella*, which is classified in the phylum *Actinobacteria*, class *Actinobacteria*, order *Bifidobacteriales*, in the family *Bifidobacteriaceae*.

Clinical Significance of Gardnerella vaginalis

G. vaginalis is a member of the normal vaginal microbiota. In the past, this organism was closely associated with the clinical syndrome called **bacterial vaginosis (BV)**. This condition is termed BV because no single organism is solely responsible for the condition and inflammatory cells (seen with both *Candida* and *Trichomonas* vaginal infections) are not observed on Gram-stained smears of the vaginal discharge. BV is characterized clinically by a malodorous vaginal discharge associated with a significant overgrowth in the numbers of *G. vaginalis* and various obligate anaerobes, including *Prevotella bivia*, *Prevotella disiens*, *Mycoplasma* species, peptostreptococci, and *Mobiluncus* species, with a concomitant decrease in the numbers of normal vaginal lactobacilli.[292] Several case-control and cohort-based studies have established that BV has a significant impact on adverse pregnancy outcomes and is a risk factor for preterm birth.[840] BV is also a risk factor for obstetrical infections and pelvic inflammatory disease. When this condition was originally described, *G. vaginalis* was hypothesized to be the etiologic agent.[398] Subsequent studies determined that other organisms were involved, and that, while *G. vaginalis* was invariably present in the vaginas of women with BV, it was also present in greater than 50% of women without bacterial vaginosis.[753] Using semiselective media, *G. vaginalis* may be found in from 14% to 70% of healthy women without BV.[1113] It appears, however, that the presence of large numbers of *G. vaginalis* in the vagina are indicative of BV. Using a radiolabelled oligonucleotide probe specific for *G. vaginalis* 16S rRNA, Sheiness and coworkers[1006] in Seattle showed that the presence of these bacteria at concentrations of $\geq 2 \times 10^7$cfu/mL of vaginal fluid, along with a vaginal pH greater than 4.5, had a sensitivity and specificity of 95% and 99%, respectively, in classifying women with and without BV when compared with strictly clinical diagnostic criteria. *G. vaginalis* may also be found in the male urethra. Using molecular methods, Schwebke and coworkers[994] found an overall prevalence of *G. vaginalis* of 25% in males and found no difference in the prevalence rates between male sexual partners of women with or without BV.

Diagnosis of BV is accomplished with clinical criteria along with either a wet mount or a Gram's stain of the vaginal discharge ("Diagnosis of Bacterial Vaginosis and Culture Characteristics of *G. vaginalis*" below). Routine culture of vaginal specimens for *G. vaginalis* for diagnosis of BV is not recommended since culture does not provide decisive evidence of infection due to the presence of *G. vaginalis* as part of the resident vaginal microbiota. *G. vaginalis* has been isolated from rectal specimens of 56% of 148 women with BV, 12% of 69 healthy women, 9% of 83 male sexual partners of women with BV, and 6% of 49 male sexual partners of healthy women.[513] These latter data suggest that *G. vaginalis* is not sexually transmitted, but probably

colonizes the vagina endogenously from the intestinal tract. Villegas and colleagues[1160] performed an ultrastructural study of the vaginal secretions from 10 women with BV and semen samples from their asymptomatic sexual partners using optical and electron microscopy. *G. vaginalis* was isolated from semen specimens of 50% of the men. Electron microscopy showed that the organisms adhered to the cell membranes and penetrated into the cytoplasm of both vaginal and male urethral epithelial cells. Asymptomatic colonization of the male lower genital tract by *G. vaginalis* may have some relevance regarding the role of the male partner in "recolonization" ("reinfection") of the female vagina.

G. vaginalis has also been isolated from female genitourinary tract infections associated with complications of pregnancy and from infants born to mothers with such

complications, particularly during and after delivery. These may include intrauterine infections and intra-amniotic infections, chorioamnionitis, postabortal pelvic inflammatory disease, and postpartum endometritis after cesarean delivery.[500,655] Surgically obtained tissues from these infections have yielded *G. vaginalis* in pure or mixed culture with other facultative and/or obligately anaerobic organisms. Postpartum and postabortal bacteremia with *G. vaginalis* and other genital tract microorganisms may also occur in these clinical settings.[123,906] Bacteremia with genital tract microflora occurs when organisms gain access to venous channels of the placental bed, which is disrupted before and during vaginal/cesarean deliveries. Systemic and localized *G. vaginalis* neonatal infections may occur in those involved with such complications and may include amnionitis, episiotomy wound infection, bacteremias, meningitis, cellulitis, conjunctivitis, and osteomyelitis (often involving fetal scalp monitors).[30,90,802] Oropharyngeal cultures, gastric aspirates, and tracheal suction specimens from neonates have also yielded *G. vaginalis*, presumably acquired during passage through a heavily colonized birth canal. *G. vaginalis* has been isolated from Bartholin's gland abscesses, postcesarean section and postsurgical wound infections, abdominal surgeries, hysterectomies, and episiotomies.[203,619]

G. vaginalis has also been a cause of rare infections in males and occasionally other infections not generally associated with the genitourinary tract. Bacteremia with *G. vaginalis* in men has been reported following transurethral prostatectomy, urogenital surgical procedures, and in association with renal calculi, and urinary retention secondary to obstruction.[74,267,625] Extragenital infections with *G. vaginalis* have included a pyogenic postcesarean liver abscess in a 23-year-old woman, bacteremia with lung abscess/empyema in an alcoholic man with aspiration pneumonia, and

vertebral disk and joint space infections and septic hip arthritis in a woman who was a renal transplant recipient.[316,431,503,660,1030] Yoon et al.[1232] reported a case of *G. vaginalis* bacteremia complicated by endocarditis and pyelonephritis is a previously healthy 39-year-old man, and Calvert and colleagues[152] reported a empyema and perinephric abscess in 50-year-old man.

G. vaginalis may also play a role in urinary tract infections in both men and women.[559,785] Given its normal ecosystem as part of the normal vaginal microbiota, it is not surprising that *G. vaginalis* has been recovered from the urinary tract specimens from women more frequently than from men. *G. vaginalis* has been isolated from both the upper (i.e., ureters, renal pelvis, and calyx) and the lower urinary tracts (i.e., bladder) of both symptomatic and asymptomatic patients. A treatment study performed in Mexico with 45 women with *G. vaginalis* UTI most frequently had symptoms of dysuria and frequency, lower back pain, suprapubic discomfort, and abdominal pain.[854] Isolation rates from urine specimens are greater among pregnant women than among nonpregnant women, and many of these patients are asymptomatic. Pyuria appears to be infrequently associated with *G. vaginalis* urinary tract infection and may be absent even in the presence of serious upper urinary tract disease. In a study of the role of *G. vaginalis* in urinary tract infections in men, two-thirds of 15 men with significant numbers of *G. vaginalis* in the urine had signs and symptoms referable to the urinary tract (i.e., urgency, frequency, hematuria).[1039] Most of these patients also had underlying diseases, including diabetes, hypertension, spinal cord injuries, prior UTIs, and cardiovascular disease. As in previous studies of *G. vaginalis* and the urinary tract, pyuria was present only in 7 of the 15 males evaluated. Significant numbers (i.e., $>10^4$ cfu/mL) of *G. vaginalis* in urine specimen from men are difficult to evaluate because such numbers may be seen in cultures from asymptomatic males and from men with underlying renal diseases such as ureteral obstruction, chronic prostatitis, and end-stage renal disease.[559] Rarely, *G. vaginalis* may cause complicated ascending urinary tract infection in healthy males, although such scenarios are more likely in compromised hosts, such as renal transplant recipients.[344] The consensus is that *G. vaginalis* may play an important role in complicated and uncomplicated urinary tract infections in both men and women and, if present in significant numbers, the organism should be identified and reported.

Diagnosis of BV and Cultural Characteristics of Gardnerella vaginalis

Diagnosis of BV usually involves a patient with a malodorous vaginal discharge; there may be with minimal irritation, particularly after sexual contact. Exposure to the alkaline pH of vaginal secretions after sexual contact and during the menses is responsible for volatization of amines that contribute to the "fishy" odor often noted by patients. This is also the basis for the whiff test, where KOH is mixed with the discharge to reproduce the fishy odor. The vaginal discharge of BV is characteristically homogeneous, white-gray, and may be frothy. Besides the clinical complaint and presentation, the vaginal pH will usually be >4.6, with the specificity of diagnosis being even greater when the vaginal pH is ≥5. Microscopically, wet mounts will usually show moderate-to-many vaginal epithelial cells with large numbers of adherent bacteria of various morphologies (the "clue" cell); in many cases, the sheer numbers of adherent microorganisms may completely obscure the margins of the epithelial cells (Image Plate 14-8B and C). Many labs use the Gram's stain criteria published by Nugent and colleagues to evaluate relative amounts of lactobacilli, *Gardnerella* morphotypes, and *Mobiluncus* morphotypes to arrive at a score that correlates with the absence, possible presence, or diagnosis of BV. In these smears, vaginal epithelial "clue" cells will be covered with the bacterial morphotypes described above, with the benefit of distinguishing their Gram reactions and morphologies, and having a permanent record for review and training/teaching purposes.

As BV is known to have a polymicrobial etiology and because *G. vaginalis* can also be isolated from vaginal cultures of more than 50% of asymptomatic women, culture of vaginal specimens for *G. vaginalis* should be discouraged. Routine use of semiselective media such as Human Blood Tween (HBT) and V agars for culture of vaginal specimens represents an unnecessary expense that contributes little useful information, given that the laboratory diagnosis of BV rests on careful examination and interpretation of properly collected wet preps or Gram-stained smears of vaginal discharge.[499,810] *G. vaginalis* can be recovered on regular CNA agar medium after prolonged incubation, and presumptive identification will usually suffice in these circumstances.

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G. vaginalis can be isolated from clinical specimens using routine SBA, CNA, and chocolate agar media (Image Plate 14-8D). Semiselective media include HBT agar or V agar.[1113] HBT agar has a bottom layer of Columbia colistin–nalidixic acid agar base supplemented with Proteose Peptone no. 3 (BD Microbiology Systems, Sparks, MD),

amphotericin B (2.0 µg/mL), and 0.0075% Tween 80, overlaid with a similar layer to which 5% human blood is added. Tween 80 improves the growth and enhances β-hemolysis produced by *G. vaginalis*. V agar contains Columbia agar base with 1% proteose peptone and 5% human blood. On HBT agar, *G. vaginalis* forms small, clear zones of β-hemolytic colonies surrounding colonies with diffuse edges after 48 hours incubation (Image Plate 14-8E). For "routine media", *G. vaginalis* grows better on Columbia agar-based media (i.e., CNA agar) than on blood agar made with a tryptic soy base. On CNA, *G. vaginalis* shows a subtle, "diffuse" hemolysis surrounding the colonies that is noted initially in confluent areas of growth or after incubation for >72 hours. Growth is best at 35°C to 37°C in a 5% to 7% CO_2 atmosphere. Most isolates are recovered after 48 to 72 hours' incubation. *G. vaginalis* will also grow in most blood culture media; however, the anticoagulant additive sodium polyanethol sulfonate (SPS) is inhibitory to some *G. vaginalis* and may compromise the recovery of this organism from blood cultures.[907] As with the pathogenic *Neisseria*, this growth inhibition by SPS may be overcome by adding gelatin (1% v/v) to blood culture bottles.

Suspect *G. vaginalis* from systemic body sites (e.g., blood, joint fluid, etc.) should be confirmed by additional testing. Presumptive identification of *G. vaginalis* includes typical cellular morphology on Gram-stained smears (small gram-positive, gram-negative, or gram-variable coccobacilli), characteristic growth on CNA agar with "diffuse" weak β-hemolysis, and negative oxidase and catalase tests. Definitive identification of *G. vaginalis* can be made based on the reactions shown in Table 14-15 Other characteristics that confirm the identification of *G. vaginalis* and help to separate it from the other so-called "unclassified, catalase-negative coryneform" (UCNC) organisms include the presence of α-glucosidase, the absence of β-glucosidase, and positive starch and hippurate hydrolysis reactions. Carbohydrate utilization tests are performed in medium containing Proteose Peptone no. 3, phenol red indicator, and 1% filter-sterilized carbohydrate. Acid is produced from glucose, maltose, sucrose, and starch, but not from mannitol, sorbitol, raffinose, rhamnose, or salicin (Image Plate 14-8F). The organism hydrolyzes hippurate, but does not possess lysine or ornithine decarboxylases or arginine dihydrolase, does not reduce nitrate, and does not produce indole, urease, or acetoin. *G. vaginalis* strains will show zones of inhibition around disks containing metronidazole (50 µg) and trimethoprim (5 µg). *G. vaginalis* is included in the database of the API Coryne system (bioMérieux, Inc., Hazelwood, MO) for the identification of gram-positive bacilli and in the databases of the *Haemophilus-Neisseria* Identification (HNID) Panel (Siemens-American MicroScan, West Sacramento, CA), the API Coryne (bioMérieux, Inc.), RapID CB-Plus (Remel), and the Vitek 2 *Neisseria-Haemophilus* (NH) identification card (bioMérieux, Inc.) for identification of fastidious gram-negative bacilli.

TABLE 14-15 Biochemical Characteristics for the Identification of *Gardnerella vaginalis*

Characteristic	Reaction
Hemolysis on human blood bilayer Tween (HBT) agar	β
Oxidase	−
Catalase	−
Hippurate hydrolysis	+
Acid production from:	
Glucose	+
Maltose	+
Sucrose	+
Mannitol	−
Starch	+
Zone of growth inhibition with:	
Metronidazole (50 µg disk)	+
Trimethoprim	+
Sulfonamide	+

+, positive reaction; −, negative reaction.

Direct nucleic acid probe and amplification technology have also been used to detect and identify *G. vaginalis* in clinical specimens, but, given the high prevalence of *G. vaginalis* in vaginal specimens, specific molecular tests solely for this organism are not particularly useful in most cases. The Affirm VP III Microbial Identification Test (Becton-Dickinson, Sparks, MD) is a commercially available, semi-automated system that uses synthetic, oligonucleotide hybridization probes for the simultaneous detection of *G. vaginalis*, *Trichomonas vaginalis* and *Candida* species from a single vaginal swab. This assay has a total hands-on time of about 5 minutes and test results are available in less than 1 hour. The test is performed on the BD MicroProbe Processor

instrument. The first product, Affirm VP, detected only *G. vaginalis* and *Trichomonas vaginalis*, but the newer Affirm VP III assay also detects *Candida albicans*. Briselden and Hillier evaluated the first assay and found that the Affirm VP test for *G. vaginalis* was positive for 97% of women who had BV based on clinical criteria and had a specificity of 71%.[139] Since the probe was more sensitive than culture on HBT medium, increased specificity could be attained by evaluating Affirm VP test results along with other diagnostic criteria (i.e., vaginal pH, presence of amine odor with KOH ["whiff test"]) for BV. Witt and colleagues[1200] found that the Affirm VP III had a specificity of more than 97% when compared to Gram-stained smears. The sensitivity of the assay varied depending on the score given the Gram-stained smear by established scoring criteria and ranged from 73.2% to 89.5%.[810] More recent evaluations have examined the currently available Affirm VP III Microbial Identification Test.

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Brown and colleagues[141] compared the Affirm VP III to wet preparations for diagnosis of BV in 425 women and found that 190 patients (45%) were positive for *G. vaginalis* with the Affirm VP III assay, while only 58 patients (14%) were positive by wet mount detection of clue cells. Symptomatic women were more likely to be positive by Affirm VP III alone or by both AffirmVP IIII and wet mount than by wet mount alone, and asymptomatic women were more likely to be negative by both Affirm VP III and wet mount. In 2008, Pappas et al.[839] evaluated the Affirm VP III assay for detection of BV in 291 consecutive female patients (193 were symptomatic and 98 were asymptomatic). *G. vaginalis* was detected in 108 of 193 symptomatic patients and in 40 of 98 asymptomatic patients when compared to clinical diagnostic criteria of Amsel et al.[32] (i.e., vaginal discharge, pruritis and irritation, foul-smelling vaginal discharge). The sensitivity of the Affirm VP III assay compared with the clinical diagnosis was 97.2% for the whole sample of women; the test had a sensitivity of 96.4% for detection of BV in symptomatic women and 100% in asymptomatic women. Specificities of the assay were 71.4%, 72.2%, and 64.4% for all women tested, for symptomatic women, and for asymptomatic women, respectively.[839]

Antimicrobial Susceptibility of Gardnerella vaginalis

Strains of *G. vaginalis* are generally susceptible to penicillin, ampicillin, erythromycin, clindamycin, trimethoprim, and vancomycin.[591] Ciprofloxacin and imipenem show variable activity against *G. vaginalis*. Some strains may be resistant to tetracycline and minocycline, and marked resistance to amikacin, aztreonam, and sulfamethoxazole is seen in most strains. Some clinical isolates may be pan-susceptible, including

susceptibility to daptomycin and linezolid.[625] Because of resistance to sulfamethoxazole, no synergistic activity against *G. vaginalis* is seen with cotrimoxazole (i.e., sulfamethoxazole and trimethoprim in a 19:1 ratio).

Lactobacillus Species

Taxonomy and Epidemiology

Lactobacilli are gram-positive bacilli that are part of the normal microbiota of the human vagina, gastrointestinal tract, and oropharynx. They are widely distributed in nature (i.e., water, sewage, milk products, and silage), and comprise part of the normal flora of many other animal species. Lactobacilli are also found in various foods (e.g., dairy products, grains, meats, fish, sauerkraut) largely because of their wide range of fermentative capabilities. Certain *Lactobacillus* species are now commonly added to foods as probiotics, presumably for their beneficial health effects. Individual cells of *Lactobacillus* species are often long and slender, although smaller "coryneform" bacilli or "coccobacilli" may be observed (Image Plates 14-8A and 14-7G). Some *Lactobacillus* species may form long chains. Most species are nonmotile, with rare motile species having peritrichous flagella. Most species are facultative, although certain species grow best under either anaerobic or microaerophilic conditions, particularly on primary isolation. Lactobacilli are so named because the major end product from glucose fermentation is lactic acid; some species may also produce acetic, formic, and succinic acids, along with CO_2. Lactobacilli are uniformly catalase-negative, oxidase-negative, do not reduce nitrate, do not produce indole or H_2S, do not liquefy gelatin, and do not form spores. The cell walls of *Lactobacillus* species contain a thick peptidoglycan and membrane-bound teichoic acids. Lysine, *meso*-diaminopimelic acid (*meso*-DAP), and ornithine are the di-amino acids comprising the peptidoglycan cross-linkages. Electron transport menaquinones are absent. Lactobacilli are classified in phylum *Firmicutes* in the class "Bacilli," proposed order "Lactobacillales." In this order, genus *Lactobacillus* and genus *Pediococcus* are included in the family *Lactobacillaceae*.

Clinical Significance of Lactobacillus *Species*

While inherently of low virulence, lactobacilli cause opportunistic infections in compromised hosts. The most common clinical presentations include bacteremia and endocarditis. *Lactobacillus* bacteremia occurs primarily in patients with underlying conditions, including immunosuppressive therapy, cancer with chemotherapy, bone marrow and solid organ (particularly liver) transplantation, diabetes, kidney disease, and prior surgical interventions.[46,155,425] *L. rhamnosus* is the most frequently isolated

species causing bacteremia; other species have included *L. gasseri*, *L. acidophilus*, *L. casei*, *L. paracasei*, *L. delbrueckii*, *L. fermentum, L. minitus*, *L. plantarum*, *L. zeae*, and *L. reuteri*.[155,425,649] Portals of entry for septic episodes with lactobacilli reflect the normal habitats of these organisms within the oropharynx, the genitourinary tract, and the gastrointestinal tract. Risk factors for the development of *Lactobacillus* bacteremia include persistent neutropenia, use of broad-spectrum antibiotics, allograft transplantation with immunosuppressive therapy, chemotherapy for cancer, and invasive gastrointestinal, gynecologic, or respiratory tract instrumentation and procedures.[155,237,356] *Lactobacillus* bacteremia has also been associated with HIV infection and AIDS.[1,943] In profoundly immunosuppressed patients, bacteremia with vancomycin-resistant *Lactobacillus* strains may occur during or shortly after administration of vancomycin (and other broad-spectrum agents). A 2010 case report described *L. casei* bacteremia in a 75-year-old woman with prostheses in the thoracic aorta and abdomen for aortic dissection 8 years before.[956] During her continuous bacteremia, the patient developed another aortic dissection despite absence of prosthetic vegetations. The authors suggested that *L. casei* may have directly infected aortic lesions that triggered the dissection. *Lactobacillus* bacteremia is also associated with gynecologic infections; women with lactobacillemia usually have underlying genital pathology (e.g., endometritis), have undergone invasive gynecological or obstetrical procedures (e.g., dilatation and curettage procedures, drainage of pelvic abscesses), or have neoplasms of the female genitourinary tract (e.g., ovarian cancer with large bowel invasion, choriocarcinoma).[36,236,1065] In these cases, the source of the organism is usually the patients' own vaginal microbiota. Lactobacilli are not part of the resident skin flora and have not been associated with intravenous catheter-related infections.

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Despite the increasing and widespread use of lactobacilli in various foods as probiotics (i.e., *L. acidophilus, L. rhamnosus* GG), the incidence of *Lactobacillus* infections or the proportion of bacteremic *Lactobacillus* infections have not increased.[972,973]However, in several reports, ingestion of probiotic lactobacilli by patients with various underlying conditions has been temporally associated with the development of lactobacillemia. In some cases of *Lactobacillus* bacteremia, heavy dairy consumption was posited as a "possible risk factor."[155,612,956,1111] LeDoux and colleagues[649] in New York described persistent *L. acidophilus* bacteremia in a 38-year-old man with AIDS and stage IV Hodgkin's disease. In addition to his antiretroviral therapy, the patient had been taking acidophilus probiotic three times a day for the previous 3 weeks. In another instance, a 24-year-old woman with a

prosthetic aortic valve developed *L. rhamnosus* sepsis from peri-operative administration of a probiotic containing three strains of *L. rhamnosus*.[612] One of these was identical to the *L. rhamnosus* strain recovered from the blood cultures by PFGE. The authors suggested that the organisms may have directly translocated across the intestinal barrier and avoided entrapment in mesenteric lymph nodes due to the patient's mesenteric ischemia resulting from heart failure. At the present time, three *Lactobacillus*-containing probiotics have received "GRAS" ("generally recognized as safe") status from the FDA, despite the absence of clinical efficacy data. These are *Lactobacillus reuteri* strain DSM 17938, *Lactobacillus casei* subsp. *rhamnosus* GG (known as *L. rhamnosus* GG), and *Lactobacillus acidophilus*/*Lactobacillus lactis*/*Pediococcus acidilactici* complex. Concerns have been raised about the administration of such probiotics in certain populations, including immunocompromised hosts, the very young, the elderly, patients with multiple comorbidities, patients with intravenous catheters or prostheses, and patients with valvular defects or short bowel syndrome.[1042]

Lactobacillus species are infrequent causes of endocarditis primarily in patients with congenital heart defects (e.g., ventral septal defect, bicuspid aortic valve defects), acquired native valve defects (e.g., prior endocarditis, rheumatic heart disease), vascular grafts, and prosthetic valves.[155,394,532,885,1218] Transient bacteremia originating from localized abscesses (e.g., periapical tooth abscesses), dental manipulations (e.g., extractions, root canals), gynecologic surgery or instrumentation, neonatal peri-omphalitis, and gastrointestinal endoscopy can seed the abnormal valves and initiate endocardial lesions.[1065] In those with native valve disease, large vegetations containing the lactobacilli may be noted by echocardiograms of aortic, mitral, tricuspid, and pulmonic valves.[885] These cardiac vegetations have a marked propensity to embolize to the brain and other organs. In the series of patients reviewed by Gallemore and colleagues,[394] 14 of 35 patients developed septic emboli to the brain; 5 of these patients died and 9 required surgery. Fradiani reported a case of *L. jensenii* endocarditis in a previously healthy 47-year-old woman who presented as a 10 mm mobile mass in the left atrium and a large vegetation on the mitral valve. The clinical course was complicated by thromboembolic complications, and the patient required replacement of the mitral valve.[352] For patients with endocarditis due to *Lactobacillus* species, synergistic therapy with a β-lactam agent plus an aminoglycoside for at least 4 to 6 weeks appears to be the optimal therapy. However, effecting a medical cure alone without relapse or the necessity for valve replacement is often difficult. In one series, cure with antimicrobials alone was achieved in only 39% of patients.[439]

Pleuropulmonary infections due to *Lactobacillus* species have been reported in systemically immunocompromised patients and those with impaired pulmonary status

(e.g., COPD) or localized underlying disease. Infections have included necrotizing pneumonia secondary to esophageal cancer, pleural empyema in a patient with esophageal squamous cell carcinoma, and community-acquired lung abscess.[943] Sriskanden, Lacey, and Fisher documented pneumonia due to *Lactobacillus* species in three patients with profound neutropenia secondary to immunosuppression from HIV infection, and from cytotoxic chemotherapy for vasculitis and subsequent bone marrow transplantation.[1053] In 2002, Wood and colleagues[1211] reported the first case of ventilator-associated pneumonia in a critically ill immunocompetent patient. Lung abscess due to *L. rhamnosus* was described in a 79-year-old man with COPD and emphysema.[1013] While the patient was being treated with a carbapenem, the abscess enlarged and ruptured into the pleural space, and the organism was isolated from the pleural effusion specimen.

Lactobacilli have been isolated from a variety of other infectious processes. Meningitis due to lactobacilli has been described as a complication of endocarditis, where septic emboli have lodged in the brain causing embolic brainstem infarction.[1203]Relapsing *L. rhamnosus* bacteremia and meningitis developed in a 10-year-old boy undergoing allogeneic hematopoietic stem cell transplantation, and a fatal meningitis caused by *L. rhamnosus* developed on an elderly female as a complication of prolapsed cervical disc surgery and cervical spine plate grafting 6 years earlier.[942,985] Lactobacilli have also been a cause of epidural abscess and vertebral osteomyelitis due to a spontaneous esophageal rupture and postresection surgical site abscesses in patients with cancer receiving radiation therapy.[749,1209] Hepatic and splenic abscesses involving *L. rhamnosus*, *L. paracasei*, and other species have occurred in patients with underlying disease (e.g., tonsillar carcinoma, uncontrolled type II diabetes, pancreatitis) and in clinically well individuals.[149,191,289,962] Lactobacilli are rare causes of septic arthritis and protheses infections.[53,192] Lactobacilli are uncommon causes of uncomplicated and complicated urinary tract infections, and have been associated with both urolithiasis and pyelonephitis.[195,256] Uncommon clinical presentations have included endophthalmitis following penetrating eye injuries, chrondritis following ear piercing, bacteremic cholecystitis, and primary peritonitis.[278,436,900,1209] Like many other opportunistic agents, lactobacilli have also been recovered from peritoneal infections in patients on CAPD.[604]

Isolation and Identification of Lactobacillus *Species*

After growth for 24 hours on SBA, colonies of *Lactobacillus* species are usually small (2 to 5 mm), convex, and smooth with entire edges; some α-hemolysis or "graying" of the media may also be observed (Image Plate 14-7H). Because of the large number of *Lactobacillus* species that have been described, isolates are not generally identified to

the species level. For species-level identification, workers have used the

phenotypic methods described by Holdeman and Moore in the *VPI Anaerobe Manual*, cross-referenced with species descriptions in *Bergey's Manual of Systematic Bacteriology*. In many publications, the API CH, a carbohydrate assimilation panel, has been used to assist in identification of lactobacilli. Highly standardized whole-cell protein patterns obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have proved helpful for identifying *Lactobacillus* species.[880] Oligonucleotide hybridization and molecular taxonomic methods (PCR, 16S rRNA sequencing) have also been developed for both genus- and species-level identification of lactobacilli.[58,296]

Recognition and confirmation of lactobacilli in the clinical microbiology laboratory can be accurately obtained with a handful of phenotypic tests. Some species of *Lactobacillus* are intrinsically resistant to vancomycin, and this characteristic is often helpful in identifying these organisms. Resistant isolates will grow up to the edge of a vancomycin test in a disk diffusion test. Vancomycin-resistant isolates must be differentiated from *Leuconostoc* and *Pediococcus* species, which are both vancomycin-resistant and can appear coccobacillary on Gram's stains. *Leuconostoc* species produce gas from glucose in Mann–Rugosa–Sharp (MRS) broth, while the majority of *Lactobacillus* species isolated in the clinical laboratory do not produce gas. Lactobacilli are also PYR-positive, while both *Leuconostoc* and *Pediococcus* species are PYR-negative. Lactobacilli are also LAP-negative, while *Streptococcus, Enterococcus*, *Pediococcus*, and *Lactococcus* species are LAP-positive. GLC from glucose broth cultures can be used to obtain a genus-level identification of isolates, due to the formation of large amount of lactic acid by these organisms. *Lactobacillus* species that have been identified in association with human infections include *L. rhamnosus*, *L. plantarum*, *L. leichmanii*, *L. fermentum*, *L. casei* subsp. *casei*, *L. paracasei*, *L. confusus*, and the *L. acidophilus* group. Strains of *L. rhamnosus*, *L. casei*, and *L. plantarum*, in particular, may be intrinsically resistant to vancomycin and teicoplanin (Image Plate 14-8A).

Antimicrobial Susceptibility of Lactobacillus *Species*

Recent surveys on the antimicrobial susceptibility of lactobacilli have shown a wide variation in susceptibility for several antimicrobial agents.[155,255,1076,1150] Danielsen and colleagues[255] in Denmark determined the susceptibility of 23 significant blood culture isolates belonging to seven different species. No differences were seen among the isolates regarding susceptibility to ampicillin (0.125 to 4 μg/mL), clindamycin

(0.032 to 1 µg/mL), erythromycin (0.032 to 2 µg/mL, and linezolid (0.5 to 4 µg/mL). High or intrinsic resistance related to individual species was observed against vancomycin, ciprofloxacin, cefotaxime, and meropenem. Vancomycin MICs were either <2 µg/mL or >256 µg/mL. In a retrospective review of over 200 cases (including reports of bacteremia, endocarditis, and localized infections), most lactobacilli were susceptible to clindamycin (90.9%) and erythromycin (94.3%), while only 22.5% of the isolates were susceptible to vancomycin.[155] In this survey, the percentage of susceptible isolates were 54.9% for penicillin, 63.6% for ampicillin, 47.8% for cefazolin, 64.3% for ciprofloxacin, and 70% for gentamicin. Individual case reports have also documented multi-resistance among *Lactobacillus* strains. For example, a *L. rhamnosus* isolate that was recovered from CSF of a stem cell transplant patient was resistant to penicillin, cefotaxime, all aminoglycosides, trimethoprim-sulfamethoxazole, all fluoroquinolones, tetracycline, and vancomycin.[942]Resistance of lactobacilli to vancomycin and teicoplanin is related to their cell wall structure. Analysis of the cell walls of vancomycin-resistant strains (e.g., *L. rhamnosus*) reveals that the peptidoglycan precursors for these species have a terminal depsipeptide "D-alanine-D-lactate," while species that are vancomycin-susceptible (e.g., the *L. acidophilus* group) have a terminal "D-alanine-D-alanine" structure.[110,468] This single amino acid difference effectively alters the target site for vancomycin, allowing peptidoglycan synthesis to occur in the presence of vancomycin.

Weissella Species

Weissella species are catalase-negative, gram-positive bacilli that were formerly members of the genus *Lactobacillus* (*L. confusus*) and *Leuconostoc* (i.e., *Leuconostoc paramesenteroides*).[229] Two additional *Weissella* species—*W. hellenica* and *W. thailandensis*—have also been described, and four former *Lactobacillus* species have also been transferred to the genus *Weissella* as *W. halotolerans*, *W. kandleri*, *W. minor*, and *W. viridescens*.[724,1089] These bacteria constitute a distinct phylogenetic group separate from the genera *Lactobacillus*, *Leuconostoc*, and *Streptococcus*. The cell wall of these organisms contains lysine and alanine joined with an intra-peptide bond and gas is produced from glucose. These properties differentiate *Weissella* species from the homofermentative lactobacilli, which contain lysine and *meso*-DAP in their cell walls. Two species—*W. confusa* (formerly *L. confusus*) and *W. cibaria*—have been associated with human infections, and these organisms are usually resistant to vancomycin.[117] *W. confusa* has been isolated as a cause of bacteremia in immunocompromised hosts, including a liver transplant patient with a history of hepatocellular carcinoma, a patient with abdominal aortic dissection and repair, and a

allogeneic stem cell transplant recipient.[473,819,969] Two patients with native valve endocarditis due to *W. confusa* have been reported. One case was detected as an incidental finding in a 65-year-old man with angina pectoris and aortic insufficiency, while the second patient had a transthoracic echocardiography that showed severe mitral insufficiency and a nodular vegetation on the mitral valve.[347,1011] In the latter instance, the patient refused antimicrobial therapy and further care, resulting in his death 4 days after discharge. Lee and colleagues in Taiwan described 10 patients with *W. confusa* bacteremia. Underlying diseases in these patients included non-Hodgkin's lymphoma, B-cell lymphoma, chronic renal failure, ischemic bowel disease, malignant peritonitis, esophageal cancer, ileus, and subarachnoid hemorrhage.[654] *W. confusa* has also been isolated from foods, clinical specimens from dogs, and human feces.[117] *W. cibaria* has been isolated from fermented food products ("chili-bo") in Malaysia, sugar cane and cheese whey in Germany, and clinical specimens from animals (i.e., otitis isolates from dogs' ears) and humans (i.e., feces).[117]

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Weissella species are nonmotile, catalase-negative, heterofermentative bacilli that produce CO_2 from glucose along with lactic acid. Both *W. confusa* and *W. cibarica* are arginine dihydrolase-positive. *W. confusa* ferments galactose and xylose, but not arabinose, while *W. cibarica* ferments arabinose but does not ferment either galactose or xylose.[117] *W. confusa* strains have been misidentified as *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Staphylococcus hominis*, and *Streptococcus bovis* II by the Phoenix Automated Microbiology System (Becton-Dickinson) and as *Pediococcus pentosaceus*, *Leuconostoc pseudomesenteroides*, *Leuconostoc citreum*, and *Leuconostoc lactis* by the Vitek 2 system (bioMérieux, Inc.).[654] Some strains of *W. confusa* are susceptible to penicillin, ampicillin, imipenem, erythromycin, clindamycin, and ciprofloxacin; high-level resistance to trimethoprim-sulfamethoxazole, metronidazole, and vancomycin is commonly found, as is high-level resistance to ceftazidime (MIC >256 μg/mL).[654,1073] Ampicillin-sulbactam, amoxicillin clavulanate, daptomycin, doripenem, and tigecycline are active against most isolates of *W. confusa*, but they vary in their susceptibility to linezolid.[654]

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CHAPTER 15

Aerobic Actinomycetes

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Introduction, Classification, and Taxonomy

The Nocardioform Group

Nocardia spp.

Non-*N. asteroides* Complex Species

Epidemiology, Pathology, and Pathogenesis

Clinical Disease

Rhodococcus spp.

Taxonomy and Classification

Epidemiology, Pathology, and Pathogenesis

Clinical Disease

Gordonia spp.

Taxonomy and Classification

Epidemiology, Clinical Disease, and Pathogenesis

Tsukamurella spp.

Taxonomy and Classification

Epidemiology and Clinical Disease

Dietzia spp.

Other Nocardioform Bacteria

The *Streptosporangineae*

Actinomadura spp.
Taxonomy and Classification
Epidemiology, Clinical Disease, and Pathology

Nocardiopsis spp.

The Streptomycetes

Streptomyces spp.

Thermophilic Actinomycetes

Miscellaneous Actinomycetes

Dermatophilus

Tropheryma whipplei
History and Taxonomy
Ecology
Clinical Disease and Pathology

Laboratory Diagnosis of Infections Caused by Aerobic Actinomycetes

Primary Isolation

Differentiation of *Nocardia* From Other Genera of Aerobic Actinomycetes

Identification of Thermophilic Actinomycetes

Identification of *Tropheryma whipplei*

In Vitro Susceptibility of *Nocardia* and Related Bacteria to Antimicrobial Agents and Therapy for Infections

Concluding Comments

Introduction, Classification, and Taxonomy

The aerobic actinomycetes are gram-positive bacteria that are characteristically more filamentous and branched than the bacteria discussed in other chapters. These bacteria commonly produce fungal mycelium-like growth that fragments into rod-shaped and short coccoid forms. These organisms, when isolated in many clinical laboratories, are most often initially isolated in the mycology or mycobacteriology sections of the laboratory and/or are referred for identification to mycology or to mycobacteriology

rather than remaining in the routine bacteriology section of the laboratory. The reasons are that most aerobic actinomycetes grow more slowly than other aerobic and facultatively anaerobic bacteria; hence, the routine bacterial media may not be incubated long enough for their isolation. They are more often seen on commonly used fungal media (e.g., Sabouraud dextrose agar [SAB] or potato dextrose agar [PDA]) or on mycobacteria isolation media (e.g., Middlebrook agar or Löwenstein–Jensen [LJ] media), which are incubated for weeks rather than a few days. These aerobic, branching, filamentous gram-positive rods are, nonetheless, bacteria, not fungi. True fungi have a eukaryotic cellular organization. In contrast to the fungi, the prokaryotic aerobic actinomycetes do not have a membrane-enclosed nucleus; they lack the intracellular organelles possessed by eukaryotic organisms (e.g., mitochondria); their cell walls contain muramic acid, diaminopimelic acid (DAP), or lysine (which fungi lack); they are inhibited by antibacterial antibiotics, but not by antifungal agents. Lastly, they do not produce hyphae although the term *branching hyphae* is often used to describe the aerobic actinomycetes; the branching filaments are narrower than fungal hyphae, measuring 0.5 to 1.2 μm in diameter (Box 15-1). Modern chemical and genetic techniques have established the great diversity of this group, and although the phenotypic characteristics described above remain an important part of the microbiologic definition, these alone are insufficient for species identification of most members of the group (Table 15-1).[41] The aerobic actinomycetes as a whole are placed taxonomically in the class *Actinobacteria*, order *Actinomycetales*, and divided among six suborders, including *Corynebacteriaceae, Streptosporangineae, Pseudonocardineae, Streptomycineae, Micrococcineae,* and *Thermoactinomcetaceae*. The flowchart in Figure 15-1depicts the relationships among these bacteria. Although the taxonomy and nomenclature of these bacteria are still being determined, a provisional, ad hoc phenotypic characterization of the group may remain useful, although in the future, more reliance will be placed on molecular taxonomy.[19,30,41,58]

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BOX 15-1

Terms Used to Differentiate and Classify the Aerobic Actinomycetes

1. Aerobic actinomycetes are prokaryotes unlike the eukaryotic fungi; hyphae are used to describe the “filaments” of fungi that make up a matt of mycelia among the fungal molds; however,*branching* and *aerial hyphae* (and at times mycelia)

are terms used to describe some of the aerobic actinomycetes, especially the *Nocardia* spp. Here are some differences between fungi and the aerobic actinomycetes.

A. *Nocardia* filaments (and those of other aerobic actinomycetes), are 0.5–1.0μm in diameter as compared to the diameter of the hyphae of fungi which varies, but has a range of 1.5 to≥15μm.
B. *Nocardia* and other aerobic actinomycetes reproduce by fission (like other bacteria) as compared to the way in which fungi reproduce asexually by mitosis-derived conidia, as well as in some instances, sexually by means of more elaborate reproductive structures and meiotically-derived spores.
C. Many of the aerobic actinomycetes possess mycolic acid in their cell wall; fungi possess chitin in their cell wall along with glucans and mannans and other fungal-specific proteins.
D. Treatment of the aerobic actinomycetes is with antibacterial agents as compared to the antifungal agents used to treat yeasts and molds.

Having listed these differences, the term "hyphae" is still used throughout the literature to describe the filaments of many of the aerobic actinomycetes.

2. Strain description of the aerobic actinomycetes:
   A. "Type strain" denotes the exact strain of the bacterium for which the original description of the species was based.
   B. "Reference strains" is name given to other strains in a collection that are thought to belong to the same species as a given type strain.
   C. *Sensu stricto* means "in the strict sense" and when used with a species name, refers to organisms belonging to that particular species. So,*N. asteroidessensu stricto* should mean isolates that by gene sequencing are identical to the type strain *N. asteroides* ATCC 19247.
3. *N. asteroides* complex: This term is used in this chapter to refer to the 8–10 *Nocardia* species, such as *N. farcinica, N. nova,* and *N. transvalensis*, that are all partially acid-fast, contain *meso*-DAP with short-chain mycolic acids in cell wall (44–64 carbon atoms), and are usually negative for hydrolysis of casein, xanthine, and tyrosine.

The scheme outlined in Figure 15-1 uses tests that had been commonly performed in clinical microbiology laboratories and in some cases are still being performed. The non–acid-fast genera present the greatest problem, because the cell wall analyses required for their identification are beyond the capacity of most clinical laboratories. Molecular techniques or mass spectrometry will probably replace the phenotypic and cell wall analyses for species-level differentiation, although cell wall analysis will remain as a tool for research and epidemiologic studies. Most of the aerobic actinomycetes are environmental bacteria that can occasionally produce infections in

humans, especially in an immunocompromised host. If an aerobic actinomycete is isolated from a patient who has a clinically compatible illness, it should be referred to a reference laboratory for complete identification, especially if your laboratory does not have the capacity to perform sequencing or employ other advanced methods for identification. Even within the group that consists of partially acid-fast organisms, increasing complex tests are required for identification. The distinction of aerobic actinomycetes from a rapidly growing *Mycobacterium* species should always be done; this is usually accomplished by performance of full and partial acid-fast stains. *Mycobacterium* spp. should be fully acid-fast positive as compared to the partial or weakly acid-fast nature of some of the aerobic actinomycetes, such as *Nocardia* spp. *Mycobacterium* spp. are usually devoid of filamentous bacillary structures seen with the aerobic actinomycetes. Both rapidly growing *Mycobacterium* spp., *Nocardia,* and other aerobic actinomycetes can cause similar diseases (see Chapter 19).

Kiska and colleagues have presented a simplified battery of tests for the identification of *Nocardia* spp.[99] This biochemical algorithm may be useful initially to determine if one has indeed isolated an aerobic actinomycetes, but are not sufficient for complete identification to the species level and at times, not even to the genus level when used alone. This edition of the text will migrate from traditional biochemical testing and will emphasize molecular and mass spectrometric means of identification, which are becoming the gold standard for identification of these organisms.

The aerobic actinomycetes are widely distributed in nature, where they participate in the decomposition of organic plant material.[75,167] Isolates of various species are made regularly from soil and marine sediments, as well as from the feces of a variety of animals. They should be considered

TABLE 15-1 Most Common Aerobic Actinomycetes Genera in the Order *Actinomycetales*[a]

NOCARDIOFORMS (SUBORDER *CORYNEBACTERINEAE*)[B]
Nocardia spp.
Rhodococcus spp.
Gordonia spp.
Tsukamurella spp.
Dietzia spp.
Williamsia spp.
Segniliparus spp.
SUBORDER *STREPTOSPORANGINEAE*

Nocardiopsis spp.
Actinomadura spp.

SUBORDER *STREPTOMYCINEAE*

Streptomyces spp.

SUBORDER *MICROCOCCINEAE*

Dermatophilus spp.

SUBORDER *PSEUDONOCARDINEAE*

Pseudonocardia spp.
Amycolata spp.
Amycolatopsis spp.
Saccharomonospora
Saccharopolyspora

SUBORDER *THERMOACTINOMYCETACEAE*

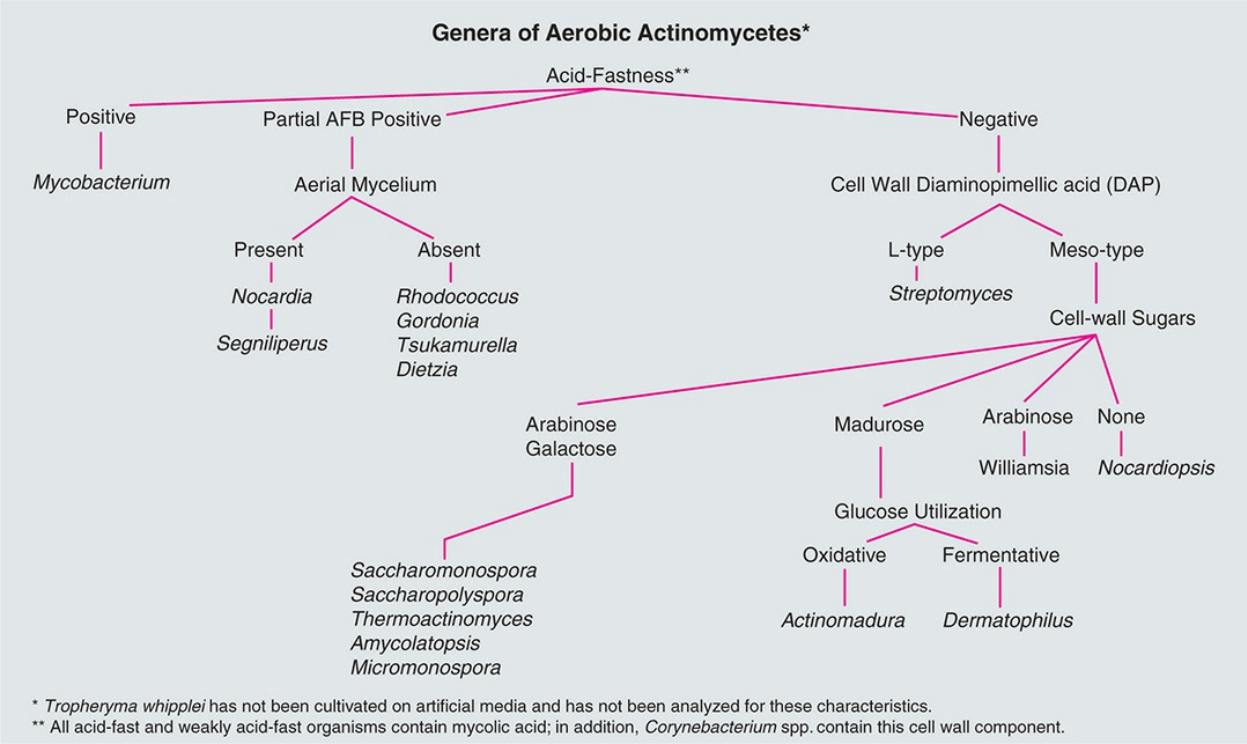
Thermoactinomyces spp.

[a] Table has been modified from reference 41, Figure 1, page 444. This list does not contain all the members of Actinomycetales, but rather the ones most commonly isolated from human disease and clinical specimens.
[b] Also in suborder *Corynebacterineae* are the *Mycobacterium* spp. and *Corynebacterium* spp.

opportunistic pathogens, causing infection primarily in individuals whose defense mechanisms have been compromised. In addition, however, these bacteria have provided some of our most important antibiotics: streptomycin (*Streptomyces* spp.), vancomycin (*Amycolatopsis orientalis*), macrolides (*Micromonospora* spp.), and aminoglycosides (*Micromonospora* spp.). By far the most important pathogens are in the genus *Nocardia*, which will be considered in detail. The detail of discussion of the other genera will be proportional to their clinical relevance.

From a practical standpoint, the aerobic actinomycetes can be recognized in the laboratory because they form glabrous, tough, adherent, waxy, or dry chalky colonies that grow after 3 days to 2 weeks of incubation. Tan, pink, orange, or gray pigments may be observed. The odor of a musty basement or freshly turned soil is an important clue by which they can be recognized, although caution should always be used in working with these isolates to minimize aerosolization and "sniffing" of any bacterial or fungal colonies. The odor from these organisms is usually evident, without sniffing the plate. The optimal temperature for growth is 30°C to 36°C, both in the CO_2 incubator (as for the culture of mycobacteria) or in ambient air (as in the incubator

used for fungal cultures). They grow well on culture media such as sheep's blood agar, chocolate agar, LJ egg-based medium, Middlebrook synthetic agar, and on most fungal recovery media that are free of cycloheximide. All species are gram-positive, although *Nocardia* spp., in particular, tend to stain with a beaded, gram-variable pattern. Thus, the finding of gram-positive or gram-variable, beaded, delicate (1 μm in diameter), branching organisms should alert one to the possibility of an aerobic actinomycete; cultures for *Nocardia* should be held for 2 to 4 weeks. As mentioned above, the most common characteristic by which *Nocardia* spp. are separated from rapidly growing *Mycobacterium* species in clinical laboratories is their "partial" acid-fastness. The acid-fast reaction for *Nocardia* is positive only when a low concentration of an inorganic acid such as sulfuric acid (H_2SO_4) is used instead of the stronger hydrochloric acid (HCl) as the decolorizer. One can have a high suspicion for an isolate being a *Nocardia* spp. if the isolate is partially acid-fast, demonstrates branching filaments, and produces the musty, dirt-like odor or appropriate colony morphology. These properties are variable, however, and not always reliable. In problem cases, analysis of the cell wall or genome analysis has been required, which necessitated sending the isolate to a specialized reference laboratory. Today, however, methods such as mass spectrometry and sequencing are becoming more available, and knowing which laboratories provide these services is very important if your laboratory cannot.[35,143,167,193,194] For completeness and during this transition period, the cell wall composition of the aerobic actinomycetes, still potentially useful both taxonomically and diagnostically, is detailed in Table 15-2.



■ **FIGURE 15-1** Classification of aerobic actinomycetes associated with human disease.

The Nocardioform Group

Nocardia *spp.*

The taxonomy of *Nocardia*, the most important genus among the aerobic actinomycetes, is ever-changing. The definition of a nocardiae species is based on molecular data, usually obtained from 16S rDNA sequencing. The *Nocardia* spp. possess tuberculostearic acid, like *Mycobacterium* spp., but also contain short-chain mycolic acids (40 to 60 carbon units); their cell wall contains peptidoglycan as do all gram-positive bacteria, but it is composed of *meso*-DAP, arabinose, and galactose. The presence of these cell wall components is fairly universal among the aerobic actinomycetes; however, the length of the mycolic acid chains may differ among the genera.[19,167] In addition, some of the aerobic actinomycetes, as shown in Table 15-2, do not contain mycolic acids.

The original isolate from infected animals with granulomatous disease (bovine farcy) was made by Nocard in 1888 on Guadeloupe Island. This isolate was subsequently named *Nocardia farcinica*, and this isolate was made the type species of the genus in

1954.[41]Interestingly, the original isolate of Nocard actually consisted of two strains that were thought to be identical, but in actuality one of the strains had mycolic acids that are characteristic of nocardiae, while the mycolic acids of the other were more typical of a mycobacteria.[19] The strain that had characteristics of a *Nocardia* sp. (American Type Culture Collection [ATCC] strain 3318) was found in 1962 to be phenotypically identical to what was then being called *N. asteroides* and since the latter nomenclature had become more commonly used in clinical laboratories, *N. asteroides* (ATCC strain 19247) replaced *N. farcinica* as the type species for the genus and continued to be used in most clinical laboratories as the name for the most commonly isolated *Nocardia* species.[41] *N. asteroides* proved to be very diverse as more genetic studies were performed and these differences led to formation of subgroups within the *N. asteroides* species. Microbiologists were eventually able to demonstrate that many of these subgroups of isolates actually were biochemically and immunologically identical to the original ATCC strain of *N. farcinica*, 3318, and interestingly not to *N. asteroides* type species 19247. The term *N. asteroides* "complex" was coined to demonstrate this diversity, and thus designations appeared like *N. asteroides* subsp. *farcinica* or *N. asteroides* subsp. *nova*, etc. to define these differences within the complex. More sophisticated molecular studies soon verified the homology of the subgroup referred to as *N. farcinica* with the ATCC-type strain 3318.[32]

As the use of polymerase chain reaction (PCR), nucleic acid sequencing, and other molecular tools continued to be applied to the *Nocardia* isolates, it became apparent that many of what had been called *N. asteroides* or even *N. asteroides* complex were not related to the type species at all.

N. asteroides complex isolates are relatively inert biochemically, and hence differentiation using phenotypic means was impossible and many infections that were reported to be due to *N. asteroides* were really not caused by this organism, when the infecting agent was identified using modern molecular tools. In addition, Wallace et al.[199] and others began to notice differences in drug susceptibility patterns among the species of the *N. asteroides* complex, and in 1988, he and his colleagues reported six different drug patterns for 12 antimicrobials (Table 15-3). This group defined as drug patterns I to VI for the 78 clinical isolates of what were referred to at the time as *Nocardia asteroides*. Steingrube and associates

TABLE 15-2 Cell Wall Composition of Aerobic Actinomycetes

Cell Wall Chemotype[a]	Composition	Nocardomycolic Acids	Genera
I	L-DAP; no diagnostic sugars	No	*Streptomyces*
II	*meso*-DAP; no diagnostic sugars	No	*Thermoactinomyces, Nocardiopsis*
III	*meso*-DAP; madurose	No	*Actinomadura, Dermatophilus*
IV	*meso*-DAP; arabinose and galactose	Yes	*Nocardia, Rhodococcus, Gordonia, Tsukamurella, Dietzia*
VI	No DAP; aspartic acid and galactose	No	*Oerskovia*[b]
NA	No DAP; madurose	No	*Actinomadura*

[a]Cell wall of all genera contain major amounts of alanine, glutamic acid, glucosamine, and muramic acid.
No significant human pathogens have the characteristics of II, V, VII, or VIII cell walls.
[b]*Oerskovia* has been found to be more closely related to the general *Cellulomonas* than to the genus *Nocardia* or other aerobic actinomycetes.
Data from references 19 and 199.

TABLE 15-3 Antimicrobial Susceptibility Profiles of 78 Isolates of the *Nocardiaasteroides* Complex

Type	Percentage (%) Isolates	Susceptible	Resistant	Species Correlation
I	20	Ampicillin, carbenicillin, broad-spectrum cephalosporins, imipenem (50%)	Imipenem (50%)	*Nocardia abscessus*
II	0	Same as type I, but kanamycin- and ciprofloxacin-susceptible	Gentamicin and Clarithromycin	*N. brevicatena/ N. paucivorans*
III	18	Ampicillin, erythromycin	Carbenicillin	*Nocardia nova*
IV	5	Ciprofloxacin	All aminoglycosides, including amikacin	*N. transvalensis* complex
V	17	Ciprofloxacin, imipenem	All penicillins and broad-spectrum cephalosporins, all aminoglycosides except amikacin	*Nocardia farcinica*
VI	35	Broad-spectrum cephalosporins	Penicillins	*Nocardia asteroides* sensu stricto, *Nocardia cyriacigeorgica*

Data from reference 199.

later defined four separate unnamed groups, designated type I, type II, type IV, and type VI, using DNA amplification and restriction fragment length polymorphism (RFLP) to characterize products derived from the 65-kDa heat-shock protein gene.[179] These groups likewise showed differences in antimicrobial susceptibility profiles, as Wallace had previously shown. Strains that match the classical description of the type species (ATCC 19247) were designated *Nocardia asteroides* sensu stricto type VI. It should be noted that "type VI" in this context is not the type VI classification of the components of the cell wall. It was later proposed that the isolates with a type VI susceptibility pattern should be named *N. cyriacigeorgica* and not *N. asteroides*.[163,219]

Roth and colleagues in 2003 redefined the phylogeny of the genus *Nocardia* based on 16S rDNA sequencing. They determined 30 established species and 10 unclassified

taxa in the genus and proposed methods for future taxonomic decisions as new species are recognized. Brown-Elliott in 2006 reviewed all of the data regarding taxonomy of *Nocardia* spp., including the work of Wallace (1988), Steingrube, and Roth, clarifying the status of the genus at that time. Many earlier reports of clinical disease did not distinguish these pathogens using currently acceptable techniques; so it is at times difficult to determine what the true identity of the infecting species.[19,163]

It turns out that the drug pattern VI isolates referred to as *N. asteroides* sensu stricto represents approximately 35% of clinical isolates of *Nocardia* spp.[19,199] It has been suggested that perhaps sometime in the future the name *N. asteroides* will return to designate this group of *Nocardia* sp. since this drug pattern represents one-third of the isolates seen clinically. Currently, *N. cyriacigeorgica* and *N. asteroides* complex both share that designation. The following lists the species that correspond to each of the six drug patterns listed in Table 15-3, which will increase as new species are being identified regularly:

I. *N. abscessus*[217];
II. *N. brevicatena*[99,163]/*N. paucivorans*[53,215] complex and an unnamed group;
III. *N. nova* complex, which is composed of *N. nova*,[196] *N. veteran,*[7,36,152] *N. africana,*[78] and *N. kruczakiae*[38];
IV. *N. transvalensis* complex (including *N. wallacei*)[39,126,222];
V. *N. farcinica*[204];
VI. *N. asteroides* complex (or sensu stricto) and *N. asteroides* Type VI (unnamed)/*N. cyriacigeorgica*.[219]

Non-N. asteroides Complex Species

Among the non-"*N. asteroides* complex" (not members of the antimicrobial susceptibility patterns I to VI), *N. brasiliensis*,[167,176] *N. pseudobrasiliensis,*[90,165,195] and *N. otitidiscavarium*[34,148] (formerly *N. caviae*) are the most commonly isolated species in human disease. Additionally, other species of *Nocardia* that have been found to be involved in clinical disease include *N. arthritidis*,[91] *N. punis*,[221] *N. asiatica*,[89] *N. beijingensis*,[142] *N mexicana,*[161] and many other named and unnamed species.[19,60,99]

Epidemiology, Pathology, and Pathogenesis. Members of what was once referred to as the *N. asteroides* complex are the most frequently isolated human aerobic actinomycetes pathogens and are distributed widely throughout the United States.[167] *N. farcinica*, *N. cyriacigeorgica,* and *N. nova* are the species most often identified;

however, some of this is geographically determined. Beaman et al.[12] estimated that 500 to 1,000 cases of nocardiosis occur in the United States each year, but that figure is likely to be an underestimate because nocardiosis is not a reportable disease. Unfortunately, this survey was done in the 1970s and there have been no other more recent surveys of the incidence of *Nocardia* in the United States, although with increasing immunosuppressed populations, the number of infections is most likely higher today.

Nocardiae are opportunistic pathogens, more often producing infections in patients who are immunocompromised or have debilitating diseases. A combination of long-term corticosteroid therapy and chronic lung disease has been suggested as a particularly important combination of risk factors.[167] Patients with nocardiosis may have no

demonstrable underlying immunologic defect, but these are not the usual hosts for *Nocardia* spp. other than in cases of traumatic introduction of the organism into the skin and soft tissues. Nocardiae may colonize the skin and respiratory tract; so isolation of a *Nocardia* sp. could potentially represent colonization rather than infection in any nonsterile site. In one Australian study, only about 20% of the isolates were not associated with clinical disease.[64]Even the recovery of *Nocardia* species from blood cultures can be insignificant. Of eight patients with *Nocardia* bacteremia reported by Esteban and associates, *N. asteroides* was thought to be clinically relevant in only two; the remaining isolates were considered clinically insignificant or of uncertain significance.[55] However, there have been many reports more recently about positive blood cultures, especially in catheter-related bacteremia cases in which the *Nocardia* spp. were indeed significant. Clinical correlation with laboratory results is always needed so that isolates are not dismissed as merely laboratory contaminants or "diphtheroids."[147,168]

The most common clinical presentation of nocardial disease, especially when caused by members of the *N. asteroides* complex, is that of pulmonary disease in the immunocompromised host; the portal of entry of the organisms is via inhalation of airborne fragments of the bacteria from the environment. Extrapulmonary disease, as a result of dissemination from primary pulmonary disease or localized infection, can occur anywhere in the body, but predominantly occur in the skin, subcutaneous tissue, and the central nervous system. Approximately 20% of patients with disseminated disease present solely with extrapulmonary disease.[19] The second most common form of nocardiosis, cutaneous and soft tissue infections, is more commonly caused by nonasteroides complex *Nocardia* spp. *N. brasiliensis* is a common cause of

subcutaneous infections and mycetoma in South America, much of Central America. In the 1960s, 85% to 95% of reported mycetomas in Mexico were caused by *N. brasiliensis*.[71] Castro and associates noted that *N. brasiliensis* was by far the most common actinomycete isolated from 41 patients seen in Sao Paulo, Brazil, between 1978 and 1989.[28]Most patients were from rural areas in the northeast regions of the country, and the majority were field laborers. Significant infection of skin and soft tissues with *N. brasiliensis* was also found in patients from Queensland, Australia, who had nocardiosis.[64] Although primarily found in tropical countries, *N. brasiliensis* is, nonetheless, among the most common of the *Nocardia* sp. isolated in the United States, where it is most prevalent in southwestern and southeastern states.[176] As mentioned above, many of the reports of *N. brasiliensis* infection are actually due to a newly described species, *N. pseudobrasiliensis*.[90,165,195] *N. brasiliensis* and *N. pseudobrasiliensis* can also cause pulmonary disease in immunocompetent and immunocompromised hosts.[103]

N. otitidiscaviarum (formerly *N. caviae*) has been isolated from soil throughout the world. It is a less common cause of disease and is less frequently recovered than other *Nocardia* spp. The distribution and clinical relevance of other rarely isolated species of *Nocardia* is less clear although molecular and mass spectrometry methods for identification may change this in the future.[57,193,202]

One of the most serious forms of nocardiosis is chronic pulmonary infection, with or without dissemination to other organs. The pathogenesis of infection with the *Nocardia* spp. is inhalation of airborne bacteria, which perhaps explains the frequency of infection in the dry southwestern states, analogous to infections by *Coccidioides* spp.[19,167] Cutaneous disease, including actinomycotic mycetoma, can result from direct introduction of bacteria after trauma, including gardening, walking barefoot, after surgical or cosmetic procedures, or as a result of disseminated disease.[45,51,167]

Nocardia spp. are facultative intracellular pathogens. Their ability to grow in macrophages and also polymorphonuclear nuclear leukocytes is undoubtedly important for their ability to produce infection. The organisms are capable of living inside macrophages by inhibiting phagosome–lysosome fusion and by their ability to produce catalase and superoxide dismutase, which inactivates products of the myeloperoxidase system of these phagocytic cells. In particular, patients with chronic granulomatous disease are at risk of serious nocardial infection, as they are for other bacteria that produce catalase.[46] The cellular immune response is critical for control of the infection, as suggested by experimental studies and the occurrence of disease in patients with compromise of cellular immunity.[11,69] Initially, nocardial infection appeared to be underrepresented in patients who are infected with HIV; however, Beaman and others note the accumulating number of case reports and unreported infections, and that the geographic variations in environmental nocardiae, which do not

match centers of HIV infection, may partially explain any of the discrepancies in incidence.[11]

The bacterial cells of *Nocardia* spp. are not usually seen in hematoxylin- and eosin-stained sections, and may not be seen in periodic acid–Schiff (PAS) stains. They are quite readily observed in Gomori methenamine–silver, Gram–Weigert, Giemsa, or tissue Gram's stains (e.g., Brown and Brenn, or Brown–Hopps stains). In a tissue Gram's stain, the organisms are usually thin, beaded, branched filamentous rods. The modified Kinyoun acid-fast stain (i.e., the partial acid-fast stain wherein destaining is done with 1% aqueous H_2SO_4 instead of the usual 3% HCl that is used in the Ziehl–Neelsen method) is useful for direct smears and frozen sections, whereas the Fite–Ferraco acid-fast stain (i.e., also a partial acid-fast stain) is recommended for paraffin sections.[209,210] When both full and partial acid-fast staining is used,*Nocardia* spp. are partially acid-fast positive/full acid-fast negative, *Mycobacterium* spp. in both the full and partial acid-fast positive, and *Actinomyces israelii* and the other anaerobic actinomycetes are negative with both the full and partial acid-fast stains. Other members of the aerobic actinomycetes, such as *Rhodococcus*, *Gordonia*, and *Tsukamurella*, for example, stain partially acid-fast positive or not at all. For all of the aerobic actinomycetes, use of the partial acid-fast stain in tissue and clinical samples directly often gives a true indication of their acid-fastness as compared to applying the stain to the in vitro colonies of the isolate when the property of acid-fastness can wane. Box 15-2 describes the method for performing the partial acid-fast stain.

Clinical Disease. The clinical disease produced by all species of the genus *Nocardia* is similar. Pulmonary disease can present as a subacute, or indolent disease lasting from days to weeks before a patient seeks medical care.[19,125] Manifestations include fever, a cough productive of thick mucopurulent sputum, sometimes accompanied by weight loss and fatigue mimicking nontuberculous mycobacterial disease. There is usually chest roentgenogram evidence of nonresolving infiltrates with or without smooth, round nodules in one or more locations. Cavitation of the infiltrate or nodule can develop.[19] There may be progressive bronchopneumonia, extensive consolidation, single or multiple abscesses, pleural effusions, empyema, and sinus tracts with involvement of the chest wall. The primary inflammatory response is neutrophilic, but in rare instances granulomas have been described.

BOX 15-2

Modified Acid-Fast Stain for *Nocardia* Species

1. Make a smear of the organism from growth media and heat-fix.
2. Flood the slide with Kinyoun carbolfuchsin for 5 minutes.
3. Pour off the excess stain.
4. Decolorize with a 1% aqueous solution of H_2SO_4.
5. Wash with tap water.
6. Counterstain with methylene blue for 1 minute.
7. Rinse with water and dry. Examine under oil-immersion optics.

Disseminated infection usually originates from a pulmonary focus, but it may also occur after a primary infection elsewhere. Bacteremic spread results in multiorgan infection, especially in the brain and skin.[19,41,80] Approximately 45% of patients with disseminated nocardiosis have infections of the central nervous system, usually in the form of one or more brain abscesses.[7,19,60,113] The prognosis for these patients is poor with a fatality rate of 7% to 44% overall and up to 85% in severely immunosuppressed patients.[125] Serious infection with *Nocardia* spp. has been documented in patients who have neoplastic disease[188] or acquired immunodeficiency syndrome[85] and in recipients of renal transplants,[207] among others. Metastatic brain abscesses, which develop in about one-third of patients, produce headaches, nausea, vomiting, changing mental status and depressed consciousness. Seizures, focal neurologic deficits or other neurologic abnormalities can also occur. Blood cultures (in spite of hematogenous spread of the organism) and cerebrospinal fluid (CSF) cultures almost invariably fail to demonstrate the bacteria.[53]

Cutaneous infections are probably underdiagnosed because they are often self-limited and adequate microbiologic studies may not be done on superficial infections.[113] Primary cutaneous infections usually present as lymphocutaneous, superficial cellulitis or localized infections on the face when presenting in children or on the lower extremities in adults.[19]When there is lymphangitic spread to the regional lymph nodes, possibly with fistula formation, it clinically resembles sporotrichosis and has been referred to as sporotrichoid nocardiosis.[175] Secondary cutaneous infection can also occur as a result of disseminated disease. Unlike pulmonary disease, the patient who develops primary cutaneous disease is most often an immunocompetent host. As previously mentioned, *N. brasiliensis* and *N. pseudobrasiliensis* are more commonly associated in cutaneous nocardiosis than members of the *N. asteroides* complex and can occur in immunocompetent as well as immunocompromised individuals.[62] A more recently recognized species in *Nocardia asteroides* complex, *N. neocaldoneniensis* was isolated from an abscess of the jaw of a 68-year-old man

receiving prednisone and methotrexate for his rheumatoid arthritis.[124] In addition, a classical cutaneous lesion, the mycetoma, may be caused by true fungi (eumycotic mycetoma) or by aerobic actinomycetes, including *Nocardia* spp. (actinomycotic mycetoma). (See discussion in the section on "*Actinomadura*," below.)

Bacteremia can occur with *Nocardia*, although not commonly, in patients with and without catheters.[2,31] *N. nova* has been the organism most often isolated in patients with cancer and was the cause of a catheter-related bacteremia; the presence of a biofilm may suggest the mechanism of its pathogenicity.[2]

Ocular nocardiosis can occur in an immunocompetent individual subsequent to trauma or as a postoperative complication. Alternatively, the eye may be infected as a result of disseminated disease in an immunocompromised host. *Nocardia* infections of the eye include keratitis,[51] uveitis, retinal abscesses, or endophthalmitis.

A variety of other infections may be caused by *Nocardia* spp. Peritonitis can occur in patients who are undergoing continuous ambulatory peritoneal dialysis, the presentation of which is indistinguishable from that of other bacteria. *N. veterana* was recently recovered from ascitic fluid in an infection in a HIV-positive individual.[36,69] *Nocardia synovitis* has also been reported, but is rare. There have been healthcare-related *Nocardia* infections, as well as rarely reported outbreaks of infections in the community. One such outbreak has involved *N. cyriacigeorgica* in which eight individuals developed soft tissue infection subsequent to an unlicensed cosmetic procedure for enhancement. All eight patients required hospitalization, surgical debridement, and prolonged antimicrobial therapy.[6]

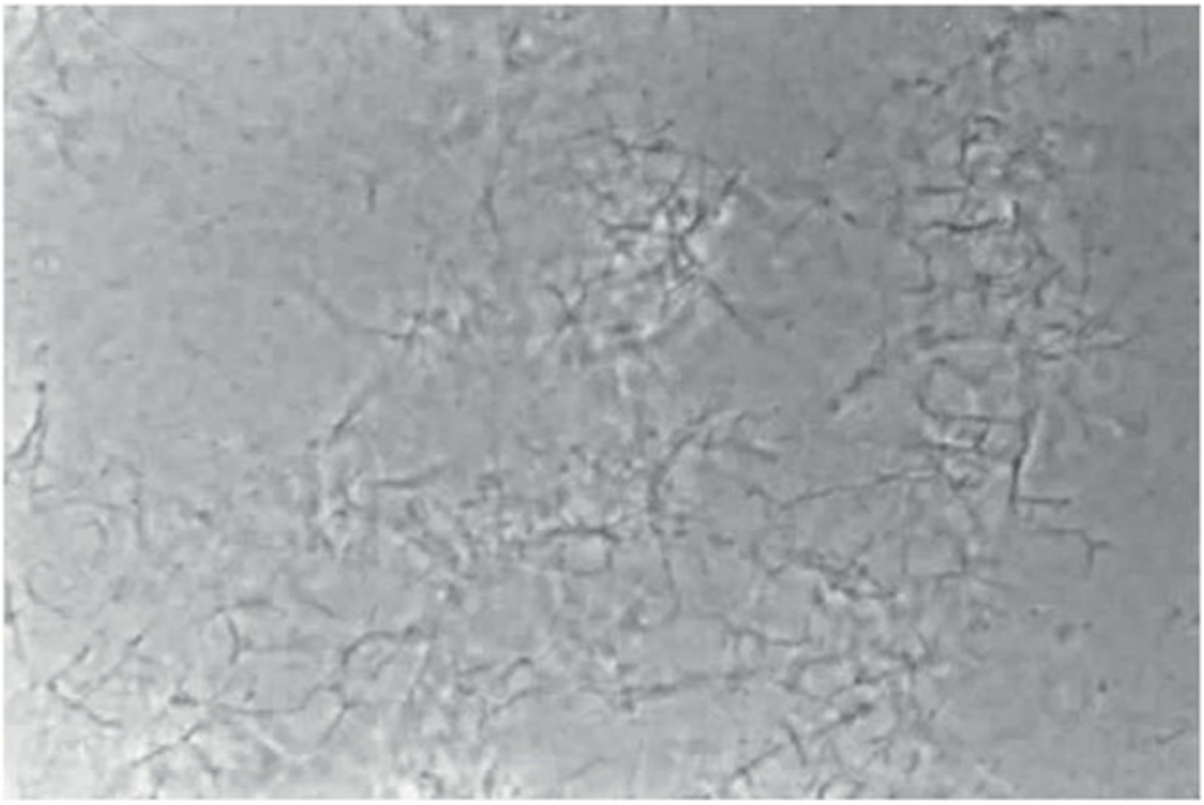
The frequency of *Nocardia* infections in organ transplant patients varies between 0.7% and 3%. A review of 5,126 transplants in the United States revealed 0.6% *Nocardia* infections. Most patients presented with pulmonary disease, although 20% had disseminated nocardial infections. The incidence of nocardiosis in transplant recipients was reported as follows: lung, 3.5%; heart, 2.5%; intestine, 1.3%; kidney, 0.2%; and, liver, 0.1%. *N. nova* was the most common species involved, followed by *N. farcinica*, *N. asteroides,* and *N. brasiliensis*. Sixty-nine percent of the patients were on prophylaxis with sulfamethoxazole–trimethoprim (SXT) and 89% were successfully treated and cured of their nocardial infection.[144] Another review of lung transplant recipients in Pittsburgh reported that 2.1% of 473 patients had *Nocardia* infection, and the major species involved was *N. farcinica*; the overall mortality was much higher than the Peleg study at 40%.[83] Of the *N. asteroides* complex species, *N. farcinica* is often the most resistant to antimicrobials, which might explain the difference in prevalence.

Rhodococcus *spp.*

Taxonomy and Classification. The genus *Rhodococcus* is a heterogeneous group of bacteria that are closely related to the *Nocardia*, although these lack the aerial "hyphae" (Fig. 15-2) that are characteristic of the *Nocardia* spp. (Fig. 15-3). Members of this group of aerobic actinomycetes are gram-positive, nonsporulating, and are usually partially acid-fast. As the name implies, the appearance of any of the members under specific environmental conditions or staining properties, may be coccoid or rod-like. The species are widely distributed in the environment, in soil, on plants, or in the gastrointestinal tract of a variety of insects or animals. There have been many changes to the taxonomy of the *Rhodococcus* genus, and many former species have been reassigned to other genera, such as *Gordonia*, *Dietzia*, *Tsukamurella,* and others.

The term *Rhodococcus* was first used by a German botanist, Zopf, in 1891 as he was classifying pigment producing bacteria and fungi; these particular bacteria produced a salmon-pink to red colony after 4 days of growth on solid media and their colonies were often mucoid. The genus name *Rhodococcus* was redefined in 1977 to include members of the "rhodochrous" complex of bacteria that contained organisms that were evolutionarily placed somewhere between the nocardioform and mycobacterial species.[214] Many of the *Rhodococcus* spp. have never been shown to cause infections.[125]

FIGURE 15-2 *Rhodococcus* on tap water agar. No aerial hyphae are produced.



■ **FIGURE 15-3** *Nocardia* on tap water agar produces aerial hyphae.

Rhodococcus equi (at one time referred to as *Corynebacterium equi* and *Mycobacteriumrhodochrous*) is the most important human pathogen in the genus. The other human pathogens, *R. erythropolis*, *R. rhodnii,* and *R. rhodochrous,* are rarely recovered from clinical specimens.[74] The biochemical characteristics of *R. equi* include production of catalase, urease, lipase, and phosphatase, but not DNase, elastase, or lecithinase. These bacteria are partially acid-fast and may demonstrate gram-positive coccoid and bacillary forms, or short branching chains; this group does not produce aerial hyphae (Fig. 15-2). *R. equi* does not grow in lysozyme, which is another differentiating feature from *Nocardia* spp. However, like most *Nocardia* complex isolates, it does not hydrolyze casein, tyrosine, or xanthine.[214] Chart 15-1addresses these hydrolysis assays and Chart 15-2 addresses the lysozyme test. Table 15-4 gives a summary of the characteristics of many of the aerobic actinomycetes.

Epidemiology, Pathology, and Pathogenesis. *Rhodococcus equi* is the cause of pulmonary disease in foals and other domestic animals. Between 1967 and the early 1980s, only a limited number of human infections were reported. Rhodococcosis is a zoonotic disease, with human infections resulting from contact with animal carriers (e.g., cattle, pig, horses, cattle, or manure), presumably by a respiratory route.[190] *R.*

equi causes a rare and often fatal, primarily pulmonary disease. Human infections most commonly occur in immunocompromised individuals, primarily those with defects in cell-mediated immunity—for example, patients with lymphoma, Hodgkin's disease, leukemia, AIDS, and following transplantation.[190,214] McNeil and Brown cited over 100 cases of *R. equi* infections in patients with AIDS in 1994 and patients with infections continue to be reported annually.[59,125,187]

The histopathology of *Rhodococcus* is unique. Although neutrophils are a component of the inflammatory response, there is also a prominent chronic inflammatory component, consisting predominantly of macrophages. These collections of histiocytes may be interspersed with microabscesses.[125] Well-formed caseating granulomas, as seen in tuberculosis, are not present. Malacoplakia, a distinctive histologic reaction in which laminated basophilic concretions (Michaelis–Gutmann bodies) are found amid the macrophages, has been described in patients with pulmonary *Rhodococcus equi* infection.[137,214] Although there may be other rare causes of pulmonary malakoplakia, this histologic finding has become almost pathognomonic for pulmonary rhodococcosis.

The pathogenesis of infections with *R. equi* is thought to be due to the ability of the organism to persist in and eventually destroy macrophages, by prevention of phagosome–lysosome fusion and the nonspecific degranulation of lysosomes in vivo.[153] In addition, the production of a biofilm has been implicated as a factor in catheter-related bacteremia, in particular in association with central lines. Removal

TABLE 15-4 Principal Features of the Major Aerobic Actinomycetes

Genus	Colony	Gram/AFB	Lysozyme	Aerial Hyphae
Nocardia spp.	Chalky or velvety, musty or soil odor; white initially, then may be any color, but often orange or salmon or tan	Beaded, branching, and filamentous; modified acid-fast positive	Growth	Present
Rhodococcus equi	Rough, smooth, although often mucoid; pigment may be delayed, but often pink, red, or orange	Coccoid and bacillary forms; often modified acid-fast positive	No growth	Considered negative; however, rudimentary aerial hyphae may be seen microscopically
Gordonia spp.	Rough and wrinkled; brown, pink, orange, red pigments	Short coryneform (diphtheroidal) without branching; usually weakly modified acid-fast positive, if at all	No growth	No aerial hyphae
Tsukamurella spp.	Small colonies, dry; may be white or pigmented orange	Straight to slightly curved long rods, singly or in pairs; no branching; some may demonstrate shorter bacilli and appear diphtheroidal; stains very weakly with the modified acid-fast stain	Growth	No aerial hyphae
Dietzia spp.	Smooth yellow colonies	Coccobacillus to bacillus without branching; modified acid-fast stain negative	No growth	No aerial hyphae
Nocardiopsis	Coarsely wrinkled or folded surface; greenish yellow or brown pigment	Aerial hyphae usually coccoid forms; best seen in slide cultures; modified acid-fast stain negative	No growth	Positive, usually abundant aerial hyphae
Actinomadura spp.	Wrinkled and often leathery colonies; may be almost any pigment	Thin, short, branching filaments; modified acid-fast stain negative	No growth	Variable; some strains may have aerial hyphae
Streptomyces spp.	Discrete, lichenoid, leathery, or butyrous; may be pigmented, sometimes gray but other colors as well depending upon species	Filamentous rods that may bead at times; can look like *Nocardia* on Gram's stains; modified acid-fast stain negative	No growth	Positive, present
Dermatophilus	Rough, heaped, opaque, or may be granular; may adhere to agar; some β-hemolysis may appear	Branching filaments and coccoid cells arranged longitudinally and parallel with other chains; modified acid-fast stain negative	Unknown	Present
Segnilaparus	Smooth and domed or rough and wrinkled; usually nonpigmented	Bacillary with V forms; no branching; modified acid-fast positive	Unknown	No aerial hyphae
Williamsia	Smooth colonies; orange to red colonies	Short bacillus or coccobacillus; modified acid-fast stain negative	Unknown	No aerial hyphae

of the catheter is important in the treatment of these patients, as well as appropriate antibiotic therapy.[1] Box 15-3 addresses treatment options.

Clinical Disease. Pulmonary infections with *Rhodococcus* may clinically mimic tuberculosis, as these are slowly progressive and may cavitate.[110,170,190] Invasive pneumonia with cavity formation is noted particularly in patients with HIV and a low CD4 count, in whom there is a high propensity for the infection to disseminate to the brain, liver, spleen, and other organs.[125,187] Clinical disease develops insidiously, with fever over several days to weeks, malaise, dyspnea, a nonproductive cough and chest pain. Hemoptysis has been reported in some patients. Chest roentgenogram examination demonstrates an infiltrative process with opaque lesions, usually in one upper lung lobe. Cavitation will occur if untreated and in rare instances a pleural effusion can occur.[170] Blood and sputum were the specimens most commonly found

positive in a review of 272 cases of HIV-associated *R. equi* disease.[187]

A 37-year-old HIV-positive patient with *Pneumocystis* pneumonia developed prostatic and scrotal abscesses with *R. equi* that required 6 weeks of antimicrobial therapy for resolution.[121] Non–HIV-positive immunosuppressed patients should also be considered at risk of *R. equi* pulmonary infections, including children with leukemia, transplant recipients—especially kidney and lung transplant recipients—and patients on immunosuppressive therapy, including treatment with alemtuzumab or corticosteroids or anti-metabolites given to patients with connective tissue disorders.[129,131,214] *R. equi* has also been reported to cause bacteremia, endophthalmitis, osteomyelitis, pleurisy with effusion and wound infections.[153,155,166,187,214] *R. equi* infection has rarely been seen in immunocompetent hosts, who have much lower mortality rates. Immunocompetent patients with rhodococcosis had a mortality rate of 11%, whereas patients with HIV had 50%

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BOX 15-3

Usual Susceptibility Patterns of the More Common Non-*Nocardia* Species of Aerobic Actinomycetes

Rhodococcus equi

Usually susceptible to erythromycin, aminoglycosides (gentamicin, tobramycin, and amikacin), rifampin, vancomycin, imipenem, and minocycline

Resistant to penicillin, ampicillin, and cephalosporins

Variable susceptibility to fluoroquinolones

There are clinical reports of success with the use of linezolid.

Gordonia spp.

There are no recommended standards for treatment of *Gordonia* spp. However, the CLSI document M-24-A2recommends that these agents can be considered for primary susceptibility testing: amikacin, amoxicillin clavulanic acid, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, SXT, and tobramycin.

There are some data from case reports that fluoroquinolones may be adequate.

Tsukamurella

There is variability to the response of different species of *Tsukamurella* and if treatment information is needed, a susceptibility test should be done on each

isolate.

In general, the following can be suggested before specific susceptibility results are known:

Susceptible to amikacin, imipenem, SXT, and ciprofloxacin

Resistant to amoxicillin/clavulanate, ampicillin, erythromycin, and minocycline

Streptomyces

Usually susceptible to amikacin, linezolid, imipenem, clarithromycin, and minocycline

Variable susceptibility to SXT

Often resistant to ciprofloxacin and ampicillin and cephalosporins

Actinomadura

Susceptible to SXT, erythromycin, linezolid, minocycline, fluoroquinolones, and imipenem

Resistant to ampicillin

to 55% mortality and non-HIV immunosuppressed patients had a mortality of 20% to 25%.[214]

Gordonia *spp.*

Taxonomy and Classification. *Gordonia* spp. (originally *Gordona* spp.) consists of aerobic actimomycetes species that were originally described by Tsukamura and revived by Stackebrandt et al. in 1988.[41] Members of this genus are difficult to identify and to separate from one another and from related genera without the use of newer molecular or mass spectrometric methods.[41] These organisms may have been dismissed in older literature as contaminating "diphtheroids" or misidentified as members of the *Nocardia* or *Rhodococcus* genera.[16]

Gordonia spp. are usually partially acid-fast, do not grow in lysozyme and possess 48 to 66 carbon atoms in their mycolic acids. Use of DNA sequencing or mass spectrometry for identification may aid in identification of the *Gordonia* spp. in the future and help clarify their role in clinical disease. There are 29 validly named species in the genus, but *Gordoniabronchialis,*[177,205] *G. sputii,*[21] *G. terrae,*[47,68,149] *G. otitidis,*[156] and *G. polyisoprenivorans*[96,192] are the species most often associated with human infections or isolated from clinical specimens.[68,96,149,174]

Epidemiology, Clinical Disease, and Pathogenesis. Infection, most often catheter-related bacteremia, and skin and soft tissue infections in immunocompromised and

immunocompetent individuals, are being reported more frequently with use of newer identification methods. Table 15-4 lists some of the main differentiating characteristics of *Gordonia* among the aerobic actinomycetes. Ramanan et al.[156] has reviewed a patient with *G. otitidis* bacteremia and reviewed additional patients with catheter-related *Gordonia* bacteremia. The patient was a 4-year-old boy with multiple medical issues, including Hirschsprung disease, who had a broviac catheter in place for TPN. Fever developed and the patient failed to thrive. He was initially treated with amoxicillin but when *Gordonia* sp., identified initially as "gram-positive diphtheroids," was isolated from multiple blood cultures on two separate episodes of fever, appropriate antibiotics were successfully instituted for *Gordonia* and cultures became negative and a clinical cure achieved.[211] Johnson et al.[86] reported a patient recently who developed a pleural infection and bacteremia; this patient did not have any intravascular catheters. This emphasizes the need for clinicians and laboratories to maintain awareness of this organism from any blood culture, but especially in immunocompromised hosts. In addition, pneumonia, osteomyelitis and central nervous system infections have been reported.[47,174,177] Catheter-related bacteremia in five pediatric patients were reviewed in Utah; three were due to *G. terrae* and one each due to *G. otitidis* and *G. bronchialis*. Fifteen other instances of invasive *Gordonia* infections in adults and pediatric patients were reviewed, 12 of which

were catheter-related. Twelve of the 15 reviewed infection occurred in immunocompromised patients as did 3 of the 5 instances reviewed in the Blaschke report.[16,68,149] *Gordoniabronchialis* has been reported to cause a recurrent breast abscess in an immunocompetent 43 year old woman. Microscopic examination of the biopsy initially revealed acute inflammatory changes and intra- and extracellular gram-positive bacteria without abscess. Within 2 weeks of inadequate antibiotic therapy, abscesses formed from which 10 mL of purulent material was drained. Despite doxycycline treatment and repeated drainage, the abscesses recurred, which appears to be consistent with other reported cases of *Gordonia* abscesses.[205] *G. terrae* has been documented as a cause of granulomatous mastitis following nipple piercing. *G. bronchialis* and *G. terrae* are indistinguishable with phenotypic identification methods, but can be differentiated by use of sequencing.[86,223] A more recently described species, *G. araii*, has been associated with infection of medical devices, which may suggest that like *R. equi*, *Gordonia* can produce a biofilm that leads to its pathogenicity in the appropriate patient and setting.[84] Box 15-3 addresses treatment options.

Tsukamurella *spp.*

Taxonomy and Classification. *Tsukamurella* are another group of aerobic actinomycetes that are being identified more often in clinical laboratories especially with newer molecular tools or mass spectrometry. These were originally named for the Japanese microbiologist Tsukamura to describe a group of actinomycetes that contained long-chain and unsaturated mycolic acids (68 to 70 carbons). Initially the organism was isolated from sputum of a patient with tuberculosis-like pulmonary disease in 1971, and was called *Gordona aurantiaca.* This same organism was later called *Rhodococcus aurantiacus*, but after 16S rDNA sequencing was employed in 1988, it was found to be homologous with an organism referred to as *C. paurometabola* and the two were combined into a new name, *Tsukamurella paurometabola,* which is now the type species of the genus.[169] Other species that have been associated with clinical disease include *T. inchonensis, T. pulmonis, T. tyrosinosolvens* and a recently described species, *T. strandjordae*.[95,216,218]

Tsukamurella are slightly acid-fast when a partial stain is applied; aerial hyphae are usually not found; rod and coccal forms, as is seen with *R. equi,* may occur with *Tsukamurella*. The bacilli of *Tsukamurella* spp. tend to be longer than most other actinomycetes.[95]

Epidemiology and Clinical Disease. Infections have been reported rarely as opportunistic disease in patients who are immunocompromised or have foreign bodies. Like *Gordonia* spp., *Tsukamurella* spp. are most often associated with catheter-related bacteremia and osteomyelitis.[17,108,169,171,173] *Tsukamurella* has been demonstrated as a cause of keratitis in a patient following corneal grafts; this mimicked infection with rapidly growing mycobacterial species and was reported by Tam et al.[184] in 2010. These authors (and others) reminded readers that correct identification of organisms in keratitis is essential to appropriate therapy.[178,184] In a review of infections caused by *Tsukamurella* spp. from 1997 to 2008, eight infections and two instances of colonization were found, all of which had been incorrectly identified as being caused by *Rhodococcus* spp. until sequencing corrected the identifications. *T. tyrosinosolvens* was the most frequent species and keratitis, followed by bacteremia, were the most common infections produced.[114] Box 15-3 addresses treatment options.

A pseudo-outbreak of *Tsukamurella* was described in Hong Kong in 2013 wherein the organism was found at the same time in four patients; it was concluded that this was the result of laboratory contamination of scissors that were used for tissue processing.[186] A combination of 16S rDNA sequencing, pulsed-field gel electrophoresis, phenotypic and metabolic tests were used to prove that all isolates were identical. When a cluster of infrequently isolated bacteria occur within a short period of time in a laboratory, then an investigation of the possibility of an outbreak or

pseudo-outbreak should be considered and investigated.

Dietzia *spp.*

Dietzia spp. are aerobic actinomycetes and like the others described above are gram-positive rods. These are usually shorter than those of *Tsukamurella*, usually non–acid-fast positive and possess short-chain mycolic acids (34 to 38 carbons); they do not grow in lysozyme. *Dietzia* are normally present in the canine intestinal tract and oral cavity as well as in environmental sources like soil and plants. Colonies may be butyrous and orange pigmented. The name *Dietzia* was given to these organism in honor of Alma Dietz, an American microbiologist.[154]The type species, *D. maris* (formerly *Rhodococcus maris*), has been isolated from the bloodstream of an immunosuppressed patient who had an indwelling intravascular catheter,[14]a pacemaker pocket infection,[146] a hip prosthesis infection,[150] papillomatosis skin lesions in an immunocompetent individual,[87] and a dog bite wound.[81] A new species of *Dietzia*, *D. aurantiaca*, was recently proposed for an isolate from CSF of a young woman.[93] In the past, most *Dietzia* spp. were probably misidentified as a member of the *Rhodococcus* genus; with molecular methods, recognition of this genus has increased.[100,141,151]

Other Nocardioform Bacteria

Segniliparus spp. are aerobic actinomycetes that are rod-shaped without branching or aerial hyphae; they are strongly partially acid-fast positive and contain very long-chain mycolic acids (70 to 90 carbon atoms). The first isolations of *S. rugosus* from cystic fibrosis patients were published in 2007.[24] In 2001, pneumonia due to *S. rotundus* was reported in a patient with bronchiectasis not associated with cystic fibrosis. Treatment with clarithromycin and ciprofloxacin was successful in eliminating the organism and resolving the clinical symptoms.[102]

Williamsia spp. are aerobic actinomycetes that appear as short, gram-positive rods to coccobacilli that are not acid-fast. These do not produce aerial hyphae and do not grow in lysozyme. Their cell walls contain mycolic acids with from 50 to 56 carbon atoms. *W. muralis* is the species most often

described from environmental sources, including surfaces in a day care centers.[92] These aerobic actinomycetes were assumed to be nonpathogens until recently.*W.*

muralis was isolated from a protected bronchial brush specimen of an 80-year-old woman with pneumonia from which she died and no other significant pathogens were recovered.[44]With the increased use of molecular techniques and mass spectrometry, laboratories may recognize more isolates of *W. muralis*.

The *Streptosporangineae*

Actinomadura *spp.*

Taxonomy and Classification. The genus *Actinomadura* is genetically diverse, and is separated into two suprageneric groups.[72,125] Once again, the terminology is confusing and in transition. Goodfellow suggests that *Actinomadura* spp. should be reserved for *A. madurae* and related species (including *A. pelletieri*), which are genetically most closely related to the *Thermomonospora* group.[73] In contrast, "maduromycetes" is reserved for a natural group of bacteria that includes *A. pusilla* and related organisms.[73] From a practical standpoint, the major human pathogens, *A. madurae* and *A. pelletieri* are categorized with the former group. Members of the genus *Actinomadura* contain *meso*-DAP in their cell walls like the aerobic actinomycetes described thus far; however, madurose is the major carbohydrate component and they do not possess mycolic acids in their cell wall.[41,73] A more recently described species in the genus, *A. sputii* sp. nov., has been isolated from the sputum of a 64-year-old man.[220] *Actinomadura* spp. are not acid-fast positive, but may produce short branching filaments. Growth in lysozyme is variable.

Epidemiology, Clinical Disease, and Pathology. *Actinomadura* spp. are soil organisms that are introduced through the skin by trauma. The infections are found primarily in tropical and subtropical countries, which may, in part, reflect the greater propensity for people who live in warmer climates to spend majority of time outdoors and walk barefoot.[125]

Mycetomas are chronic, penetrating, progressively destructive lesions that destroy skin, subcutaneous tissue, and underlying structures, such as bone, muscle, and fascia. Not surprisingly for infections caused by soil organisms, they tend to occur on the extremities, with the foot being a particularly common site. The chronic, granulomatous inflammation often results in multiple draining sinuses, from which granules (or grains) are extruded. These granules, which usually measure less than 1 mm in diameter, represent colonies of the infectious agent. The granules vary in color depending on the nature of the infecting agent—for example, white grain mycetoma. Microscopically, granules consist of masses of filamentous bacteria or fungal hyphae embedded in a cement-like matrix, which imparts the color that is seen

macroscopically. An immunologic reaction of deposited proteins and immunoglobulins surrounding the organism, known as the Splendore–Hoeppli phenomenon, may be present histopathologically. Fibrosis is often the end result of mycetoma, although not to the extent seen in actinomycosis caused by *Actinomyces israelii*. Bone is commonly involved in *Actinomadura* mycetoma infections.

Mycetomas are caused by true fungi (eumycotic mycetoma) or by aerobic actinomycetes (actinomycotic mycetoma). Colloquially, actinomycotic mycetoma is sometimes called "Madura foot." The most common etiologic agents and their associated characteristics are summarized in Table 15-4. In a study of 366 isolates of aerobic actinomycetes from mycetomas submitted to the Centers for Disease Control and Prevention (CDC) in the late 1980s, *A. madurae* was second in incidence only to *N. asteroides* (11.5% vs. 26%).[127] Most infections occur in tropical countries, particularly in India and Tunisia (*A. madurae*) or in Senegal, Chad, and Somalia (*A. pelletieri*). As noted above, *Nocardia asteroides* causes infections worldwide, whereas *N. brasiliensis* is limited to the southern United States and Central and South America. An uncommon nocardial species, *N. harenae*, was reported as a cause of mycetoma in two patients from Mexico in 2012. Before the isolate was sequenced, it was assumed to be *N. brasiliensis*, but the isolate was susceptible to imipenem and treatment was successful after a combination of amikacin and imipenem was used followed by SXT, minocycline, and dapsone for 2 years.[104]

The vast majority of *A. madurae* infections are superficial, and can be found on any part of the host, depending on where the organisms have entered the host, most commonly the foot,[185] but mycetomas have occurred on the tongue,[133] on the neck, back, and chest of two family members in West Bengal[119] and perianally in another reported patient.[30] An unusual scenario involved a man who had experienced "Madura foot" 10 years prior to its appearance on his spine, abdominal wall, and retroperitoneal space.[27] Systemic infections have been reported in patients with compromised host defenses. *A. madurae* peritonitis has been reported in a patient undergoing continuous peritoneal dialysis.[212] Disseminated *A. madurae* infection has occurred in a patient with AIDS who was also a habitual user of heroin.[128] The diagnosis of mycetoma may be challenging at times because of the difficulty to grow the organism involved; discussion of these challenges can be found in the article by Liu et al.[115] in 2008. Box 15-3 addresses treatment options.

Nocardiopsis *spp.*

Nocardiopsis dassonvillei, the most important species in the genus, forms zigzag chains of spores within a sheath-like structure and has a type III cell wall (*meso*-DAP) with no diagnostic sugars present. The members of the genus *Nocardiopsis* were

removed from the genus *Actinomadura* to accommodate related organisms that lack the unique cell wall carbohydrate madurose. These organisms lack mycolic acids in their cell wall, are non–acid-fast but can produce aerial hyphae. They do not grow in lysozyme.[41] *N. dassonvillei*, ordinarily a soil saprophyte, has been recovered from animals and rarely from human infections. When it has been recovered, the infection has been primarily a mycetoma.[70] Nasal vestibulitis due to *N. dassonvillei* was described recently in an adult diabetic patient.[164] In 1997, Yassin and colleagues reported the isolation of a new species, *N. synnemataformans* from the sputum of a renal transplant recipient[216]; the role of this new species in human disease is unclear.

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The Streptomycetes

Streptomyces *spp.*

Streptomyces spp. are soil organisms that have considerable importance in the industrial and pharmaceutical fields and are responsible for production of two-thirds of the world's naturally occurring antibiotics, including streptomycin. Clinically, *Streptomyces* species, of which there are over 500, are chiefly responsible for actinomycotic mycetoma. In a study of 366 aerobic actinomyces isolates from clinical mycetomas submitted for study at the CDC in the late 1980s, only *N. asteroides* and *A. madurae* were recovered more frequently than *Streptomyces* species.[127] *S. somaliensis*, which can cause actinomycotic mycetoma, has essentially a worldwide distribution, having been recovered from patients with mycetoma in Saudi Arabia, Nigeria, Niger, Sudan, Somalia, South Africa, Venezuela, India, and Mexico.[125] A high proportion of these infections involved the head and neck, producing what is known as "Madura skull." One woman with actinomycotic scalp abscesses due to *Streptomyces* spp. also had blood cultures positive for the same *Streptomyces* spp. for which she was successfully treated with SXT and penicillin.[88] Grains from streptomycete mycetomas are large (2 to 4 mm) and yellow-brown. *Streptomyces anulatus,* also referred to as *S. griseus*, is an organism that causes subcutaneous mycetomas in felines and dolphin, also has been recovered from human mycetomas.[29]

Streptomyces spp. are most often saprophytes, and when isolated in clinical laboratories, they are usually considered as contaminants in clinical specimens. However, a number of nonactinomycotic human infections have been described especially in immunocompromised individuals.[94] Invasive pulmonary disease has been described in HIV patients, in a splenectomized patient with sarcoidosis and in

otherwise immunocompetent hosts.[48,101,160]This organism was found to cause peritonitis, probably contracted from multiple paracenteses, in a chronic alcoholic patient who presented with fever and abdominal pain; no other pathogens were found to explain his symptoms.[43] A brain abscess was found due to *Streptomyces* sp. in a patient who experienced penetrating cerebral trauma with an object heavily contaminated with soil.[162] Rare instances of bacteremia, with and without the presence of an indwelling catheter, have been reported due to *Streptomyces* spp.[25,54,65,134] In one of these patients, the use of a holistic preparation as treatment for breast cancer was implicated as the source of the infection; although the catheter tip was negative for the organism, no other pathogens were isolated and the patient's systemic symptoms disappeared with treatment for the *Streptomyces* sp.[25] A Saudi Arabian patient was reported with endocarditis that followed a Carpentier–Edwards valve replacement for aortic stenosis. Multiple blood cultures were positive for *Streptomyces* and treatment was successful with a combination of antibiotics directed to the isolate in conjunction with valve replacement.[135]Repeated isolation of any aerobic actinomycetes from sterile specimens such as blood cultures needs to be further investigated before being considered as merely a contaminant or saprophyte. Box 15-3 addresses treatment options. With the increased use of molecular techniques and mass spectrometry for microbial identification, and with increasing immunocompromised patient populations, more of these infections may be recognized and reported correctly.[94,122]

Thermophilic Actinomycetes

Thermophilic actinomycetes cause human disease, but they are rarely encountered in the clinical microbiology laboratory. They are more commonly a cause of allergic reactions rather than productive infections. In 1963, Pepys and colleagues defined the antigen present in moldy hay as *Saccharopolyspora rectivirgula*.[145] Subsequently, *Thermoactinomyces vulgaris* was recognized as an additional allergen. The clinical disease, farmer's lung, is an example of hypersensitivity pneumonitis or extrinsic allergic pneumonitis caused by these organisms. The illness can be chronic and disabling, with deterioration of lung function until the offending antigen is removed or avoided. Other similar situations occur after exposure to moldy compost, sugar cane (bagassosis), air conditioners or ventilation ducts. There are a few recent review articles describing the presentation, epidemiology and pathology of hypersensitivity pneumonitis.[76,80,106] Various members of the *Saccharopolyspora*,*Micropolyspora* (*Faenia*) and *Thermoactinomyces* genera are responsible. The taxonomy of these organisms has undergone multiple changes over the years. The use of molecular

techniques for identification may enable more of these isolates to be recognized and classified to further assist in proper diagnosis and treatment.[213]

Miscellaneous Actinomycetes

Dermatophilus

Dermatophilus congolensis is an interesting actinomycete that causes a pustular, exudative dermatitis known as dermatophilosis. This disease is also known as foot rot, pitted keratolysis, or streptotrichosis in many species of animals, including cattle, sheep, horses, goats, deer, swine, squirrels, and domestic cats. A similar presentation has been found in human infections, however, it infrequently produces disease in humans.[67] *D. congolensis* has been associated with hairy leukoplakia on the tongue.[22] The exact mode of transmission is unknown, although most infections occur following direct contact with infected materials, and possibly from the bites of ectoparasites and flying insects. Occupations and avocations that appear at particular risk are primarily those that involve extensive contact with animals, such as veterinarians, abattoir workers and hunters.[5,41] Recently, a 15-year-old girl returning from horseback riding camp developed pustular dermatitis which was due to *Dermatophilus*.[23] Pathologists may encounter these organisms within hair follicles or keratin layers of the soles of the feet in the forms of masses of non–acid-fast, branching, filaments.

Tropheryma whipplei

History and Taxonomy. This bacterium is the latest addition to the aerobic actinomycetes, but the associated disease, intestinal lipodystrophy (Whipple's disease), was first described in 1907 by George Whipple. The bacteria had been identified in affected tissues by light and electron microscopy, but the nature of the etiologic agent remained a mystery until 1991, when Wilson and colleagues used

nucleic acid amplification and sequencing of 16S rDNA in a duodenal biopsy from a patient with Whipple's disease to delineate the nature of the etiologic agent.[123] The morphologic appearance of the bacteria in tissue was not considered typical for either gram-positive or gram-negative bacilli, perhaps because of the intracellular location of the organisms.[123] By molecular analysis, however, the organism aligns best with the gram-positive family. It is most closely related to *Rothia*,[123] *Rhodococcus*, *Arthrobacter,* and *Streptomyces*, and less closely related to the mycobacteria.[208] Less

than a decade later the bacterium was cultivated in human fibroblasts[158] and named *Tropheryma whipplei* (an emended designation from the original *T. whippelii*).[109] The genome of *T. whipplei* has now been sequenced completely, providing useful insights into its nature.[15] It has a surprisingly small genome, demonstrating characteristics of other intracellular bacteria that require external amino acids and are deficient in energy metabolism. The amount of genetic material devoted to coding surface structures is, nonetheless, relatively large, suggesting that interactions between the external aspect of the bacteria and the host are critical to its survival. Finally, there is considerable genetic variability, including phase variation, which may be advantageous for adapting to changing intracellular environments.[15]

Ecology. The ecologic niche of *T. whipplei* remains unknown. It has been detected in human feces and in sewage. It is unclear whether human excrement is a source of infection for humans or these findings simply are the result of excretion by infected individuals.[123] This organism has been detected in human saliva[182] and gastric secretions[52] by nucleic acid amplification methods. It has been detected in duodenal biopsy specimens from patients who do not have Whipple's disease,[52] although other investigators have found *Tropheryma* DNA only rarely in the absence of clinical evidence of the disease.[50,98,120] It is possible, therefore, that humans are reservoirs for the bacteria, but much remains to be determined. Potential problems with the specificity of amplification procedures and the presence of extraintestinal Whipple's disease without overt involvement of the bowel[77] complicate the analysis.

Clinical Disease and Pathology. Whipple's disease is a rare, chronic, multisystem disease.[49,123] The classical triad of infection consists of diarrhea, weight loss, and malabsorption, which reflects the frequency with which the gastrointestinal tract is affected. Other organ systems are also involved, particularly joints and the central nervous system (Box 15-4). The heart may also be affected, as *T. whipplei* is a documented cause of culture-negative bacterial endocarditis.[58,77]

The pathogenesis of Whipple's disease is poorly understood. It is likely that defects in cellular immunity and/or macrophage function are important, but they appear to be specific to this agent, as patients are not usually infected with other opportunistic pathogens. *T. whipplei* is a facultative intracellular pathogen. As is true for such bacteria, the pathologic hallmark of infection is the macrophage. Foamy macrophages, which contain numerous bacteria within vacuoles, were responsible for the yellow lacteals that George Whipple observed in the small bowel. The bacteria in these cells can be stained by the PAS method, but this technique is not specific. Other bacteria, particularly *Mycobacterium avium* complex, are PAS-positive and occur within macrophages, particularly in patients with advanced HIV infection. *Tropheryma* and

M. avium may be differentiated with an acid-fast stain, as *Tropheryma* is not acid-fast. *Rhodococcus equi*, present in macrophages, may also be considered in the differential diagnosis usually outside of the intestinal tract. Ultimately, immunologic staining of tissues, culture, or nucleic acid amplification is required for diagnostic resolution. A recent review of Whipple's disease by Mendolara et al.[4] includes an unusual scenario wherein a young girl was recognized during emergency surgery for gastrointestinal obstruction and perforation. In extraintestinal tissues, noncaseating, sarcoid-like granulomas may be observed.[123] There appears to be an increase in the reports of patients with cardiac infection with *T. whipplei* without any evidence of gastrointestinal involvement.[117,206] With the use of molecular techniques, including quantitative PCR applied to gastrointestinal tissue biopsies to delineate the degree of otherwise undiagnosed disease and also as a result of the many microbiome projects, the scope of Whipple's disease and infection with *T. whipplei* may increase and be more fully understood.[50,118]

BOX 15-4

Extraintestinal Sites of Whipple's Disease: An Analysis of 52 Cases[a]

Extraintestinal Site	No. (%) of Cases
Joints	43 (83)
Nervous system	11 (21)
Skin and mucous membranes	9 (17)
Heart and blood vessels	9 (17)
Lung and pleura	7 (13)
Eye	5 (10)

[a] Multiple sites affected in some patients.
Adapted from reference 21.

Laboratory Diagnosis of Infections Caused by Aerobic Actinomycetes

Primary Isolation

N. asteroides and other aerobic actinomycetes are aerobic organisms that grow on a variety of bacteriologic media, including sheep's blood agar, brain–heart infusion agar, or SAB without antibiotics. They can be inhibited, however, by chloramphenicol, penicillin and streptomycin present in selective media. They also grow on media designed for the recovery of mycobacteria. *N. asteroides* grows well at 25°C, 35°C to 37°C, and at 42°C to 45°C. Incubation at the higher temperature permits growth of *N. asteroides* complex, whereas many other bacteria will be inhibited. Growth may take from 2 days to 4 weeks of incubation. However, in many instances of true infection, *Nocardia* can be isolated in ≤5 days. Growth is enhanced by incubation in 10% CO_2. *Nocardia* species can

be recovered from contaminated specimens using modified Thayer–Martin (MTM) medium.[139] Shawar and associates took advantage of the peculiar ability of these bacteria to grow with paraffin as a sole source of carbon to develop a selective, chemically defined medium that contained paraffin on a coated glass rod placed into a carbon-free broth.[172] If *Nocardia* is present in this system, then growth appears on the rod just above the surface of the broth. Similarly, Ayyar and colleagues found paraffin agar an inexpensive selective medium for *Nocardia* spp., being superior to either MTM or the paraffin bait technique of Shawar.[9]

Selective buffered charcoal-yeast extract agar (BCYE), commonly used for the recovery of *Legionella* spp. from respiratory specimens, also facilitates the recovery of *Nocardia* spp. from sputum and other specimens that may be contaminated with mixed bacteria.[63,97] The use of selective BCYE agar, to which polymyxin B, anisomycin, and vancomycin have been added, and pretreatment of the specimen by an acid wash (Chart 10-1) improved recovery of *Nocardia* spp. from 8% to 33% for the former and to 67% for the latter.[97]

N. asteroides may survive the usual *N*-acetyl-L-cysteine digestion procedure (without NaOH) that is used for recovery of mycobacteria from sputum or bronchial washings.[138] It has been recommended that cultures of sputum and bronchoalveolavage specimens submitted for *Nocardia* culture be done both before and after the digestion procedure, although this approach doubles the work.

Colonies on LJ media often develop within 1 to 2 weeks. They may be similar in appearance to atypical mycobacteria (see Chapter 19). *Mycobacterium* spp., however, do not produce aerial hyphae, are fully acid-fast, and differ biochemically from nocardiae. The branched filaments of *Nocardia* that may not always be seen in smears prepared from growth on solid media can sometimes be demonstrated from growth in

broth cultures, whereas mycobacteria do not usually branch, although some rods may be elongated.

Differentiation of Nocardia *From Other Genera of Aerobic Actinomycetes*

Infections of humans with the genera *Streptomyces*, *Nocardiopsis*, *Rhodococcus*, *Actinomadura*,*Gordonia*, *Tsukamurella,* and *Dermatophilus* are far less common than infections caused by *Nocardia* spp. Some characteristics that may aid in differentiating among and within genera of aerobic actinomycetes and other related bacteria are given in Table 15-4. The presence of *Nocardia* and *Streptomyces* spp. can be suspected on the basis of a few simple characteristics. The typical colonies of *Nocardia* and *Streptomyces* spp. are dry to chalky in consistency, and usually heaped or folded. *Nocardia* spp. are commonly some shade of yellow or light orange, whereas *Streptomyces* spp. are most frequently gray-white. (Image Plate 15-1A) Both groups produce colonies with a pungent musty basement odor. The colonies of *Rhodococcus equi* lack the musty odor and often have a pink or salmon color on agar media (Image Plate 15-1B). *Gordonia* spp. produce rough and wrinkled colonies with some pigment, whereas *Tsukamurella* often produces smaller colonies that are dry, wrinkled, and without pigment.

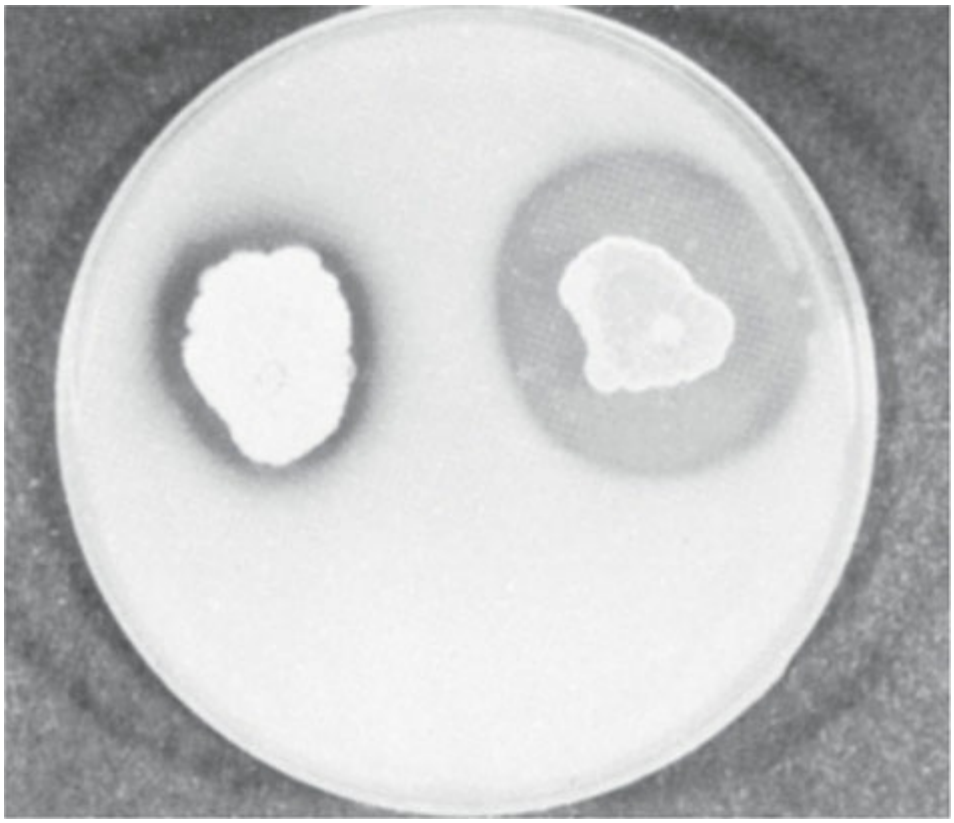
Typically, Gram's stain of material from a colony of *Nocardia* or *Streptomyces* will show delicate, branching filaments no more than 1 μm in diameter (Image Plate 15-1C). *Nocardia* spp. are partially acid-fast (i.e., they do not decolorize when treated with 1% H_2SO_4, but will decolorize when the more active HCl decolorizer used in the Ziehl–Neelsen or Kinyoun stains is applied) (Box 15-2). *Streptomyces* spp., in contrast, are not partially acid-fast. The acid-fastness property of *Nocardia* may be enhanced by growing the organism on certain media, such as Middlebrook 7H11 agar or casein agar (see discussion of identification techniques below). If the unknown organism is not acid-fast, a report of "non–acid-fast aerobic actinomycete" can be made, but such a report should be delayed until adequate attempts to improve the acid-fastness have been made.

Rhodococcus equi do not form the long filaments seen in isolates of *Nocardia* and *Streptomyces*. They are gram-positive coccobacilli that may cluster like Chinese characters, reflecting their former taxonomic home in the genus *Corynebacterium* (Image Plate 15-1D). Isolates can be partially acid-fast, but this depends on the media and age of the colony (Image Plate 15-1E). If *R. equi* is grown in broth cultures, the coccoid appearance of the bacteria may be more apparent.

Growth in the presence of lysozyme is useful for identifying *Nocardia* spp. (Table 15-4; Chart 15-2), particularly for those strains that are weakly acid-fast. All *Nocardia* species are resistant to lysozyme and will grow in the presence of lysozyme within 5 to 10 days. A control tube of glycerol broth without lysozyme should always be

simultaneously inoculated and included in addition to positive and negative controls. For specific directions about preparation of lysozyme and how to perform the test, refer to the Manual of Clinical Microbiology, 2011.[8]

The differential ability of the various nocardiae and related aerobic actinomyces to hydrolyze casein, tyrosine, xanthine, and hypoxanthine is one of the mainstays of conventional phenotypic identification protocols in clinical laboratories. The procedure is described in Chart 15-1. The differential plates are inoculated with the unknown organism and incubated for up to 3 weeks at 30°C and observed for hydrolysis. This appears as clearing of the medium around the colonies. An example of casein hydrolysis by a colony of *Streptomyces* spp. is shown in Figure 15-4. Differentiation of certain species cannot be accomplished solely with the analysis of



■ **FIGURE 15-4** Casein agar plate illustrating the hydrolytic action of two species of *Streptomyces*.

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hydrolysis. Characterization of the ability to decompose carbohydrates or use them as a sole carbon source may be necessary.[132]Differentiation of some members of the

Nocardia complex is facilitated by analysis of the temperature of growth,[12] by the ability to opacify Middlebrook 7H10 or 7H11 media (Table 15-5),[26,61,99] and by the antimicrobial susceptibility profile (Table 15-6).[33]

Molecular tools for specific identification of the aerobic gram-positive rods, including the aerobic actinomycetes, are becoming more widely available as is the use of mass spectromety.[20,143,193,194] Mishra and colleagues have devised a key for more detailed differentiation of the nocardiae and the streptomycetes than has been modified and presented in Table 15-5.[132] The use of carbohydrate utilization in test

TABLE 15-5 Biochemical and Physiologic Tests for the Identification of Selected Medically Important Aerobic Actinomycetes

Organism	Modified Acid-Fast	Growth in Lysozyme	Decomposition of					
			Casein	Tyrosine	Xanthine	Hypoxanthine	Urease	Gelatin Hydrolysis
Nocardia asteroides	+	+	–	–	–	–	+	–
Nocardia brasiliensis	+	+	+	+	–	+	+	+
Nocardia otitidiscaviarum (*N. caviae*)	+	+	–	–	+	+	+	–
Nocardia transvalensis	+	+	–	–	–	+	+	–
Nocardia farcinica	+	+	–	–	–	–	+	–
Nocardia nova	+	+	–	–	–	–	V	–
Nocardia pseudobrasiliensis	+	+	+	+	–	+	+	+
Rhodococcus equi	+	–	NA	–	–	–	NA	NA
Rhodococcus erythropolis	+	+	NA	–	–	–	NA	NA
Rhodococcus rhodnii	+	+	NA	+	–	–	NA	NA
Rhodococcus rhodochrous	+	–	NA	–	–	–	NA	NA
Gordonia bronchialis	+	–	–	–	–	–	NA	NA
Gordonia rubropertincta	+	–	NA	NA	NA	NA	NA	NA
Gordonia sputi	+	–	NA	NA	NA	NA	NA	NA
Gordonia terrae	+	–	NA	NA	NA	NA	NA	NA
Tsukamurella inchonensis	+	+	+	–	–	+	NA	NA
Tsukamurella paurometabola (*Rhodococcus aurantiacus*)	+	+	–	–	–	–	+	NA
Tsukamurella pulmonis	+	+	–	–	–	–	NA	NA
Tsukamurella strandjordae	+	+	–	–	–	–	NA	NA
Tsukamurella tyrosinosolvens	+	+	–	+	+	+	NA	NA
Tsukamurella wratislaviensis	+	+	NA	+	+	+	NA	NA
Dietzia (*Rhodococcus*) *maris*	–	–	–	–	–	–	+	NA
Dermatophilus spp.	–	NA	+	–	–	NA	+	+
Actinomadura madurae	–	–	+	+	–	+	–	+
Actinomadura pelletieri	–	–	+	+	–	+	–	+
Streptomyces somaliensis	–	–	+	+	–	–	–	+
Streptomyces griseus	–	–	+	+	+	+	V	+
Nocardiopsis dassonvillei	–	–	+	+	+	+	+	+

+, positive; –, negative; V, variable; NA, not available.

TABLE 15-6 In Vitro Susceptibility Profiles of *Nocardia* spp.

Drug Pattern (DP)/Species	Amk	Amp	A/C	Carb	CTX	CFTR	Cipro	Clari	Eryth	Gent	IMI	Kana	Linez	Mino	Sulfa	Tobra
***N. ASTEROIDS* COMPLEX**																
DP I	S	S	S	S	S	S	R	R	R	—	R/S	—	S	S/I	S	—
DP II	S	S	—	S	—	S	S	—	—	R	S	S	S	S/I	S	S
DP III	S	R	R	R	S	S	R	S	S	—	S	—	S	S/I	S	—
DP IV	R	R	S/R	—	S	S	S	R	R	R	S	R	S	S/I	S	R
DP V	S	R	S	—	R	R	S	R	R	R	S	R	S	S/I	R/S	R
DP VI	S	R	R	—	S	S	R	R	R	—	S	S	S	S/I	S	S
N. brasiliensis	S	R	S	S	S/R	S/R	R	R	R	S	R	R	S	S/I	S	S
N. pseudobrasiliensis	S	R	R	S	S/R	S/R	S	S	—	S	R	R	S	S/I	S	S
N. otitidiscavarium	S	R	R	R	R	R	S	—	—	—	R	R	S	—	S	—

DP I, *N. abscessus*; DP II, *N. brevicatena/paucivorans* complex and unnamed group; DP III, *N. nova* complex, which includes *N. nova*/*N. veteran*/*N. africanum*/*N. kruczia-kiae*; DP IV, *N. transvalensis* (which includes *N. wallacei*); DP V, *N. farcinica*; DP VI, *N. asteroides* sensu stricto or *N. asteroides* complex and *N. cyriacigeorgica* and unnamed group.
Amk, Amikacin; Amp, Ampicillin; A/C, Amoxicillin-Clavulanate; Carb, Carbenicillin; CTX, Cefotaxime; CFTR, Ceftriaxone; Cipro, Ciprofloxacin; Clari, Clarithromycin; Eryth, Erythromycin; Gent, Gentamycin; IMI, Imipenem; I, Intermediate; Kana, Kanamycin; Linez, Linezolid; Mino, Minocycline; R, Resistant; Sulfa, Sulfamethoxazole; Tobra, Tobramycin; —, Not Determined or Not Commonly Used.
Modified from personal communication with Barbara Brown–Elliott and Dr. Richard Wallace. These are "usual patterns and subject to variations, as resistance may appear in isolated strains."

tubes or with a kit system has been assessed for the identification of species of *Nocardia,* but as more and more species of *Nocardia* are identified, it will be more difficult to identify *Nocardia* to the species level with phenotypic means alone.

Thin-layer chromatography has been helpful alone or in conjunction with phenotypic or molecular methods for identification. Differences in the cell wall composition are used in some research laboratories or reference laboratories for taxonomic purposes. The primary constituents of the cell walls are summarized in Table 15-2 (Fig. 15-1). Most of the nocardioform organisms, including *Nocardia* and *Rhodococcus*, have a type IV cell wall composition (i.e., the significant constituents are *meso*-DAP, arabinose, and galactose, with mycolic acids present). *Streptomyces* species have type I cell walls (i.e., no L-DAP and no diagnostic carbohydrates). Other chemotaxonomic tests, in addition to determining cell wall type, include analyses of menaquinones, long-chain fatty acids, phospholipids, and mycolic acids. These studies are not practical, nor are they necessary for differentiation of most isolates of *Nocardia* and *Rhodococcus* in the clinical microbiology laboratory. If definitive identification is necessary, microbiologists may wish to refer isolates to a reference laboratory.

Nucleic acid amplification techniques and DNA sequencing are useful both for direct detection of aerobic actinomycetes in clinical specimens and for identification of isolated bacteria.[13,42,100,107,111,143,163,181,201–203] Cloud and colleagues have suggested that sequencing of 16S rDNA is more accurate than a combination of conventional biochemical tests and antimicrobial susceptibility profiles for identification of *Nocardia*

spp.[35] Patel et al. used partial 16S rDNA gene sequencing as compared to biochemicals, fatty acid analysis, drug susceptibility testing, and PCR-restriction analysis of the 65 *hsp* gene for the identification of 74 *Nocardia* isolates; some species could not be entirely differentiated and hence the introduction of the "complex" identification that many laboratories used.[143] Partial rRNA sequencing was used to successfully identify *R. equi*, to distinguish among species of *Gordonia* and *Streptomyces* and identify *Actinomadura* to the genus level. Some difficulty was found with *Tsukamurella* strains.[143] Molecular methods have been used to identify *Gordonia,*[16] *Dietzia,*[150,151] *Tsukamurella,*[173,186] and *Nocardiopsis dansonvillei*.[13] As with any molecular sequencing methods, the validity of the database used is essential to obtaining the most appropriate identifications.[40,130]

All clinical microbiologists are becoming very familiar with the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for the identification of aerobic and anaerobic bacteria; so it is not surprising that identification of aerobic actinomycetes would also benefit from use of this new technique. Farfour et al. used Bruker MS with Andromas software to identify 659 aerobic gram-positive bacilli. Over 98% of the strains, which included 46 *Nocardia* spp. and two *R. equi,* were identified correctly to the species level.[57] Verroken et al. demonstrated that MALDI-TOF could be used successfully to identify *Nocardia* sp. in <1 hour with an inexpensive method[193]; Vila et al. reported the use of MALDI-TOF for identification of *R. equi* as well as relevant *Corynebacterium* spp. and *Arcanobacterium hemolyticum*.[194]

It is important that clinical microbiologists keep abreast of these advanced methods of identification, since they will provide often species-level identification of difficult-to-identify microorganisms. The appropriate identification, in turn, affords more targeted decision-making by clinicians, which

should translate into and the prompt administration of appropriate therapeutics. It will be increasingly difficult if not impossible for laboratories to rely on phenotypic methods alone for the identification of the ever-increasing aerobic actinomycetes.

Identification of Thermophilic Actinomycetes

The thermophilic actinomycetes are rarely encountered in the clinical laboratory because they are environmental organisms that produce disease in humans by an allergic reaction, rather than productive infection.[145] Their ability to grow at elevated temperatures provides a useful means by which to evaluate their presence in

environmental specimens, if desired. Protocols for identification of these bacteria have been suggested.[82,105] As mentioned above, use of 16S rRNA sequencing and other molecular techniques for identification of these unusual organisms will increase in the future.[140,213]

Identification of Tropheryma whipplei

This actinomycete can be cultured, but only with difficulty.[159] In most instances, the diagnosis will be made by clinical and histologic criteria, and through the use of molecular methods. Immunohistochemical identification of the organism in sectioned tissue has been demonstrated.[10,112,159] Alternatively, the organism can be recognized by nucleic acid amplification techniques.[157] Although experience is limited, it has been suggested that molecular methods may be more useful than histology for monitoring the course of patients with Whipple's disease.[157] Although PCR-based methods are important diagnostic tools, it is important to recognize that *T. whipplei* DNA can be detected in the absence of overt clinical disease.[52,182] The significance of these results is unclear, but the diagnosis of Whipple's disease should not be based solely on molecular detection of bacterial DNA. The results of molecular studies should be correlated with clinical and, if available, histopathologic findings. These techniques are currently used primarily in research or large reference laboratories; microbiologists must remain alert to the future availability of commercial products.

In Vitro Susceptibility of Nocardia *and Related Bacteria to Antimicrobial Agents and Therapy for Infections*

The antimicrobial susceptibility patterns of aerobic actinomycetes are useful for taxonomists, clinical microbiologists (as a clue to the identification of an isolate), and clinicians for treatment of infected patients. The pattern of susceptibility for various *Nocardia* species is summarized in Table 15-6. Standardization of susceptibility testing for clinical use has been published by the Clinical and Laboratory Standards Institute (CLSI) and includes methods for performing the susceptibility test via a broth microdilution and breakpoints for interpretation of the results.[33] These are the guidelines recommended for use.

If the results of sulfonamide testing are difficult to interpret in broth microdilution and a disk diffusion test, then an Etest is recommended. The inocula for performing a susceptibility test with the aerobic actinomycetes should be prepared from a blood agar or trypticase soy agar plate that contains sufficient growth. The final organism concentration should approximate 1.0×10^4 to 5.0×10^4 colony-forming units (CFU)/well. For many of the *Nocardia* spp., which tend to clump, care must be taken to emulsify the inoculum as well as possible. The broth microdilution panels should be

incubated in ambient air at 35°C ± 2°C for 3 days; some species such as *N. nova* may require up to 5 days. However, *R. equi* and some *Tsukamurella* spp. can be read within 24 to 48 hours. Examples of interpretation of the wells of the microtiter plates are given in the CLSI document and should be referred to frequently, especially when first beginning to perform susceptibility testing for the aerobic actinomycetes. Table 9 in the CLSI document provides breakpoints for *Nocardia* spp. For *R. equi* it is recommended that breakpoints for *S. aureus* should be used but should be considered tentative until more data is generated. The footnote in Table 9 of the CLSI M24-A2 document states that the breakpoints provided are to be reported as "tentative" for other aerobic actinomycetes pending accumulation of further information in the future.[33]

A multisite study demonstrating the reproducibility of the CLSI broth microdilution method for susceptibility testing of *Nocardia* sp. was published in 2012. In addition, it provided isolates that could be used as reference isolates for laboratories to use in order to successfully perform the test.[37] For laboratories that perform these tests rarely, it is judicious to send isolates to a reference laboratory that has considerable experience with aerobic actinomycetes, however it behooves all laboratorians to understand the methods and how to interpret the results.

Many β-lactamases have been characterized in various *Nocardia* spp. and these are mainly penicillinases with less activity against cephalosporins.[200] Clavulanic acid can inhibit many of the β-lactamase enzymes and combination of β-lactam antibiotics plus β-lactamase inhibitors do demonstrate activity against some *Nocardia* spp. The enzymes found in *N. farcinica* are distinct from those found in other species of *Nocardia* and are homogeneous as compared to the diversity of β-lactamases found in *N. brasiliensis*.[180,197] The drugs of choice for treatment of nocardiosis, even in immunosuppressed patients, remains the sulfonamides (e.g., sulfadiazine, sulfisoxazole, and triple-sulfonamide combinations). The combination of SXT is preferred by most clinicians,[19,198] but the question of whether the combination is more effective than a sulfa drug alone remains open.[198] In vitro studies may be needed to aid in the selection of antimicrobial agents for patients who are allergic to sulfonamides or who are infected with *N. otitidiscaviarum* and some species of the *Nocardia* complex that demonstrate inconsistent susceptibility to sulfonamides. Imipenem, minocycline, amikacin and linezolid are potential choices, based on in vitro studies and some clinical trials along with clinical experiences in the literature.[136]

Alternatively, desensitization to sulfonamides may be considered. There has been a recent report from the CDC[189] that data from 10 years of testing demonstrated as much as 61% sulfamethoxazole resistance among strains of *Nocardia* spp.; however, a subsequent article by Brown–Elliott et al. suggested that those results could be due to a misinterpretation

of in vitro testing results, since their review of testing by six large laboratories in the United States demonstrated only a 2% resistance. Inclusion of quality control strains to assist in correct reading of microtiter trays will be very useful.[18] Resistance to amikacin was recently described in a new species of *Nocardia*, *N. amikacinitolerans*.[56] Resistance to imipenem is common among strains of *N. brasiliensis*.

For mycetomas, a combination of surgical and pharmacologic approaches, often empirical, is indicated. To prevent antibiotic resistance and cover all clinical isolates of *Nocardia* spp., a combination of SXT, amikacin, and ceftriaxone or imipenem is preferred for patients with local or disseminated disease.[19] A recent article tested strains of *Streptomyces* from patients with a mycetoma and found that all were resistant to sulfamethoxazole, but were susceptible to novobiocin, doxycycline, and gentamicin.[79] Other articles have suggested use of oxalidinones such as linezolid based on in vitro data.[162]

Rhodococcus equi is susceptible to vancomycin, which is considered by some to be the therapy of choice; most isolates are susceptible to erythromycin, aminoglycosides, rifampin, and imipenem; there are data available about the successful use of linezolid in management of *R. equi* infections.[3,116,214] Experience with treatment is limited. However, recent review articles suggest that a combination of surgical debridement and antibiotics is appropriate. In vitro synergy testing was reported recently with many drug combinations including macrolides and rifampin; no clinical relevance was made to correlate with in vivo results, however.[66]There are no treatment recommendations for *Gordonia* infections. CLSI does recommend that breakpoints for *Nocardia* can be used for other aerobic actinomycetes such as *Gordonia* and recommends that testing for the latter should include amikacin, amoxicillin clavulanic, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, SXT, and tobramycin.[33,211] In a recent article from China, three species of *Tsukamurella*, responsible for eight infections, had different drug susceptibility patterns; *T. pulmonis* was the most resistant pathogen, with higher minimum inhibitory concentrations of clindamycin (>2 mg/L), erythromycin (2 mg/L), and tetracycline (8 mg/L) than those for *T. tyrosinolvens* or *T. spumae*.[114] The in vitro susceptibility of 24 strains of *Actinomadura madurae* to a new oxazolidinone (DA-7867), gatifloxacin, moxifloxacin, and garenoxacin by using a broth microdilution method was determined; the most active was DA-7867.[191] An article about a rare instance of brain abscess caused by *Streptomyces* sp. includes information from the CDC on successful use of linezolid and amikacin based on in vitro susceptibility data.[162] A review article of invasive

*Streptomyces i*nfections found that isolates were consistently susceptible to amikacin; frequently susceptible to imipenem, clarithromycin or erythromycin, minocycline, and SXT; and infrequently susceptible to ciprofloxacin and ampicillin.[94] Less information is available for treatment and susceptibility testing of other aerobic actinomycetes.

The recommended therapy for Whipple's disease is prolonged (at least 1 year) treatment with SXT.[123] Tetracycline therapy, previously used commonly, is associated with an unacceptably high frequency of relapses. Especially in seriously ill patients, an initial course of parenteral therapy with ceftriaxone or a combination of penicillin and streptomycin is administered before long-term oral SXT therapy is begun.[123]

Concluding Comments

Although uncommonly isolated in clinical laboratories and rarely encountered in clinical practice as compared to other aerobic bacteria, the aerobic actinomycetes are a very diverse group of bacteria that have traditionally been difficult to identify and consequently may have been misidentified, signed out as "unknown" or discarded as contaminants.[168,183]Unfortunately, conventional phenotypic methods will not be sufficient to allow for species-level and sometimes not even for genus-level identification of many of these. Susceptibility testing is not performed in all laboratories for these isolates, so in addition, the appropriate agents might not be recognized to assist the clinician in making appropriate therapeutic decisions. There is good evidence, however, to believe that the newer molecular and mass spectrometry methods will be very helpful in overcoming these difficulties. At the moment, only some reference laboratories are capable of performing these tests, but that will hopefully change in the future.

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CHAPTER 16

The Anaerobic Bacteria

thePoint For additional ancillary materials related to this chapter, please visit the point.

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Anoxomat

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Summary

This chapter includes information about the present taxonomy and clinical relevance of anaerobes in human infections; traditional methods for isolation and identification of anaerobic bacteria; nontraditional methods for identification of anaerobes, including DNA sequencing and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometery; in vitro susceptibility testing; and management of infections involving anaerobes. An Appendix has been added to the chapter that contains some of the more conventional phenotypic procedures for identification of anaerobes that have been included in past chapters of this text. Greater emphasis is placed on the nonphenotypic classification and identification of anaerobes, but we have kept the traditional methods as much as possible or provided references for presumptive identification of anaerobes. Updates on anaerobic diseases such as Lemierre's disease, botulism, tetanus, bacterial vaginosis, and *C. difficile* enterocolitis are included. A summary of recent changes in the susceptibility testing of selected anaerobes concludes the chapter.

Introduction to Anaerobic Bacteria

Classification of Bacteria Based on Their Response to Oxygen

Although defined in various ways by different authors, a practical working definition is that *obligately anaerobic bacteria* are those bacteria that grow in the absence of free oxygen but fail to multiply in the presence of oxygen on the surface of nutritionally adequate solid media incubated in room air or in a CO_2 incubator (i.e., 5% to 10% CO_2 in air). The amount of O_2 in a CO_2 incubator, or in a candle extinction jar, is considerable (i.e., about 18% to 19%). In practice, anaerobic bacteria are most often recognized in the clinical laboratory following aerotolerance tests of colonies that grew on primary isolation plates incubated anaerobically (see below). Thus, most anaerobes identified in the clinical laboratory grow initially on anaerobic blood agar, or on one of

the selective anaerobic media, or in an enrichment broth incubated anaerobically, but not on blood agar or chocolate agar plates incubated aerobically or in a CO_2 incubator.

It is an oversimplification to discuss the anaerobes as if they fit uniformly in one large group, just as it is an oversimplification to refer to all bacteria that grow in room air as aerobes. Thus, several terms, including *obligate aerobe, facultative anaerobe, microaerophile, aerotolerant anaerobe,* and *obligate anaerobe* (strict and moderate), have been used to subdivide bacteria based on their relationships to oxygen. These terms reflect a continuous spectrum of bacteria that cannot tolerate oxygen to those that require it for growth.

Obligate aerobes, including species of *Mycobacterium* and *Pseudomonas*, require molecular oxygen as a terminal electron acceptor, resulting in the formation of water, and do not obtain energy by fermentative pathways. However, it is not uncommon to find *P. aeruginosa* growing scantily on anaerobically incubated media in the clinical laboratory, since these bacteria can use nitrate in the medium as a terminal electron acceptor, through anaerobic respiration, in place of O_2. In contrast, molecular oxygen varies in its toxicity to different species of anaerobic bacteria and is not a terminal electron acceptor for the anaerobic bacteria. In general, the clinically important anaerobes obtain their energy through fermentative pathways, in which organic compounds such as organic acids, alcohols, and other molecules serve as final electron acceptors.

Anaerobes are divided into two major groups: the obligate anaerobes and the aerotolerant anaerobes. The obligate anaerobes have been further subdivided into two groups based on their ability to grow in the presence of or to tolerate small amounts of oxygen. Strict obligate anaerobes are not capable of growth on agar surfaces exposed to O_2 levels above 0.5%. Atmospheric oxygen is highly toxic for these microorganisms for reasons that are not entirely known. Examples of these bacteria include *Clostridium haemolyticum, C. novyi* type B, *Selenomonas ruminatium,* and *Treponema denticola.* The second group of obligate anaerobes, the moderate obligate anaerobes, is bacteria that can grow when exposed to oxygen levels ranging from about 2% to 8% (average, 3%). Examples of these bacteria include members of the *Bacteroides fragilis* and the pigmented *Prevotella-Porphyromonas* groups (i.e., formerly the pigmented *Bacteroides* group), *Fusobacterium nucleatum, and C. perfringens*.[299]

The term *aerotolerant anaerobe* is used by some microbiologists to describe anaerobic bacteria that will show limited or scant growth on agar in room air or in a 5% to 10% CO_2 incubator, but show significantly better growth under anaerobic conditions. Examples of these bacteria include *Clostridium carnis, C. histolyticum,* and *C. tertium*. Most of the anaerobes isolated from properly selected and collected specimens in the clinical laboratory fit into the moderate

obligate anaerobe or aerotolerant category. These organisms are more tolerant to the toxic effects of oxygen than the strict obligate anaerobes, but are still killed by oxygen unless anaerobic conditions are maintained properly during specimen collection and transport to the laboratory and during the steps required for processing of specimens and isolation and identification, as discussed in later sections of the chapter. Strict obligate anaerobes are rare in infections of humans, but both the moderate and the strict obligate anaerobes are found in a variety of nonpathogenic habitats (e.g., the gastrointestinal [GI] tract from and the oropharynx to large intestine), as part of the normal microbiota.

The facultative anaerobes (e.g., *Escherichia coli* and *Staphylococcus aureus*) grow under either aerobic or anaerobic conditions. They use oxygen as a terminal electron acceptor or, less efficiently, can obtain their energy through fermentation reactions under anaerobic conditions. Grown aerobically, facultative anaerobes obtain much more energy when they completely catabolize a molecule of glucose to CO_2 and H_2O, than when grown anaerobically. The microaerophiles require oxygen as a terminal electron acceptor, yet these bacteria do not grow on the surface of solid media in an aerobic incubator (i.e., 21% O_2) and grow minimally, if at all, under anaerobic conditions. An example of a microaerophile is *Campylobacter jejuni*, which grows optimally in 5% O_2; the gas mixture of the incubation environment commonly used for recovering this organism in clinical laboratories is 5% O_2, 10% CO_2, and 85% N_2. *Campylobacter* spp. are discussed in another chapter as are *Capnocytophaga* spp. For this edition, they have been removed from tables in this chapter, although you will find them listed in older editions.

Reasons for Anaerobiosis

Through the years, bacteriologists working on the development of improved media and systems for cultivation of anaerobes have focused on two fundamental limiting factors that may affect the growth of anaerobes. The first and most important of these are the inhibitory effects of atmospheric oxygen and its toxic derivatives. The second limiting factor of concern is the oxidation–reduction potential of the culture medium. Some of the strictest obligate anaerobes (e.g., *Clostridium haemolyticum* and *C. novyi* type B) are killed when exposed to atmospheric oxygen on the open laboratory bench for 10 minutes or longer. Conversely, most of the moderate obligate anaerobes encountered in human infections tolerate exposure to oxygen for longer times, from 25 minutes to a few hours. There are likely multiple reasons why anaerobes vary in their tolerance to oxygen, but one idea is that the oxygen tolerance of many moderate obligate anaerobes depends on their production of superoxide dismutase (SOD),

catalases, and possibly peroxidase enzymes, which are protective against toxic oxygen reduction products.[238,470]

A popular notion is that exposure to atmospheric O_2 results in a series of reactions within the bacterial cells, possibly reactions mediated by flavoproteins, results in production of the negatively charged superoxide radical (O_2-), hydrogen peroxide (H_2O_2), and other toxic oxygen reduction products.[325] The superoxide anion and H_2O_2 may react together to produce free hydroxyl radicals (OH.), the most powerful biologic oxidants known, and there may also be the production of toxic singlet oxygen ($1O_2$) through the reaction of superoxide anions with free hydroxyl radicals. SOD catalyzes the conversion of superoxide radicals to less toxic hydrogen peroxide and molecular oxygen. Catalase catalyzes the conversion of hydrogen peroxide to form water and oxygen. Several species of oxygen-tolerant moderate obligate anaerobes were shown to produce SOD and the level of SOD produced correlated with the level of oxygen tolerance and virulence of the organism.[470] Other studies have not shown such correlations. Several species of anaerobes have been shown to produce catalase (e.g., members of the *Bacteroides fragilis* group, *Propionibacterium acnes,* and others), as well as SOD, and some anaerobes that are relatively oxygen-tolerant or aerotolerant (e.g., *Clostridium tertium*) produce neither SOD nor catalase. Rolfe et al. observed that the degree of oxygen tolerance of anaerobic bacteria is related to the proportion of the bacterial cells in the population that survive following exposure to oxygen.[398]

The oxidation–reduction potential (abbreviated "redox" potential or Eh) of a culture medium, expressed in volts or millivolts, is affected by pH (i.e., the hydrogen ion concentration); thus, redox potential is commonly expressed at neutral pH (pH 7) as Eh. Reducing agents, such as thioglycolate and L-cysteine, may be added to anaerobic transport media and to certain culture media to help maintain reduced conditions (i.e., a low Eh) in the medium. A positive Eh (e.g., as indicated by pink color of resazurin indicator in certain media, or a blue color of methylene blue indicator in other media) means that the medium is oxidized. In nature, the upper limit of Eh is +820 mV, which might be found in some environments that have considerable oxygenation. Oxidizing conditions prevail in healthy human tissue that is well-oxygenated and has an intact blood supply (e.g., the Eh is about +150 mV). In contrast, the lower limit of Eh in nature is about −420 mV. An anaerobic environment (e.g., an abscess or necrotic tissue) or a culture medium rich in hydrogen might have such a low Eh. The large bowel of humans, containing enormous numbers of strict obligate anaerobes, has an Eh of about −250 mV. In an excellent study reported by Walden and Hentges,[500] some of the misconceptions about Eh effects on anaerobic bacteria were dispelled. They determined that oxygen inhibited multiplication of *C. perfringens, B. fragilis,* and *P. magnus* whether the medium was at a negative redox potential (Eh =−50 mV) or at a

positive redox potential (Eh near +500 mV). In the absence of O_2, these organisms were able to multiply even when the Eh was maintained at +325 mV, by the addition of potassium ferricyanide (an oxidizing agent). From their work, a practical conclusion was drawn that the purging of oxygen from the cultural environment, to avoid oxygen toxicity, is probably more important than the establishment of a low Eh.[500] Thus, the rapid achievement and maintenance of a low oxygen tension, or the absence of oxygen, is an essential requirement for the successful cultivation of anaerobes in modern anaerobic systems (e.g., anaerobic jars and glove boxes used for incubation of anaerobes in the clinical laboratory).

Habitats for Anaerobes

Anaerobic bacteria are widespread in soil, marshes, lake and river sediments, the oceans, sewage, foods, and animals.

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In humans, anaerobic bacteria normally are prevalent in the oral cavity around the teeth, in the GI tract, especially in the colon, where they outnumber coliforms by at least 1,000:1, and in the orifices of the genitourinary (GU) tract, and on the skin.[160,217] Most of these anaerobic habitats have both a low oxygen tension and reduced Eh resulting from the metabolic activity of microorganisms that consume oxygen through respiration. If the oxygen is not replaced, anaerobic conditions are maintained in the environment. A brief summary of commonly encountered anaerobes in the normal microbiota of the human body is given in Box 16-1.

Taxonomic Classification and Nomenclature

The anaerobes include essentially all morphologic forms of bacteria: gram-negative rods; curved, spiral-shaped, and spirochete forms; gram-positive and gram-negative cocci; gram-positive non–spore-forming (NSF) rods; and spore-formers. Based on their morphologic features as observed in Gram-stained preparations and the absence or presence of spores, the anaerobic bacteria are broadly classified as shown in Table 16-1. Several of the genera in this list have been found only in nonpathogenic habitats (e.g., sites harboring normal microbiota such as the oral cavity, lower intestinal tract [as evidenced in feces], GU tract, and skin of humans or various animals) and have not been reported to occur in diseases of humans. Many of these genera will not be discussed further.

There have been many recent developments in the taxonomic classification and

nomenclature of anaerobes since the sixth edition[519] of this book was published. Genetic studies utilizing polymerase chain reaction (PCR) and 16S ribosomal (r) RNA, 16S rDNA sequencing, 16S–23S rRNA spacer-region (SR)-PCR, and other molecular methods have more definitively classified anaerobes than was possible using phenotypic characterization and the older, less sophisticated genetic methods available in the past.[449,450] These studies have resulted in the descriptions of many new species and other taxa; at times, the genera into which an organism is then placed may not relate to Gram reaction, rod or coccus morphology, the ability to form spores, relationships to oxygen, or other phenotypic characteristics traditionally used to classify anaerobes. Fortunately, some excellent taxonomic updates have simplified the task of keeping up with ongoing changes in nomenclature.[158,162,236,237] Table 16-2 provides a compendium of taxonomic changes, compiled over the past several decades, for clinically significant anaerobic bacteria. The most clinically important anaerobes and/or those that are most commonly encountered in properly selected and collected specimens are listed in Table 16-3. Up-to-date, correct species identifications can have important implications related to prognosis, treatment, and clinical outcomes of patients with anaerobic infectious diseases.[158,160,162]

Taxonomy of the Anaerobic Gram-Negative Bacilli

The anaerobic gram-negative bacilli include some of the most commonly isolated and pathogenic anaerobes from clinical specimens. Tables 16-1 and 16-2 provide a summary of the latest taxonomy of this group of anaerobes, although not all anaerobic bacteria are listed because many are not known to be clinically relevant or part of the human microbiota. Members of the *Bacteroides fragilis* group make up a significant proportion of the normal microbiota of the GI tract in which the number of bacteria can be as high as 10^{11} to 10^{12} colony-forming units (CFU)/g feces; anaerobes far outnumber the aerobes >1,000:1. *B. thetaiotaomicron* and *B. ovatus* are the most common members of the *B. fragilis* group in the GI tract. *B. fragilis*, although only comprising about 0.5% of the human colonic microbiota, is the most common species involved in clinical disease because of its known virulence factors.[238] It is the most common cause of

BOX 16-1

Anaerobes Found in Normal Human Microbiota

Oral Cavity and Upper Respiratory Passages

Pigmented *Prevotella* species; *Porphyromonas* species

Nonpigmented *Prevotella* species (e.g., *P. oralis*)

Bacteroides species (e.g., *B. ureolyticus*)

Fusobacterium species (e.g., *F. nucleatum*)

Anaerobic gram-positive cocci

Veillonella species

Actinomyces species and *Propionibacterium* species

Stomach (During Fasting)

Lactobacilli

Small Intestine (Proximal Portion)

Microaerophilic streptococci

Lactobacilli

Large Bowel (and Terminal Ileum)

Bacteroides fragilis group

Porphyromonas species

Fusobacterium species

Anaerobic gram-positive and gram-negative cocci—many species

Clostridium species

Eubacterium species

Eggerthella species

Bifidobacterium species and related genera

Propionibacterium species

Other anaerobic gram-positive bacilli

Genitourinary Tract; Vagina and Cervix

Pigmented *Prevotella* species; *Porphyromonas* species

Nonpigmented *Prevotella*

Bacteroides species

Anaerobic gram-positive cocci

Eubacterium species

Eggerthella species

Clostridium species

*Veillonell*a species

Lactobacillus species

Propionibacterium species

Mobiluncus species

Atopobium species

Urethra (Male and Female)

Propionibacterium species

Anaerobic gram-positive and gram-negative cocci

Bacteroides species

Prevotella species

Fusobacterium species

Skin

Propionibacterium species

Anaerobic cocci

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TABLE 16-1 Classification of the Genera of Anaerobic Bacteria

Gram-Negative Bacilli (Include Curved, Spiral, and Spirochete Forms)	Gram-Positive Cocci	Gram-Negative Cocci	Gram-Positive Bacilli (Non–Spore-Forming)	Gram-Positive Spore-Forming Bacilli
Alistipes	*Anaerococcus*[a]	*Acidominococcus*	*Alderreutzia*	*Clostridium*[a,b]
Anaerobiospirillum[a]	*Atopobium*[a]	*Anaeroglobus*	*Alloscardovia*	*Desulfomaculum*
Bacteroides[a]	*Coprococcus*	*Megasphaera*	*Anaerofustis*	*Desulfosporosinus*
Barnesiella	*Finegoldia*[a]	*Negativococcus*	*Anaerostipes*	*Caloramator*[b]
Bilophila[a]	*Gallicola*[1]	*Veillonella*[a]	*Anaerotruncus*	*Filifactor*[b]
Butyrivibrio[c]	*Gemmiger*		*Actinobaculum*	*Moorella*[b]
Catonella[a]	*Murdochiella*		*Actinomyces*[a]	*Oxobacter*[b]
Desulfuromonas	*Peptococcus*		*Atopobium*[a]	*Oxalophagus*[b]
Desulfovibrio	*Peptostreptococcus*[a]		*Bifidobacterium*[a]	
Dialister[a]	*Ruminococcus*		*Bulleidia*	
Faecalibacterium	*Sarcina*		*Catabacter*	
Fusobacterium[a]	*Peptoniphilus*[a]		*Catenibacterium*	
Johnsonella[a]	*Staphylococcus*[a]		*Cryptobacterium*	
Jonquetella	*Streptococcus*[a]		*Collinsella*	
Leptotrichia	*Gemella*[a]		*Eggerthella*[a]	
Megamonas			*Eubacterium*[a]	
Mitsuokella			*Faecalibacterium*	
Odoribacter			*Gordonibacter*	
Parabacteroides[a]			*Holdemania*	
Paraprevotella[a]			*Lachnospira*	
Parasutterella[a]			*Lactobacillus*[a]	
Phocaeicola			*Marvinbryantia*	
Porphyromonas[a]			*Methanobacterium*	
Prevotella[a]			*Mobiluncus*[a]	
Pyramidobacter			*Mogibacterium*	
Selenomonas			*Olsenella*	
Sneathia			*Oribacterium*	
Spirochaeta			*Paraeggerthella*	
Succinomonas			*Parascardovia*	
Succinivibrio			*Propionibacterium*[a]	
Sutterella[a]			*Propionimicrobium*[a]	
Tannerella[a]			*Pseudoramibacter*	
Tissierella			*Roseburia*	
Treponema[d]			*Scardovia*	
			Shuttleworthia	
			Slackia[a]	
			Solobacterium	
			Turicibacter	
			Varibaculum	

[a] In the majority of properly collected clinical specimens, only these genera will need to be considered by the clinical microbiologist. However, on rare occasions, serious illness may involve *Anaerobiospirillum*,[208] *Leptotrichia*,[156] *Selenomonas*,[2778] *Desulfovibrio*[93] or one of the other genera that is not listed above.
[b] The genus *Clostridium* continues to be under major taxonomic revision. Thus, in 1994, five new genera of spore-forming anaerobes were proposed by Collins et al.[49] With a single exception, none of these is known to be pathogenic for humans. *Filifactor alocis* has been isolated from patients with endodontic infections.[236]
[c] *Butyrivibrio* is discussed with the gram-negative bacilli in Vol. 1 and with the irregular non–spore-forming, gram-positive anaerobic rods in Vol. 2 of Bergey's Manual.[153,241] Although they are gram-negative with the Gram's stain, some strains have atypical gram-positive cell wall features by electron microscopy.
[d] *Treponema* spp. will not be discussed in this chapter, but is included here because they are a strict anaerobe.
Adapted and modified from *Bergey's Manual of Systematic Bacteriology*[153,241] and from very recent volumes of the International Journal of Systematic and Evolutionary Microbiology that contain numerous publications related to recent changes in anaerobe taxonomy.

TABLE 16-2 Present and Former Names of Anaerobic Bacteria

Present Name	Former Name/Comments
Anaerobic Gram-Negative Rods	
Genus Bacteroides—*Bacteroides fragilis* Group[507,a]	
Bacteroides caccae	Fecal microbiota
B. eggerthii	Fecal microbiota—not common in infections
B. fragilis	Most common anaerobe in infections
B. ovatus[370]	Human fecal microbiota; occasional infections
B. stercoris	Human fecal microbiota
B. thetaiotaomicron	Second to *B. fragilis* in causing infections
B. uniformis	Occasional clinical isolate
B. vulgates[370]	Common fecal microbiota; occasional fecal isolate
B. dorei[22,370]	New species; rarely isolated from blood
B. finegoldii[21]	New species; occasional clinical infection
B. massiliensis[156]	New species; isolated rarely from blood
Also Considered *B. fragilis* Group Members, but in *Parabacteroides* Genus	
Parabacteroides distasonis[403]	Human infections; formerly *B. distasonis*
P. goldsteinii[403]	Formerly *B. goldsteinii;* bacteremia
P. gordonii[19,408]	New species; blood culture isolate
P. johnsonii[405]	New species; fecal microbiota
P. merdae[403]	"T4-1," formerly *B. merdae;* human fecal microbiota
Other Species Remaining in the Genus *Bacteroides*, but Not a Member of the *Bacteroides fragilis* Group	
B. coagulans	Rare in clinical specimens
B. galacturonicus	Human fecal microbiota
B. pectinophilus	Human fecal microbiota
B. ureolyticus[485,511]	Human oral cavity; oral infections, head and neck infections
Genus *Prevotella*	
P. amni	New species; genitourinary (GU) infections
P. baroniae[131,397]	New species; oral cavity; infected root canal and dental abscess infections
P. bergensis[130]	New species; skin and soft tissue infections
P. bivia[372]	*Bacteroides bivia*; vaginal microbiota; GU infections
P. buccae	*Bacteroides buccae;* oral cavity; head, neck and chest infections; human bite wound
P. buccalis	*Bacteroides buccalis;* oral cavity
P. copri[213]	New species; fecal isolates
P. corporis	*Bacteroides corporis*; oral cavity; GU infections; pigmented
P. dentalis[514]	*Mitsuokella dentalis; Hallela seregens*; oral cavity

P. denticola[263]	*Bacteroides denticola*; oral cavity; carious lesions sputum of CF patients; some are pigmented
P. disiens[372]	*Bacteroides disiens;* vaginal microbiota and oral microbiota; GU infections
P. enoeca[337]	Oral cavity
P. heparinolytica[6]	*Bacteroides heparinolytica;* animal bite wounds
P. histicola	New species; some are pigmented
P. intermedia[427,428]	*Bacteroides intermedius*; head, neck, and abdominal infections; many oral infections; human bite wounds; pigmented
P. loescheii[461]	*Bacteroides loescheii*; oral cavity; pigmented; wounds
P. maculosa	New species
P. marshii[131]	New species; oral cavity
P. melaninogenica[263]	*Bacteroides melaninogenica;* pigmented; oral, pulmonary, skin and soft tissue infections; sputum of patients with cystic fibrosis
P. micans[124]	New species; oral cavity; pigmented
P. multiformis[404]	New species; nonpigmented; gingival plaque
P. multisaccharivorax[397]	New species; endodontic infections
P. nanceiensis[4]	New species; nonpigmented; many clinical specimens
P. nigrescens[428,529]	Indistinguishable from *P. intermedia*; oral cavity; many oral infections; pigmented
P. oralis[6]	*Bacteroides oralis*; oral cavity; abscesses; osteomyelitis
P. oris	*Bacteroides oris;* oral cavity; odontogenic infections
P. oulorum	*Bacteroides oulorum;* oral cavity; *P. oulora*
P. pallens[262]	New species; pigmented
P. pleuritidis[406]	New species; isolated from pleural fluid
P. saccharolytica[132]	New species; oral cavity
P. salivae[407]	New species; oral cavity; sputum of patients with cystic fibrosis
P. shahii[407]	New species; oral cavity; pigmented
P. stercorea[213]	New species; fecal isolates
P. tannerae[337]	New species; oral cavity; carious lesions; some strains pigmented
P. timonensis[183]	New species; breast abscess
P. veroralis	*Bacteroides veroralis;* oral cavity
P. zoogleoformans[337]	*Bacteroides zoogleoformans*
Genus *Porphyromonas*	
P. asaccharolytica[469]	*Bacteroides asaccharolyticus*; many nonoral clinical specimens
P. bennonis[466]	New species; nonoral infections; wounds and abscesses

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***Porphyomonas* spp.**	
Porphyromonas catoniae[263]	*Oribaculum catoniae*; oral cavity; not pigmented; abdominal abscess
P. endodontalis[165]	*Bacteroides endodontalis*; dental infections
P. gingivalis[47]	*Bacteroides gingivalis*; periodontitis and dental abscesses and nonoral infections
P. somerae[467]	Lower extremity skin and soft tissue infections related to human-like *P. levii* strains
P. uenonis[165]	New species; appendicitis, peritonitis; decubitus ulcer
The following are species that are normal microbiota or pathogens in animals; any could potentially be involved in human bite wound infections.[1,6]	
P. cangingivalis	Dogs
P. canoris	Dogs
P. cansulci	Dogs
P. circumdentaria	Cats
P. crevivoricanis	Dogs
P. gingivicanis	Dogs
P. gulae	Cat, coyote, dogs, bear, wolf
P. levii	Cattle
P. macacae	*Bacteroides macacae;* monkeys and cats
***Fusobacterium* spp.**[263]	
F. gonidiaformans	Human infections
F. mortiferum[98,238]	Human infections
F. naviforme	Human infections
F. necrogenes	Human fecal microbiota; also from oral cavity
F. necrophorum subsp. *fundiliforme*[24,376,394]	Human microbiota; virulent species; Lemierre's and other oral and many other nonoral infections
F. necrophorum subsp. *necrophorum*[394]	More commonly isolated from animals; less virulent of the two subspecies
F. nucleatum[376,465]	Most common clinical *Fusobacterium* sp.; normal microbiota in oral cavity and GI tract
F. periodonticum[465]	Oral cavity; also found in GI tract
F. russii	Human fecal microbiota
F. ulcerans[98]	New species; tropical ulcers
F. varium[98,376]	Various human infections
Other Genera of Anaerobic Gram-Negative Bacilli	
Alistipes finegoldii[157,388]	New species; human appendicitis, bacteremia
A. onderdonkii[447]	New species; pigmented; intra-abdominal specimens
A. putredinis[388]	*Bacteroides putredinis*; rarely found in intra-abdominal infections

Alistipes.shahii[447]	New species; pigmented; intra-abdominal infections
Anaerobiospirillum succiniciproducens[250,377,472]	Human fecal microbiota; bacteremia
Anaerobiospirillum thomasii[263]	New species; human fecal microbiota
Anaerostipes caccae	New species; human fecal microbiota
Anaerorhabdus furcosa	*Bacteroides furcosus; Anaerohabdus furcosus*
Barnesiella intestinihominis[341]	New genus and species
Bilophila wadsworthia[30,484]	Peritonitis, appendicitis, brain abscess
Catonella morbi[329,340]	New species; prosthetic valve endocarditis
Centipeda periodontii[415]	Oral isolates
Cetobacterium somerae[164]	New species; human feces
Desulfovibrio piger[303]	*Desulfomonas pigra;* human feces
D. desulfuricans[192,495]	Fecal microbiota
D. vaginalis[228]	Vaginal microbiota
Dialister pneumosintes[135,340]	*Bacteroides pneumosintes*; oral isolate; odontogenic infection
Faecalibacterium prausnitzii[333]	*Fusobacterium prausnitzii*; normal GI microbiota; may help to balance immunity in GI tract
Filifactor alocis[193]	*Fusobacterium alocis;* oral cavity; endodontic disease
Jonquetella anthropi[241]	New genus and species
Johnsonella ignava[340]	New genus and species; human gingival crevice
Genus *Leptotrichia*	
L. amnionii	New species; amniotic fluid
L. buccalis[40,198]	*Leptothrix buccalis;* oral microbiota; bacteremia
L. goodfellowii[69,146]	New species; blood isolates
L. hofstadii[146]	New species; oral cavity
L. shahii[146]	New species; gingivitis
L. trevisanii[270]	New species; oral isolate; bacteremia
L. wadeii[146]	New species; oral cavity
Other Genera	
Megamonas fumiformis[409]	New genus and species; related to *Sutterella*; human feces
Odoribacter splanchnicus[210]	Formerly *Bacteroides splanchnicus*
Oxalobacter formigenes[251]	Colon colonizer; reduces calcium oxalate stones
Paraprevotella spp.[342]	New Genus; human feces

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Parasutterella secunda[343]	New genus; human feces
Phocaeicola abscessus[11]	New genus and species; isolated from brain abscess
Pyramidobacter piscolens[133]	New genus; oral cavity
Pseudoflavonifractor capillosus[70]	Formerly *Bacteroides capillosus*; rarely in clinical specimens
Genus *Selenomonas*[415]	
S. artemidis	Oral microbiota
S. dianae	Human gingival crevice
S. flueggei	Human oral microbiota
S. infelix	Human gingival crevice
S. noxia	Human gingival crevice
S. sputigena[415]	Oral microbiota
Sneathia sanguinegens[103]	*Leptotrichia sanguinegens*; bacteremia; bacterial vaginosis-associated
Sutterella wadsworthensis[336,346,508]	Formerly, strains of *Campylobacter (Bacteroides) gracilis;* GI microbiota
Tannerella forsythensis[193,396]	*Bacteroides forsythus*; oral cavity; periodontitis
Tissierella praeacuta[151]	*Bacteroides praecutus*
Anaerobic Cocci	
Gram-Positive Cocci	
Peptococcus niger	Only species in genus
Peptostreptococcus anaerobius[108,350]	Only species in genus; many clinical specimens; endocarditis
Anaerococcus prevotii[230,351]	*Peptostreptococcus prevotii*; GU infections
A.tetradius[351]	*Peptostreptococcus tetradius;* GU infections
A. hydrogenalis[351]	*Peptostreptococcus hydrogenalis*
A. vaginalis[351]	*Peptostreptococcus vaginalis;* GU infections
A. lactolyticus[351]	*Peptostreptococcus lactolyticus;* GU infections
A. murdochii[448]	New species
A. octavius[351]	*Peptostreptococcus octavius;* GU microbiota; skin microbiota
Finegoldia magna[170,291,349]	*Peptostreptococcus magnus;* most common anaerobic coccus in specimens; bacteremia; endocarditis
Gallicola barnesae[148]	*Peptostreptococcus barnesae*
Parvimonas micra[351,396,446]	*Micromonas micros; Peptrostreptococcus micros;* many clinical specimens; oral cavity
Peptoniphilus asaccharolyticus[351]	*Peptostreptococcus asaccharolyticus;* many clinical specimens
P. gorbachii[448]	New species

P. harei[351]	*Peptostreptococcus harei;* rare clinical specimens
P. indolicus[351]	*Peptostreptococcus indolicus*
P. ivorii[351]	*Peptostreptococcus ivorii*; leg ulcer
P. lacrimalis[351]	*Peptostreptococcus lacrimalis*; lacrimal gland abscess
P. olsenii[448]	New species
Other Anaerobic Gram-Positive Cocci	
Atopobium parvulum[128]	*Streptococcus parvulus;* oral cavity; dental abscesses
Coprococcus catus	Human feces
Coprococcus comes	Human feces
Coprococcus eutactus	Human feces
Murdochiella asaccharolyticus[351,483]	New species
Sarcina ventriculi	GI microbiota
Staphylococcus saccharolyticus[8,187,256]	*Peptococcus saccharolyticus*; bacteremia; contaminated platelets
Staphylococcus aureus subsp. *anaerobius*[141,368]	Abscesses
Gram-Negative Cocci	
Acidaminococcus fermentans[312]	Human feces; rare clinical specimens
Anaeroglobus geminatus[72]	New species; oral and GI microbiota
Megasphaera elsdenii[50]	Human feces; endocarditis
Megasphaera micronuciformis	New species
Negativicoccus succinicivorans[94,312]	New species
Veillonella atypica	Human oral microbiota; rare clinical isolates; prosthetic joint infections
V. dispar	Human microbiota; rare human specimens
V. parvula[41,45,166]	Human microbiota; most common species
V. montpellierensis[240]	New species; rare clinical specimens
Anaerobic Gram-Positive, Non–Spore-Forming Bacilli	
***Actinomyces* spp.**	
A. cardiffensis[203]	New species; abscesses
A. dentalis[208]	Oral cavity; dental abscess
A. europaeus[174,402]	New species; breast abscesses
A. funkei[283]	New species; clinical samples
A. georgiae[235]	Human gingival crevice
A. gerencseriae[235]	Human periodontal microbiota
A. graevenitzii[274]	New species; clinical specimens
A. hongkongensis[521]	New species; clinical specimens
A. israelii[222,365,383,499]	Cause of actinomycosis
A. johnsonii[216]	New species; related to *A. naeslundii*
A. massiliensis[392]	New species; blood culture isolate

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A. meyeri[153,200,238]	Head and neck infections; brain abscess
A. naeslundii[216,235,499]	Can cause actinomycosis; oral microbiota
A. nasicola[206]	New species
A. neuii subsp. *neuii*[175,176,310,497]	CDC Coryneform group 1
A. neuii subsp. *anitratus*[175]	CDC coryneform 1-like
A. odontolyticus[238,499]	Human oral cavity; rare actinomycosis
A. oricola[205]	New species; dental abscesses
A. oris[216]	New species; related to *A. naeslundii*
A. radicidentis[102,247,402,438]	New species; infected root canals
A. radingae[402,526]	New species; clinical specimens
A. turicensis[402,486,526]	New species; many clinical specimens
A. urogenitalis[144,357]	New species; urogenital microbiota
A. viscosus[235,499]	Human oral cavity; occasional actinomycosis
Genera Related to *Actinomyces* spp.	
Actinobaculum massiliense[356]	New species; isolated from urine
Actinobaculum schaalii[282,356]	New species; blood and urine isolates
Actinobaculum urinale[204]	New species; urine isolates
Varibaculum cambriense[93,207]	New genera and species
Genus *Bifidobacterium* spp.[499]	
B. adolescentis	Oral cavity
B. breve[307]	Oral cavity
B. dentium[307]	Oral cavity; involved in dental infections
B. gallicum[307]	Human feces
B. longum[307]	Dental caries
B. scardovii[221]	New species; oral cavity; dental caries
Genus *Eubacterium*[238,499]	
E. brachy	Periodontitis
E. infirmum	New species; oral microbiota
E. limosum[145]	GI microbiota; skin & soft tissue and GI infections
E. minutum	*Eubacterium tardum*
E. nodatum	Oral microbiota; periodontitis
E. saphenum	Dental infections
E. sulci	*Fusobacterium sulci*
E. tenue[281]	Oral microbiota; bacteremia
E. yurii	Dental plaque

Genus *Lactobacillus*	
L. casei[68,195]	Normal microbiota mouth, vagina, GI tract; many clinical specimens
L. coleohominis	New species; facultative anaerobe
L. oris	Saliva
L. paraplantarum	New species; fecal microbiota
L. rhamnosus[68,195]	Normal microbiota of mouth, vagina and GI tract; many clinical specimens
Genus *Propionibacterium* spp.	
P. acidifaciens[134]	Oral microbiota; associated with dental infections
P. acnes[295,328,371]	Most common species; skin microbiota; involved in many infections
P. avidum[39,331]	Skin microbiota; rare but increasing reports of clinical infections
P. granulosum[91,360]	
P. propionicum[89,438,499,524]	
New Genera Related to *Propionibacterium* sp.	
Propionimicrobium lymphophilum[457]	
Other Anaerobic Gram-Positive, Non–Spore-Forming Bacilli	
Alloscardovia omnicolens[227,307]	New genus and species; related to *Bifidobacterium* spp. Isolated from many clinical sites
Anaerofustis stercorihominis[499]	New genus and species; fecal microbiota
Anaerotruncus colihominis[499]	New genus and species; fecal microbiota; bacteremia
Atopobium minutum[105,319]	*Lactobacillus minutus*
Atopobium rimae[13,105,128]	*Lactobacillus rimae*;oral microbiota; periodontitis; bacteremia
Atopobium vaginae[85,479]	New species; association with bacterial vaginosis
Blautia wexlerae[296]	New species
Bulleidia extructa[129,260]	New genus and species; oral cavity
Catabacter hongkongensis[278,442]	New genus and species; bacteremia
Catenibacterium mitsuokai	New genus and species; fecal microbiota
Collinsella aerofaciens[245]	*Eubacterium aerofaciens*; fecal microbiota most abundant bacterium in intestine
Collinsella stercoris[244]	New species; fecal microbiota
Collinsella intestinalis[244]	New species; fecal microbiota
Cryptobacterium curtum[353]	*Eubacterium curtum;* Oral cavity; periodontitis
Dorea formicigenerans[471]	*Eubacterium formicigerans*; fecal microbiota

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Dorea longicatena	New species; fecal microbiota
Eggerthella lenta[238,493,498,499]	*Eubacterium lentum;* bacteremia; the most commonly isolated species of *Eubacterium/Eggerthella* from nonoral sites
Eggerthella sinensis[280]	New species; bacteremia
Flavonifractor plautii[70]	*Clostridium orbiscindens* and *Eubacterium plautii*; fecal microbiota; blood, abdominal wounds
Holdemania filiformis[517]	New genus and species; fecal microbiota
Mobiluncus curtisii[120,185,218,456]	Vaginal microbiota; associated with bacterial vaginosis
Mogibacterium vescum	New genus and species; oral cavity
Mogibacterium diversum	New species; oral cavity
Mogibacterium neglectum	New species; oral cavity
Mogibacterium timidum[211]	*Eubacterium timidum*
Moryella indoligenes[71]	New genus and species; probably GI microbiota; isolated from abdominal abscess
Olsenella profusa[118]	New species oral cavity; periodontitis; gingivitis
Olsenella uli[118,281]	*Lactobacillus uli;* gingival crevice; periodontitis; gingivitis; bacteremia
Paraeggerthella hongkongensis[280,525]	*Eggerthella hongkongensis*; bacteremia
Parascardovia denticolens[233]	*Bifidobacterium denticolens*
Pseudoramibacter alactolyticus[439,516]	*Eubacterium alactolyticum*
Roseburia intestinali[137]	New species; fecal microbiota
Scardovia inopinata[126,233]	*Bifidobacterium inopinatum;* oral cavity; dental caries
Scardovia wiggsiae[126]	New species; oral cavity; isolated from wound
Shuttleworthia satelles[127,430,508]	New genus and species; oral cavity; endocarditis
Slackia exigua[211,257,498]	*Eubacterium exiguum*
Slackia heliontrinireducens[381,498]	*Peptostreptococcus helionitrinireducens*
Solobacterium moorei[37,246]	Fecal isolate; bacteremia
Turicibacter sanguinis[46]	New species; bacteremia
Gram-Positive, Spore-Forming Bacilli[464]	
Clostridium aldenense[502]	New species; one of *C. clostridioforme* group
C. argentinense[181]	*C. botulinum* Type G
C. baratii[181]	*C. paraperfringens, C. perenne;* can produce *C. botulinum* Type G toxin

C. bartlettii[452]	New species; fecal microbiota
C. bolteae[451]	New species; one of the *C. clostridioforme* group; bacteremia
C. botulinum[181]	Cause of food, infant, and wound botulism; toxin-producer
C. butyricum[238,464]	*C. pseudotetanicum;* can produce *C. botulinum* Type G toxin
Clostridium celerecrescens[48]	
C. citroniae[502]	New species; one of the *C. clostridioforme* group
C. clostridioforme[163]	One of many species in *C. clostridioforme* group; bacteremia
C. hathewayi[293,461]	New species; fecal microbiota; one of the *C. clostridioforme* group; bacteremia and liver abscess proposal to change name to *Hungatella hathewayi*
C. hiranonis	New species; fecal microbiota
C. hylemonae	New species; fecal microbiota
C. innocuum[424]	Common clostridia species in clinical specimens; bacteremia
C. lavalense[121]	New species; one of the *C. clostridioforme* group
C. methylpentosum	Fecal microbiota
C. neonatale[7]	New species; isolated from patient with necrotizing enterocolitis
C. perfringens[334,463,498]	*C. welchii;* gas gangrene; food poisoning; bacteremia
C. ramosum[167]	*Eubacterium filamentosum, Ramibacterium ramosum, Actinomyces ramosus, Eubacterium ramosus*
C. scindens[143]	New species; fecal microbiota; intra-abdominal abscess
C. septicum[253,416]	Bacteremia; necrotizing fasciitis
C. sordellii[15,258,463]	Bacteremia; necrotizing fasciitis
C. symbiosum[478]	*Fusobacterium symbiosum;* bacteremia
C. tertium[173,330]	Bacteremia and other infections; aerotolerant

[a] There are now >23 species of the *B. fragilis* group, for example. Most of those not in this table have been recently named and/or have not as yet been found in clinical specimens nor associated with human infections.
This is not intended to be an all-inclusive list. A number of species of no or doubtful clinical relevance have not been included, especially in the genera *Bacteroides, Eubacterium, Lactobacillus* and *Clostridium.*
Some species that might have been in past editions may have been removed from this edition, such as *Propioniferax*, which is an aerobe, although related to *Propionibacterium* spp.
This table is based on information compiled predominantly from many references 238, 263, 446, 464, and 499 and the International Journal of Systematic and Evolutionary Microbiology.

TABLE 16-3 The Most Clinically Important Anaerobes and/or Those Most Frequently Encountered in Clinical Specimens

Gram-Negative Rods
Bacteroides fragilis group (especially *B. fragilis, B. thetaiotaomicron, B. vulgatus,* and *B. ovatus*)
Parabacteroides distasonis
Other *Bacteroides* (*B. ureolyticus*)
Porphyromonas (especially *P. asaccharolytica, P. gingivalis* and *P. endodontalis*)
Pigmented *Prevotella* (*P. melaninogenica, P. loescheii, P. denticola, P. tannerae, P. intermedia, P. nigrescens* and *P. corporis*)
Nonpigmented *Prevotella* (*P. oralis* group, *P. oris/buccae, P. bivia* and *P. disiens*)
Fusobacterium (especially *F. nucleatum, F. necrophorum, F. mortiferum* and *F. varium)*
Bilophila wadsworthia
Anaerobic Cocci
Gram-positive cocci (especially *Peptostreptococcus anaerobius, Finegoldia magna, Parvimonas micra, Peptoniphilus asaccharolyticus, Anaerococcus prevotii*, *Staphylococcus saccharolyticus,* and *Atopobium parvulum*)
Gram-negative cocci (*Veillonella* spp.)
Anaerobic Gram-Positive, Non–Spore-Forming Rods
Actinomyces (especially *A. israelii, A. meyeri, A. naeslundii, A. odontolyticus*, *A. turicensis, A. graevenitzii* and *A. viscosus*)
Atopobium vaginae
Propionibacterium (*P. acnes* and *P. propionicum*)
Bifidobacterium dentium
Eubacterium limosum

Eggerthella lenta
Paraeggerthella spp.
Lactobacillus sp.
Gram-Positive, Spore-Forming Rods (*Clostridium* spp.)
C. perfringens
C. clostridioforme group (especially *C. clostridioforme, C. hathewayi,* and *C. bolteae, C. citroniae, C. aldenense* and *C. lavalense*)
C. innocuum
C. ramosum
C. difficile
C. bifermentans
C. sporogenes
C. septicum
C. sordellii
C. novyi
C. histolyticum
C. botulinum
C. tetani
C. tertium

anaerobic bacteremia.[58,238,420] There are over 20 species in the *B. fragilis* group that are related genetically, as well as by their phenotypic characteristics: 20% bile resistance, Gram's stain morphology, and innate and increasing resistance to a wide variety of antibiotics. Table 16-2 lists the members of the *B. fragilis* group species. One group of *B. fragilis* complex (*B. merdae, B. goldsteinii,* and *B. distasonis*) has been reclassified into the genus *Parabacteroides*.[263,403] Most of the non-*B. fragilis* group *Bacteroides* spp. have also been reclassified, although *B. ureolyticus* presently

remains in the *Bacteroides* genus; however, there is a proposal to reclassify it as *Campylobacter ureolyticus*.[485] Many of the other one-time *Bacteroides* spp. members are listed with their new genera in the Table. *B. splanchnicus,* for example, has been placed in the genus *Odoribacter* and *B. capillosus* in a new genus, *Pseudoflavonifractor*, as *P. capillosus*.[70,210]

Since 1992, the genus *Porphyromonas* has changed markedly with a total now of at least 16 species (Table 16-2). Many have been isolated from the oral cavity and may be responsible for endodontic infections. Five of the *Porphyromonas s*pp. have been isolated from oral cavities of dogs only (*P. cangingivalis, P. canoris, P. cansulci, P. crevioricanis,* and *P. gingivicanis*); one has been isolated from the oral cavities of cats only (*P. circumdentaria*); another has been isolated from both monkeys and cats (*P. macacae*, which includes those previously classified as *P. salivosa*); another is from cattle (*P. levii*); one species was isolated from the human oral cavity *(P. catoniae*), and *P. gulae*, described in 2001, was isolated from various animals, including bear, cat, coyote, dog, wolf, and monkey.[169,515] Presumably, although their clinical importance in humans is unknown, the species isolated from the mouths of animals could be encountered in bite wound infections.[1] In 1995, not only did Willems and Collins reclassify *Oribaculum catoniae* as *Porphyromonas catoniae*, but they emended the genus *Porphyromonas* as well. In accordance with their emended description, some species are now included that are saccharolytic (*P. levii, P. macacae,* and *P. catoniae*); *P. catoniae* alone does not form pigment.[467,515]

There are over 37 species of the genus *Prevotella,* which consists of pigmented and nonpigmented strains. They make up the bulk of the gram-negative anaerobic normal microbiota of the oral cavity, but are also involved in many oral as well as nonoral infections.[263] Table 16-2 lists the most common species of *Prevotella*.

Fusobacterium spp. are another common genera of gram-negative bacilli, and many of them have retained their genus and species names; a number of newly named *Fusobacterium* are also shown in Table 16-2.[95] *Fusobacterium nucleatum* has the characteristic Gram's stain morphology of long, thin pointed rods (i.e., a fusiform shape) and a few other species also demonstrate this trait, but many of the species produce shorter rods, and some rather bizarre morphologies that are quite distinctive for their identification. The virulence of the *Fusobacterium* spp. has been recognized and reported on with increasing frequency in the literature as will be discussed in the sections on human infections.[95,395]

Faecalibacterium prausnitzii is a very common bacterium in the normal GI microbiota and often identified in fecal material.[238,333] *Leptotrichia* is an unusual gram-negative bacillus, the cells of which are much longer than other anaerobic gram-negative bacilli; this will be included for discussion later as well.[40,198] *Bilophila wadsworthia* is very similar to the *B. fragilis* group in its resistance to bile and

susceptibility to antibiotics, and has become increasingly recognized as a pathogen as methods of isolation and identification improve. *B. wadsworthia* will be discussed further in this chapter.[30,484] Some of the other genera of anaerobic gram-negative bacilli will not be further covered, but if references are available, these will be provided in text or tables so readers can obtain more information, if desired.

Taxonomy of the Anaerobic Gram-Positive Non–Spore-Forming Bacilli

The gram-positive NSF bacilli are a very heterogeneous group of anaerobes, often difficult to specifically identify and characterize. One of the most commonly isolated anaerobes, *Propionibacterium acnes*, belongs to this group, as do members of the *Actinomyces* genus that include the well-known pathogen *A. israelii*. Members of both of these genera share a trait of "aerotolerance" in that, although often isolated in primary cultures only under strict anaerobe conditions, they often grow quite well in the presence of oxygen upon transfers from the primary plates. *Propioniferax* spp., a genus related to *Propionibacterium* spp., is actually an aerobe, and will not be included in this chapter. *Propionibacterium propionicum*, *P. acidifaciens,* and *Propionimicrobium lymphophilum* (related to the *Propionibacterium* spp.) are not aerotolerant. For the aerotolerant species of *Actinomyces* and *Propionibacterium*, drugs such as metronidazole, which are only active against anaerobes, have no effect on their growth; the nonaerotolerant strains vary in their response to metronidazole.[100,238,263]

P. acnes is very common as a part of the normal skin microbiota, but can be a pathogen in infections in which the entry of the organisms from the skin is enhanced by placement of foreign devices through or near the skin. *Propionibacterium propionicum* (formerly referred to as *Arachnia propionica*) has been implicated in actinomycosis and other infections, as will be discussed in the next section.[55]

A. israelii is an anaerobic gram-positive, branching bacillus that has long been associated with the condition "actinomycosis," a chronic granulomatous infection that can affect the cervicofacial, thoracic, abdominal, or pelvic regions of the body. Disease is most commonly caused by *A. israelii, A. gerencseriae,* and *A. gravenitzii*. The presence of "sulfur granules" (masses of branching bacilli), seen in histopathology or on Gram's stains of clinical specimens in the microbiology laboratory, are regarded as the hallmark of such *Actinomyces* infections; in fact, actinomycosis in most instances is actually a polymicrobial infection.[55,238,263] In recent years, extensive taxonomic revisions have been made within the genus *Actinomyces* (Table 16-2). These changes have included the designation of several new or additional species including *A. cardiffensis,*[203] *A. europaeus*,[402] *A. funkei*,[283] *A. georgiae*,[235] *A. gerencseriae*,[235] *A. graevenitzii,*[385] *A. hongkongensis*,[521] *A. nasicola,* [206] *A. neuii*,[175,176,310] *A.*

oricola,[205] *A. radicidentis*,[102,247] *A. radingae*,[402,486,526] *A. turicensis,*[402,486,526] and *A. urogenitalis*.[357] Other more recently described genera that are closely related to the genus *Actinomyces* are *Actinobaculam* (e.g., *Actinobaculam schaalii* and *A. urinale*),[204,282] *Arcanobacterium* (e.g.,*Arcanobacterium bernardiae* and *A. pyogenes*),[386] and *Varibaculum cambriense*.[93,207] There will

be no further discussion of *Arcanobacterium* in this chapter because it is not an anaerobe.

Table 16-2 lists the most recent taxonomy of the anaerobic, gram-positive NSF bacilli. Using newer molecular tools for identification, many of the once *Eubacterium, Bifidobacterium,* and *Lactobacillus* have been reclassified into new genera. A very common anaerobe, *Eubacterium lentum*, has been renamed *Eggerthella lenta*; there are now at least four or five other species of clinically significant *Eggerthella* spp. or the related *Paraeggerthella* spp.[292,493,498,499] Most of the other *Eubacterium* have been reclassified into over 11 new genus/species, as can be seen in Table 16-2. Most of these have been isolated from fecal or oral microbiota, as well as, from many clinical specimens, and in some instances have been implicated in diseases either as the sole cause of disease or as part of a polymicrobial infection.[128]

Many of the once *Bifidobacterium* spp. have been reclassified into the genera *Scardovia, Alloscardovia* or *Parascardovia*; *B. breve, B. longum, B. scardovia,* and *B. dentium* remain in the *Bifidobacterium* genus. All are part of the normal oral or GI microbiota, and may contribute to the normal oral or GI health, rarely playing a pathogenic role in dental caries and periodontitis.[482,499]

The lactobacilli are common inhabitants of the human mouth, GI tract, and female genital tract. They are used as a marker of "normal" vaginal microbiota when seen in scored Gram's stains of vaginal specimens from women who are being screened for bacterial vaginosis. Some of the lactobacilli are strict anaerobes, but others are facultative and this trait can make it difficult in their recognition and identification, unless newer molecular methods of identification are utilized. The most common species involved in clinical specimens are *L. rhamnosus* and *L. casei*.[68,195] Because lactobacilli are often incorporated in probiotic treatments, the possibility of their pathogenicity in nonimmunocompromised patients is often questioned. Some of the one-time *Lactobacillus* spp. have been reclassified and placed in the genera *Atopobium*, for example, *A. rimae* (formerly *L. rimae*) and *A. minutum* (formerly *L. minutus*).[13,105,319] Two other members of the genus are *A. parvulum* (formerly *Streptococcus parvulus*) and *A. vaginae*, a newly described species which has been associated with GU tract infections including bacterial vaginosis.[85,479] *Lactobacillus*

uli has been reclassified as *Olsenella uli*; another member of this genus is *O. profusa.* Both are microaerotolerant anaerobes found in the oral cavity and have been associated with dental plaque and endodontic disease.[118]

There are also many other new genera of anaerobic gram-positive NSF bacilli as seen in Table 16-2. Some of these have only been isolated from feces, suggesting they may play a role as normal microbiota of the GI tract. These include *Anaerofustis, Anaerostipes*, *Anaerotruncus, Catenibacterium, Colinsella, Dorea, Holdemanii,* and *Roseburia*. *Mogibacterium* is new genera with species primarily found in oral cavity; *M. timidum* is the new name for *Eubacterium timidum.*[211] The other newer genera that might have some clinical relevance will be discussed further below in the section on Human Infections.

Taxonomy of the Clostridium *spp.*

Clostridium spp. are a group of anaerobic spore-forming bacilli that can be part of the normal microbiota primarily of the GI tract; these are clinically relevant as the cause of both endogenous and exogenous infections. *Clostridium tetani* is the causative agent of tetanus; *C. botulinum*, the cause of many different forms of botulism; *C. perfringens*, one of the causes of gas gangrene, a cause of necrotizing skin infections, as well as a cause of food poisoning; and *C. difficile,* the cause of a syndrome of diarrheal conditions ranging from antibiotic-associated diarrhea (AAD) to pseudomembranous colitis (PMC) and associated complications. All of these are strict anaerobes, with the exception of *C. perfringens*. These produce spores readily in cultures from clinical specimens so that identification as a *Clostridium* spp. is usually not difficult. There are many other species of *Clostridium*, as seen in Table 16-2, and probably more will be identified with increased use of molecular and mass spectrometry techniques in clinical laboratories. One of the more commonly isolated species, *C. clostridiofome*, is now considered a complex of organisms, including *C. clostridioforme,*[163,518] *C. hathewayi,*[293,461] *C. bolteae,*[451] *C. aldenense,*[502] *C. citroniae,*[502] and *C. lavalense*.[121,518] The aerotolerant *Clostridium* sp., which includes *C. tertium, C. histolyticum,* and *C. carnis,* can easily be mistaken for aerobes.[173,330,464] There are species of *Clostridium* that have been newly designated and/or newly recognized as human pathogens, such as *C. disporicum*, an unusual *Clostridium* that produces a double spore, and *C. celerescrescens*, which is sometimes mistaken as a *C. clostridioforme* group isolate.[48,379] A few of the *Clostridium* spp. appear in Gram's stains only as gram-negative bacilli, such as *C. ramosum*[167] and *C. symbiosum*[478]; others that stain gram-negative have been reclassified in non-*Clostridium* genera. *Clostridium* spp. and related genera are a complex group of organisms responsible for a diverse group of infections, some of which have yet to be elucidated. In addition, as is shown in Table 16-1, there are other genera of spore-

forming anaerobic gram-positive bacilli, but thus far none of these has been demonstrated to be pathogenic nor related to any human infections, with the exception of *Filofactor alocis*, which has been isolated in endodontic infections.[437]

Taxonomy of the Anaerobic Gram-Positive and Gram-Negative Cocci

The anaerobic cocci are part of the normal microbiota of the oral cavity, GI tract, GU tract, and skin. As can be seen in Table 16-2, the more commonly isolated anaerobic cocci have undergone significant taxonomic changes.[351] The only remaining members of the genus *Peptostreptococcus*, once the predominant genus of anaerobic gram-positive cocci, are *P. anaerobius* and *P. stomatis*.[350] *P. anaerobius*, along with *Parvimonas micra* (once called *P. micros* and *Micromonas micros*) and *Finegoldia magna* (once called *Peptostreptococcus magnus*), are the most commonly isolated anaerobic gram-positive cocci today.[291,349,351] Four species that were once members of the genus *Peptostreptococcus* have now been placed in the *Peptoniphilus* genus and six in the *Anaerococcus* genus. The commonly isolated members of these genera include *Peptoniphilus assacharolyticus* (formerly *Peptostreptococcus assacharolyticus*), which is isolated from the oral cavity along with *F. magna,*[348,351] and *Anaerococcus prevotii* (formerly *Peptostreptococcus prevotii*), which is a common inhabitant of the GI and GU tracts.[230,351,446] Other new

genera that now contain former *Peptostreptococcus* spp. include *Gallicola, Slackia,* and *Blautia*. A few newly named species of anaerobic gram-positive cocci can be found in Table 16-2 and include *Blautia wexlerae, Ruminococcus gauvreauii, Anaerococcus murdochii,* [448] *Peptoniphilus* pathogen *Murdochiella assacharolytica*.[483] *Atopobium parvulum* is the new name of what was once referred to as *Streptococcus parvulum*, an anaerobic gram-positive coccus that is part of the normal microbiota of the oral cavity.[128] It should be noted that members of the genus *Atopobium* which are morphologically bacilli and not cocci will be discussed in the section on gram-positive rods. Lastly, two *Staphylococcus* spp., are considered to be anaerobes. They appear initially as strict anaerobes, but upon transfer can become aerotolerant. These are *Staphylococcus saccharolyticus* (once called *Peptotreptococcus saccharolyticus*) and *Staphylococcus anaerobius* subsp. *anaerobius.*[446] The former can be isolated quite frequently from anaerobic cultures, although clinical relevance is not always easy to determine. The genus *Peptococcus* contains only one species, *P. niger*, which is infrequently isolated from human clinical

specimens and will not be further discussed in this chapter.

The anaerobic gram-negative cocci, although not infrequently isolated in clinical specimens, are usually not associated with clinical disease. When these are associated with disease, it is usually as a component of a polymicrobial infection. There are now six genera (Table 16-2) of these anaerobes that are part of the normal human microbiota, with *Veillonella* spp. being the most commonly isolated.[72,312,446]

Human Infections Involving Anaerobes

Overview

Anaerobic bacteria have been demonstrated to cause infections in virtually every organ and anatomic region of the body. Some of the more commonly involved sites are illustrated in Figure 16-1.[519] Based on other reports in the literature, the relative incidence of anaerobes in infections is summarized in Table 16-4.[56–60,160,161,238]

Most deep-seated abscesses and necrotizing lesions involving anaerobes are polymicrobial; these may include obligate aerobes, facultative anaerobes or microaerophiles as concomitant microorganisms.[160,161,238] These microorganisms, acting in concert with trauma, vascular stasis, and/or tissue necrosis, lower the oxygen tension and the Eh in tissues, and provide favorable conditions for the propagation of obligate anaerobes. Historically, infections and diseases involving anaerobes from exogenous sources are the ones that have been best known (Box 16-2). Within the past few decades, however, endogenous anaerobic infections have become far more common. There are two probable explanations. One is that the laboratory recovery of anaerobic bacteria has improved so that endogenous infections are no longer misdiagnosed or overlooked as they were in the past. The other is that a larger proportion of the patient population is receiving immunosuppressive drugs for malignancy and other disorders, resulting in populations of compromised hosts in which members of the normal microbiota, including anaerobes, can become opportunistic pathogens.[160,161] Primary anaerobic infections easily become established in areas of tissue damage. Bacteremia may ensue, with metastatic spread of bacteria and the formation of distant abscesses. This progressive chain of events sometimes results in a fatal outcome. The more common causes of endogenous anaerobic infections are listed in Box 16-3.

Not surprisingly, the most common anaerobic infections involve intra-abdominal abscesses, because of the predominance of anaerobes in the GI tract as normal microbiota; *B. fragilis* is the most common anaerobe in these infections, in particular due to the role of its capsule in abscess formation.[507] Most of the abscesses are polymicrobial, involving aerobes and anaerobes. Peritonitis, due to a break in the

barriers of the GI tract (often referred to as secondary peritonitis) is most often due to a mixture of aerobes (most commonly *E. coli*) and anaerobes (most commonly *B. fragilis*). A study of children whose peritonitis results from a perforated appendix determined that 81% of the peritoneal cultures were positive for *E. coli*, which were nearly always susceptible to amoxicillin/clavulanate, and 54% for anaerobes, all of which were found susceptible to metronidazole. Their study corroborated the use of these two agents for treatment.[361] In contrast, spontaneous bacterial peritonitis is rarely an anaerobic infection; likewise, peritonitis associated with peritoneal dialysis is rarely an anaerobic infection, however, when it does occur, it is due to contamination of the catheter, is most often polymicrobial and carries a good prognosis.[87] Pyogenic liver abscesses, most of which have a cryptogenic source, can be due to a mixture of aerobes and anaerobes. One study showed a predominance of *K. pneumoniae* (45%) and *E. coli* (32%); however, anaerobes were found in only 9% of positive cultures.[309]

Anaerobes are a significant component of the oral cavity and contribute greatly to periodontitis and other oral/dental infections. However, anaerobic bacteria are infrequent pulmonary pathogens, except in selected infections such as aspiration pneumonia, lung abscess, necrotizing pneumonia and empyema. Because laboratories do not process most specimens for anaerobes that are sent to the laboratory for diagnosis of pneumonia, ie sputum, bronchoalveolar lavage and endotracheal aspirates, due to the contamination of the specimen by the upper airway microbiota, which includes anaerobes. Recognition of the probable presence of anaerobes on direct Gram's stains of sputum can be very helpful to the clinicians who might not otherwise suspect aspiration pneumonia; if aspiration or necrotizing pneumonia is expected and identification of the anaerobes is requested, collection of appropriate specimens for anaerobic cultures, using a protected fiberoptic collection device or sending lung tissue aspirates is needed.[37] Bartlett's review of anaerobic infection of the lung in 2012 suggested that pleural fluid and transthoracic needle aspiration are the best specimens for the meaningful recovery of anaerobes since transtracheal aspirates are no longer performed, and fiberoptic bronchoscopy specimens are not often handled appropriately.[36] The most common anaerobes involved in these usual polymicrobic pulmonary infections, are *F. nucleatum*, the pigmented *Porphyromonas* spp. (<10%),*Prevotella* spp. (30% to 50% and these are often β-lactamase positive) and the anaerobic streptococci. The *B. fragilis* group is involved in <10% of pulmonary infections.[238] Antibiotics that cover these anaerobes and drainage of the area is necessary for treatment.[36,37]

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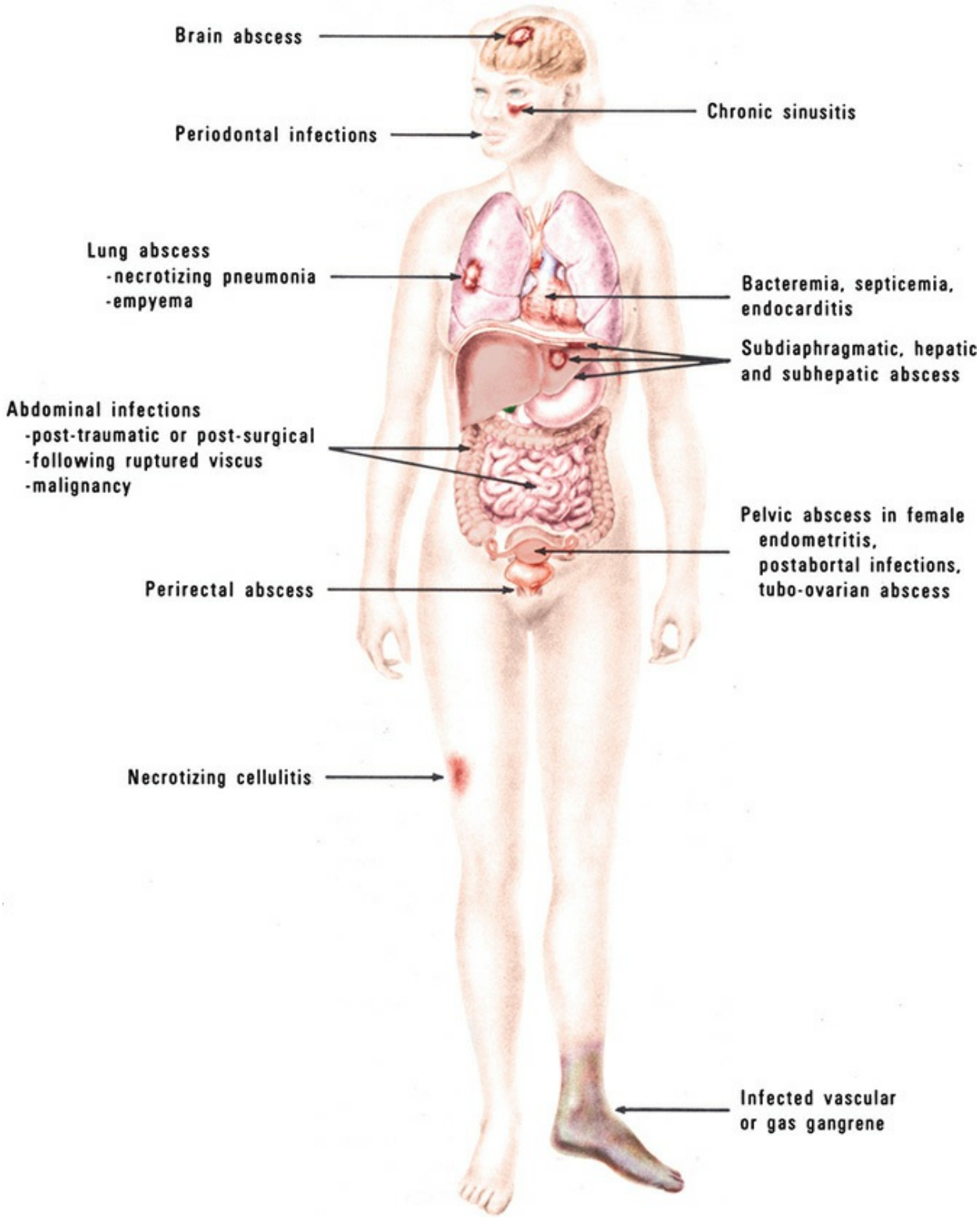

■ **FIGURE 16-1** Common locations of infections involving anaerobic bacteria.

Skin and soft tissue infections are often caused by aerobic gram-positive cocci that

predominate as normal microbiota; however, complicated skin and soft tissue infection may involve anaerobes, and result from endogenous or exogenous sources.*Clostridium* spp. such as *C. perfringens* predominate in exogenous infections; animal and human bite wounds often involve anaerobes. A review of the microbiology of dog and cat bite wounds, as well as bites from other more exotic animals demonstrated that 48% of dog bite wounds resulted in a polymicrobial infection that often included anaerobes, but anaerobes alone were responsible for disease in only one patient with a mixed infection caused by *Porphyromonas/Bacteroides* (Image Plate 16-2B). The most common anaerobe isolated from dog bite wounds was *F. nucleatum*, followed by species of *Bacteroides, Porphyromonas, Prevotella*, *Peptostreptococcus,* and *P. acnes*. From cat bite wounds, 78% were found to be mixed anaerobic/aerobic infections and again *F. nucleatum* was the most common isolate. Other organisms recovered included other species of *Fusobacterium*, *Bacteroides* spp., *Porphyromonas, Prevotella* and a wide variety of other anaerobic species.[1]

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TABLE 16-4 Percent of Positive Cultures That Yielded Anaerobes in Infections

Type of Infection	Percentage (%) of Positive Cultures With Anaerobes
Pulmonary	
Aspiration pneumonia	62–93
Lung abscess	58–100
Empyema (thoracic)	22–36
Bacteremia	1–17[a]
Brain abscess	62–83
Sinusitis	
Chronic sinusitis	48–100
Acute sinusitis	<10
Dental, oral, facial	67–100
Abdominal[b]	

Intra-abdominal	60–100
Liver abscess	<60
Pelvic[c]	50–100
Soft tissue infections	
Bite wounds	50–70[d]
Nonclostridial crepitant cellulitis	75
Gas gangrene (clostridial)	100
Diabetic foot ulcers	50–90[e]
Miscellaneous infections	
Urinary tract infection	<5[f]
Meningitis	<10

[a] From a review of anaerobic bacteremia by Brook, 2010.[58] Others have reported incidence of 7%–9% in 2008 and 2013.[155,354]

[b] Varies depending upon whether the patients are postsurgical or not.

[c] Varies considerably; pelvic inflammatory disease, <50%; pelvic or vaginal cuff abscess, 98%; postpartum endometritis, 66%.

[d] Depends on whether animal or human.

[e] Depends on methods of culture collection.

[f] May change using molecular or other new methods of direct specimen detection.

Modified from Jousimies-Somer et al.[238]

Anaerobes are responsible for 1% to 17% of bacteremia as reported in a review by Brook in 2010. The most common anaerobes were *B. fragilis*, followed in lesser incidence by anaerobic gram-positive cocci, *Clostridium* spp. and *Fusobacterium.*[58] A Canadian population-based surveillance study of anaerobic bacteremia between 2000 and 2008 found an incidence of 8.7/100,000 per year, with the predominant organisms as follows: *B. fragilis, Clostridium* (non-*perfringens*), *Peptostreptococcus* (includes *F. magna*), and *C. perfringens*. Male sex, increasing age, and a host of underlying illnesses and malignancies were predisposing factors; 30 day mortality was 20%.[354] In literature from the 1980s and 1990s, anaerobes were reported to cause 1% to 11% of

cases of endocarditis.[413,505] In a more recent review of the still infrequent anaerobic causes of endocarditis, the most common anaerobes were microaerophilic streptococci, *P. acnes, B. fragilis* group and *Clostridium* spp; mortality was high ranging from 21% to 43%.[57]

BOX 16-2

Anaerobic Infections of Exogenous Origin

Clostridium difficile hospital-acquired (nosocomial) diarrhea

Food-borne botulism

Infant botulism

Wound botulism

Clostridium perfringens gastroenteritis

Myonecrosis (gas gangrene)

Tetanus

Crepitant (anaerobic) cellulitis

Benign superficial infections

Infections following animal or human bites

Infections of injection drug abusers

Septic abortion

BOX 16-3

Anaerobic Infections of Endogenous Origin

Abscess of any organ

Actinomycosis

Aspiration pneumonia

Bacteremia

Bacterial vaginosis

Complications of appendicitis or cholecystitis

Crepitant and noncrepitant cellulitis

Dental and periodontal infections

Endocarditis

Endophthalmitis, usually following cataract surgery

Lemierre's syndrome (*Fusobacterium necrophorum*): also known as necrobacillosis

Meningitis, usually a complication of a communicating brain abscess

Necrotizing fasciitis

Necrotizing pneumonia

Osteomyelitis

Chronic otitis media

Peritonitis

Septic arthritis

Chronic sinusitis

Subdural empyema

Thoracic empyema

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It is essential to isolate and identify anaerobic bacteria because these infections are associated with high morbidity and mortality, and the treatment of the infection varies with the bacterial species involved. Antibiotic therapy for certain anaerobic infections

is different from that used for many infections caused by aerobic or facultatively anaerobic bacteria.[160,443,444] Prompt surgical intervention, including debridement of necrotic tissue or amputation of a limb, may be of extreme importance, particularly for patients with clostridial gas gangrene or in loculated abscesses into which antibiotics penetrate poorly until the exudate is drained.

Infections Involving Anaerobic Gram-Negative Bacilli

Prior to the mid-1960s, clostridial infections predominated. In recent years, however, <15% of all anaerobes were species of *Clostridium.*[159] Currently, more than three-fourths of anaerobes isolated from properly selected clinical specimens are accounted for by the *Bacteroides fragilis* group, *Prevotella, Porphyromonas, Fusobacterium*, the anaerobic cocci, and the anaerobic gram-positive NSF rods (Table 16-3).

The most common disease-producing, anaerobic gram-negative NSF rods isolated from clinical specimens are members of the *Bacteroides fragilis* group (including *Parabacteroides* spp.), the *Prevotella-Porphyromonas* (formerly members of the pigmented *Bacteroides*) groups and *Fusobacterium nucleatum*. *B. fragilis, B. thetaiotaomicron* and other species of the *B. fragilis* group are especially common. They are particularly important not only because they may be isolated from a variety of life-threatening infections, but also because of their resistance to the action of penicillin and its analogs and their resistance to many cephalosporins (including the third-generation cephalosporins) and the tetracyclines; the emergence of resistance to several of the newer quinolones; and increasing resistance to clindamycin.[149,188,190,355,364,443,444,507] Lorber has recently reviewed the role of anaerobes, especially *Bacteroides*, in the maintenance of health and their role in clinical disease.[301]

As mentioned above, members of the *Bacteroides fragilis* group are responsible for a variety of clinical infections, but the species *B. fragilis* has the highest incidence. A number of virulence factors have been demonstrated to explain the enhanced virulence and pathogenicity of this particular species: the presence of agglutinins and fimbriae that provide adhesion ability; protection against the host immune response due to the presence of a capsule to evade phagocytosis; mechanisms to ameliorate oxygen toxicity; and, destruction of host tissue via production of histiocytic enzymes. *B. fragilis* is most commonly responsible for intra-abdominal abscesses and sepsis, but is also involved in gynecological abscesses, skin and soft tissue infections, pericarditis (as a result of hematogenous spread to the heart), bacteremia often associated with malignancies or in postsurgical infections and rare instances of endocarditis, meningitis and septic arthritis.[420,507] At one time, the incidence of bacteremia following post-GI and post-GU surgery was high and carried a very high mortality (up to 60%), but with prophylaxis directed toward anaerobes and aerobes, that incidence has decreased. The

mortality associated with *B. fragilis* infection, however, can be as high as 19% and even higher when the patient has endocarditis, for example.[507] *B. thetaiotaomicron* and *B. ovatus* are two other members of the *B. fragilis* group that are more commonly involved in anaerobic infections than other members of the group (other than *B. fragilis*); they constitute a significant proportion of the microbiota of the GI tract, much more so than *B. fragilis*. Therefore, it is not surprising that breaks in the GI mucosa afford entry for these bacteria and consequent intra-abdominal infections. Many times in clinical laboratories, anaerobic gram-negative bacilli that grow on *Bacteroides fragilis* selective media (like *Bacteroides* bile esculin) may be reported out as "*B. fragilis* group"; so it is difficult to know how common some of the other members are in infections. With increased use of molecular methods and MALDI-TOF in the future, we should expect to be able to better determine what species really are involved in clinical infections; two of the more recently identified species using molecular techniques, *B. dorei* and *B. finegoldii*, have been specifically identified in blood cultures.[21,22,263]

Bacteroides ureolyticus, the corroding anaerobic gram-negative bacillus that remains as one of the few members of the non-*Bacteroides fragilis* group at least presently, has been associated with periodontal disease and has been isolated from a patient with sinusitis, but the relevance in this instance was unknown; rarely, it has been implicated in nonoral infections. There is at least one report of bacteremia and meningitis.[60,511]

Many of the pigmented *Bacteroides* are now in the *Porphyromonas* genus. Three are members of the oral microbiota, but may also be pathogens there, causing infections, such as periodontitis, dental abscesses and infected root canal. The oral species are *P. gingivalis*, the most significant and frequently isolated, *P. endodontalis* and *P. catoniae*, a recently named species.[263] *P. catoniae* has not been involved in oral infections, but has been isolated from an abdominal abscess.[263] Skin and soft tissue infections, including diabetic foot infections, have been caused by *P. asaccharolytica*[469] and *P. somerae.*[467] A new *Porphyromonas* sp., *P. uenonis,* has been found in appendicitis, peritonitis, sacral decubitus ulcers, pilonidal abscesses and other wounds of buttocks and groin.[165,466] Another oral pathogen closely related to the genus *Porphyromonas* is *Tannerella forsythia* (formerly *Bacteroides forsythus*). It plays a substantial role in periodontal disease. It has also been isolated from vaginal specimens from women suspected of having bacterial vaginosis and synovial specimens from patients with arthritis.[193,263,396]

The genus *Prevotella* has over 37 species of what were once *Bacteroides* spp. or have been newly named. About one-third of the strains are pigmented and the remaining are nonpigmented. Most members of the genus are found in the oral cavity as commensals (Table 16-2); however, many have also been associated with oral and

even nonoral infections. *P. intermedia* and *P. nigrescens*, related oral species which are difficult to distinguish phenotypically, are commonly detected in cases of gingivitis, root canal infections, dental abscesses, and other oral and dental infections. *P. intermedia* has a large role in periodontitis.[263]*P. nigrescens* has also been isolated

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from a patient with cellulitis.[529] *Prevotella bivia* and *P. disiens* have been isolated from blood, head and neck infections, GU tract infections in both males and females, and other sites of infection.[263] One recent study found higher bacterial counts of *P. bivia* and *P.disiens* in patients with bacterial vaginosis and gingivitis as compared to patients with bacterial vaginosis without gingivitis.[372] *P. melaninogenica* (formerly *B. melaninogenica*, and a very commonly identified pigmented oral anaerobe) has been found in the sputum of patients with cystic fibrosis, as have *P. denticola, P. oris,* and *P. salivae*.[238,263] Isolates of *P. bivia, P. disiens, P. buccae, P. denticola*, and *P. nigrescens* have been found in bloodstream infections.[263,436] An observational study of the incidence and characteristics of gram-negative bacilli from many tertiary hospitals in Greece (2005 to 2006) found that the majority of infections were intra-abdominal, skin and soft tissue, or pelvic; the majority were polymicrobial. *Prevotella* spp. were more frequently isolated from respiratory tract infections, superficial wounds and patients with diabetes as compared to *Bacteroides* spp. (primarily *B. fragilis* group), which were predominant in intra-abdominal infections and bacteremia. Many more of the patients with infections with *Prevotella* spp. were treated as outpatients, with most being skin and soft tissue infections, possibly indicating less serious diseases. Clindamycin was used more successfully for *Prevotella* spp. than when infections included members of the *B. fragilis* group in the intra-abdominal infections. *Prevotella* spp. did demonstrate more resistance to metronidazole (16%) than did *Bacteroides* spp. or *Fusobacterium* spp. (both less than 1%).[367] One of the non-*B. fragilis* group, *B. ureolyticus,* is a significant component of the normal oral microbiota, but has been involved in oral infections, infected sinuses, and head and neck space infections.[511]

F. nucleatum and *F. necrophorum* are the most commonly isolated species of the *Fusobacterium* genus. *F. nucleatum* is part of the microbiota of the oral cavity, along with *F. periodonticum* and *F. simiae*, but is responsible for production of biofilms in periodontal pockets and hence involved in periodontal disease. *F. nucleatum* is the species most commonly found in clinical specimens. Recent attention has been called to severe systemic infections caused by *F. nucleatum* in patients with neutropenia and mucositis following chemotherapy.[67,150] *F. nucleatum* along with members of the *Prevotella-Porphyromonas* group are the organisms most frequently involved in

anaerobic pleuropulmonary infections (e.g., aspiration pneumonia, lung abscess, necrotizing pneumonia, and thoracic empyema). In addition, *F. nucleatum* can cause bacteremia, abscesses, and infections of the GI tract, joints, brain, and GU tract.[263] The second most commonly isolated species, *F. necrophorum,* is divided into two subspecies,*fundiliforme* and *necrophorum*, but only *F. necrophorum* subsp. *fundiliforme* is recognized as a cause of human infection. *F. necrophorum* subsp. *fundiliforme* has been shown to be the cause of as many as 20% of acute pharyngitis cases especially in adolescents and young adults, and up to 20% of cases of recurrent or persistent, chronic sore throats. It can cause abscesses in adolescents, otitis media and mastoiditis in children, peritonsillar abscesses in adolescents and young adults and sinusitis in adults aged 30 to 50 years.[289,530] Perhaps more significantly, it is the primary cause of the severe and possibly life-threatening condition, Lemierre's syndrome, which has a greater incidence, morbidity and mortality than even rheumatic fever in adolescents and young adults in the Western Hemisphere.[51,56,384,393,394,474]

Lemierre's syndrome (anaerobic postanginal septicemia or necrobacillosis) usually begins with pharyngotonsillitis, followed by unilateral swelling and tenderness along the sternocleidomastoid muscle due to septic thrombophlebitis of the jugular vein. This proceeds to high fevers and metastatic lung abscesses. If recognized early and treated appropriately, it is responds to therapy, but it remains a challenging clinical and laboratory diagnosis.[51,92] Lemierre's and other disseminated *F. necrophorum* infections were prospectively investigated from 1998 to 2001 in Denmark. Fifty cases were found with an incidence of 14.4 cases per million annually in patients that ranged in age from 15 to 24 year olds. The infection originated mainly from an oropharyngeal infection but there were other instances originating from ear, sinus or teeth. The overall mortality was 9%. In older patients, Lemierres was found to have originated from foci in lower parts of body and patients often had predisposing diseases, leading to a mortality of 26%.[268] An immunocompetent patient with fulminant meningitis that developed subsequent to *F. necrophorum* otitis media has been reported, as well as a young adult patient with endocarditis.[12,339] Lastly, an epidural abscess in an elderly gentleman was found to contain *F. necrophorum* using molecular methods.[412]

Fusobacterium prausnitzii, a gram-negative anaerobe that makes up to 5% of the human GI tract normal microbiota and appears to be responsible for balancing the immune responses in the gut, has been reclassified as *Faecalibacterium prausnitzii*.[333]There is a recent review article about the significance of *Fusobacterium* spp. in serious infections. The incidence of *Fusobacterium* was 0.76 cases/100,000 in this study, and *F. necrophorum* was the most common pathogen. *F. nucleatum* and *F. varium* were described in this study as well.[376] This reference should be referred to for more information about the relevance of *Fusobacterium* spp.

Among the more recently described genera of anaerobic gram-negative bacilli, some

references are included in Tables 16-1 and 16-2, but only a few of these organisms are considered further here. The bile-resistant and pigmented *Allistipes* spp., for example, have been recently linked to appendicitis in children and species of the genus have been isolated from blood, intra-abdominal fluid, abscesses, and urine.[263] *Bilophila wadsworthia,* a non-*B. fragilis* group organism that is bile-resistant and a strict anaerobe, has become recognized as a significant human pathogen. The fastidious nature of this organism, however, may limit our ability to recover it from clinical samples and hence decrease its routine recognition. It is also an isolate found in normal fecal microbiota. Baron brought this organism to our attention in 1997 describing its involvement in polymicrobial intra-abdominal infections, especially appendicitis, and more recently, two patients in Hungary. One of these patients had mastoiditis, whereas the other had a brain abscess.[29,484] With the use of better collection procedures and culture methods for the isolation of anaerobes, as well as the use of molecular methods or mass spectrometry, laboratories may begin to more frequently detect this often antimicrobial resistant pathogen. Box 16-4 describes cultural and clinical characteristics of *B. wadsworthia*.

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BOX 16-4

Characteristics and Clinical Significance ofBilophilaspp.

Gram-negative, non–spore-forming, nonmotile, pleomorphic rods; 0.7–1.1μm in width by 1–10 μm in length

Growth enhanced by 20% bile and by 1% pyruvate; grows slowly on BBE medium; colonies on BBE after 4 days of incubation were described as 1–2 mm (diameter), either as circular, erose, umbonate, and translucent with dark black centers, or as irregular, low convex, and opaque black colonies

Colonies on Brucella agar formed slowly (4–7 days), were punctate, less than 1 mm in diameter, circular, erose, translucent, and gray

Strongly catalase-positive (using 15% H_2O_2)

Asaccharolytic

Urease-positive

β-lactamase-negative (nitrocefin test), but nonetheless, resistant to β-lactam antibiotics

Clinical Relevance: GI microbiota; common isolate in human specimens if culture conditions are optimal; perforated and gangrenous appendicitis, surgical abdominal infections; bacteremia.[30,263,484]

Desulfovibrio, a curved, often motile, gram-negative bacillus is in the same family as *B. wadsworthia*. It is a human fecal isolate, but *D. desulfuricans* and *D. fairfieldensis* have also been isolated from clinical specimens, including blood cultures.[263,495] Like *B. wadsworthia*, species of *Desulfovibrio* are often difficult to recover in laboratories without special media and the capability of molecular methods for identification, but they should be kept in mind when a curved gram-negative anaerobe is isolated from a blood culture.[292]

Leptotrichia spp. are large gram-negative "fusiform-shaped" nonmotile bacilli that are part of the normal oral microbiota. It is infrequently isolated in clinical specimens, but can cause disease in patients with underlying malignancies who have neutropenia and may have some form of mucositis/gingivitis. It has been isolated from blood and peritoneal fluid; at least one instance of endocarditis has been attributed to *L. buccalis* as well.[40,198] A few newly named species of *Leptotrichia, L. trevisanii,* and *L. goodfellowei* have also been reported to cause bacteremia and endocarditis, respectively, in immunocompromised hosts.[69,270] In addition, *Sneathia sanguinegens* (formerly *Leptotrichia sanguinegens*) has been associated with bacteremia, as well as, bacterial vaginosis.[103,120] Lastly, a long spiral-shaped gram-negative rod, *Anaerospirillum* spp., has been reported from patients with bacteremia and diarrhea; these patients had underlying disorders, including alcoholism, malignancy, diabetes, and poor dentition.[377,472] The pathogenicity and clinical relevance of this organism was recently reviewed by Kelesidis.[250]

Box 16-5 provides information on the clinical relevance of some species of anaerobic gram-negative bacilli that were not discussed above.

BOX 16-5

Clinical Relevance of Selected Anaerobic Gram-Negative Bacilli

Species	Clinical Relevance
Alistipes spp.	GI flora; isolated from abdominal infections and urine[157,447]
Anaerobiospirrilum succiniproducens	Bacteremia[250]
Catonella morbi	Endocarditis[329]
Dialister pneumosintes	Endodontogenic infections and sinusitis[135]

Filifactor alocis	Endodontic infections[193]
Leptotrichia spp.	Bacteremia[270]; peritonitis[198]; endocarditis[69]
Sneathia sanguinegens	Bacteremia[103]; associated with bacterial vaginosis
Sutterella wadsworthensis	Peritonitis; intra-abdominal abscesses; bacteremia[336]
Tannerella forsythensis	Endodontic infections[193,396]
Centipeda sp.,*Johnsonella* sp.; many *Leptotrichia* sp., *Pyramidobacter* sp., *Selenomonas* spp.	Oral flora; occasional case reports of infection
Anaerostipes sp.,*Barnesiella* sp., *Cetobacterium* sp., *Desulfovibrio* spp., *Faecalibacteriumprausnitzii, Megamonas* sp.,*Oxalobacter* sp.,*Paraprevotella* sp., *Parasutterell* sp.	Fecal flora; occasional case reports of infection

Information from reference 263 and other references as noted in table.

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Infections Involving Anaerobic Gram-Positive NSF Bacilli

Actinomycosis is an acute and chronic infectious disease characterized by suppurative lesions, abscesses and draining sinus tracts. The disease most often presents as cervicofacial, thoracic, abdominal or pelvic actinomycosis, but it can occur in other regions of the body.[109,186,378,382,401,499] *Actinomyces israelii* is the major cause of all forms of actinomycosis. Other species that are established causes of actinomycosis, though less frequently encountered in this condition than *A. israelii*, are *A. naeslundii, A. odontolyticus, A. meyeri, A. viscosus, A. gerencseriae,* and *Propionibacterium propionicum* (formerly *Arachnia propionica*). Although encountered in relatively low frequency in routine specimens, all of these species are well-documented pathogens. Members of the genus were first described in the 19th century; *A. bovis* was isolated from purulent drainage of a condition referred to as "lumpy jaw" in cattle. The association of actinomycosis and the use of an intrauterine device (IUD) have been recognized for many years. Although there has been controversy about how the diagnosis is made, the presence of abdominal pain in a

patient with an IUD should raise suspicion of pelvic actinomycosis.[383] There have been a number of reports in which actinomycosis cases are mistaken for tumors, sometimes leading to difficult and unnecessary surgical resections.[365] Many of the species of *Actinomyces*, such as *A. odontolyticus* and *A. naeslundii,* are part of the normal oral microbiota, and although they can be associated with human disease, their isolation from the respiratory tract may not always indicate that they are involved in any infectious processes. Correlation with the clinical picture, finding of *Actinomyces*-like organisms on Gram's stain, and isolation of the organism from sterile sites increase the likelihood of their role in the disease.

A. meyeri, although an uncommon cause of actinomycosis, can be associated with pulmonary disease more often than other species of *Actinomyces* and appears to have a predilection for dissemination. A series that reviewed 32 patients, disclosed poor dental hygiene and alcoholism as predisposing factors. The patients did respond well to a combination of penicillin therapy and surgical debridement.[153] An elderly patient with poor oral hygiene who developed meningitis caused by *A. meyeri* has also been reported; no anaerobic cultures of cerebrospinal fluid (CSF) were performed, although gram-positive rods were seen on Gram's stain; a blood culture was positive for *A. meyeri* in this patient.[200] More recently described *Actinomyces* species, using sequencing or mass spectrometry, have been associated with a variety of infection. *A. europaeus* has been associated with breast abscess, *A. cardiffensis* with septicemia in conjunction with lung and hepatic abscesses and *A. funkei* with endocarditis.[203,423,435,506] A report by Clarridge and colleagues aids in assessing the significance of several recently described species.[99] *A. turicensis* and *A. neuii* are two more recently described species that are responsible for a wide variety of clinical infections including breast abscess, endophthalmitis, bacteremia, and endocarditis.[310,402,497] Although the new species listed in Table 16-2 have been isolated from various human clinical sources, information still is lacking for many of them as to their clinical importance, but where reports were found, the references are included in the table. Two new genera, *Actinobaculum* and *Varibaculum*, are related to *Actinomyces* spp. Isolates *Actinobaculum* have been associated with urinary tract infections and also have been isolated from blood.[204,282,356]

Propionibacterium acnes, at one time often considered a contaminant when isolated from clinical specimens, has proven to be a pathogen when recovered from sterile sites such as ocular fluid in patients with postsurgical endophthalmitis, from blood in patients with endocarditis and in infections associated with surgically implanted prosthetic devices.[295,324,328,501] The latter includes infections following shoulder, hip and knee replacement, as well as and shunt-related infections of the central nervous system.[290,324,501] The association of *P. acnes* in lumbar disc herniation and infection has been reported when *P. acnes* was found in the excised herniated disc

tissue of 38% of the 64 patients in the study.[399] It can be difficult to determine the significance of the isolated *P. acnes*, but in true infections, the organism is repeatedly isolated, often seen on direct Gram stains of the specimens, and is the only isolate from the source of infection. Response to appropriate antibiotics can also play a role in determination of its significance.[371] *P. propionicum* (formerly *Arachnia propionicus*) is part of the normal oral cavity microbiota, but has been associated with infections of the lacrimal ducts and has been reported to cause actinomycosis-like infections in the pelvis.[499,524]*P. acidifaciens* is a member of normal oral microbiota but also has been associated with dental caries.[134,499] *P. granulosum* has been associated with infections following arthroplasty along with *P. acnes* and other *Propionibacterium* spp.[331,360] A related gram-positive bacillus, *Propionimicrobium lymphophilum*, has been isolated from lymph node of a patient with Hodgkin's disease.[457]

Eubacterium spp. are part of the normal GU and GI tracts. Many of the species are rarely isolated from clinical specimens as significant pathogens. This, however, may change with advances in molecular and mass spectrometry methods for identification. Tables 16-2 and 16-20 list the few remaining members of the genus *Eubacterium* as well as the newer genera. One of the more common clinical isolates, *Eggerthella lenta* (formerly *Eubacterium lentum)* has been associated with numerous patients with bacteremia, both as the sole pathogen and as part of a polymicrobial infection.[287,292,493] In a review of 25 patients with bacteremia caused by *E. lenta* wherein this was the only organism isolated in blood culture, 52% of the patients had fever, hypotension or leukocytosis during the bacteremia and in 44% an abdominal source was found. Mortality was high with 39% of the patients dying within 30 days of the diagnosis.[493] In addition, *Paraeggerthella hongkongensis* (formerly *Eggerthella hongkongensis)* and *Eggerthella sinensis* have recently been described as responsible for bacteremia, and like *E. lenta*, associated with significant morbidity and mortality.[280] *E. lenta* has also been described to cause multiple brain abscesses, liver abscesses, necrotizing pneumonia and osteomyelitis in an immunocompetent young man.[410] *Eubacterium limosum* made up 6% and *E. lenta* 24% of all anaerobes isolated in infections in patients with leukemia and solid tumors in a study from Egypt; in this study, anaerobes overall were responsible for 5% of the infections in this population.[145] It may be that the fastidious nature of these anaerobic gram-positive NSF bacilli has been overcome with newer isolation techniques and better

methods for identification; their relevance may continue to increase and laboratories should not dismiss their isolation especially from sterile sites.[281] *Solobacterium*

moorei, another *Eubacterium*-like gram-positive NSF bacillus, has been found responsible for oral infections and is associated with halitosis. It is not often found outside the oral cavity, but has been isolated from a few patients with bacteremia.[246,369] The taxonomy and phenotypic characteristics of other members of *Eubacterium*-related genera of anaerobic gram-positive NSF are given in Tables 16-2 and 16-20.

The genus *Bifidobacterium* has undergone taxonomic changes and now includes many species in the newly named genera or genera of *Bifidobacterium*-like anaerobes. They are normal microbiota in the human GI tract and are functionally important to the health. They can, however, be found in polymicrobial infections in which their significance may be greater than previously appreciated. *Bifidobacterium dentium* (previously *Bifidobacterium eriksonii)* is primarily associated with dental caries,[161,307] and like other bifidobacteria (e.g., *B. longum, B. adolescentis,* and *B. breve*) is isolated rarely from clinical specimens; it can, however, be involved in infections of immunocompromised hosts.[499] Many of the *Bifidobacterium* spp, along with *Lactobacillus* spp, are important in development of probiotics used for health nutrition and maintenance. *B. scardovia* has caused recurrent urinary tract infections in the elderly and has also been isolated from blood and other sites.[25,499] *Bifidobacterium*-like gram-positive anaerobic bacilli, now in other genera, such as *Parascardovia denticolens, Scardovia inopinata,* and other *Scardovia* spp., have been reported as potential pathogens when isolated from clinical material, especially in association with dental caries. Their significance in other infections has not been delineated.[126,233,499] The related *Alloscardovia omnicolens* has been isolated from a variety of sites, including urine, the GU tract, blood, lung, and the aorta, where it produced abscesses; it is felt to have pathogenic potential.[227,307]

Lactobacilli are commensals of the human microbiota, but are not regularly associated with clinical disease. *Lactobacillus* spp. rarely may cause endocarditis, but their pathogenicity when isolated from blood cultures not in association with endocarditis, or when isolated from other sites is not well defined.[68] Analysis of *Lactobacillus* spp, isolated from many sites over a 5-year period of time in a French hospital, demonstrated that *L. rhamnosus* was the most commonly isolated species and was isolated from blood cultures of 16 patients. The majority of these patients were immunosuppressed and/or had a catheter-related bacteremia. A review of 45 additional patients from the literature supported these findings.[195] The pathogenic potential of over 200 patients with *Lactobacillus* bacteremia, endocarditis and localized infection was reviewed in 2005. A 30% overall mortality was reported, although this was not deemed attributable mortality. Therefore *Lactobacillus* bacteremia may be an indicator of a serious underlying illness and/or a marker of poor long-term prognosis. In this study, the mortality rate for *Lactobacillus* endocarditis

was 23%. The localized infections in which *Lactobacillus* spp. were isolated included pulmonary infection, abscesses, endophthalmitis, meningitis, and vascular graft infections, among others.[68]

Some of the lactobacilli have been reclassified. For example, *L. uli*, which resides in gingival crevices, is now in genus *Olsenella. O. uli* has been associated with periodontitis and necrotizing gingivitis.[118] One instance of bacteremia due to this organism has been described.[274] *O. profusa* is a newly recognized species which has is also found in gingival crevices.[118] Another *Lactobacillus*-related group of organisms are now in the genus *Atopobium*. *A. minutum* (formerly *L. minutus*) and *A. rimae* (formerly *L. rimae*) are both found in gingival crevices.[105] *A. vaginae*, a new species, has been found in the vagina in association with GU infections.[85,479]

In 1984, Spiegel and Roberts proposed the name *Mobiluncus* for a new genus of gram-variable or gram-negative, motile, curved, NSF anaerobic rods, which occur singly or in pairs and have a "gull wing" appearance.[456] Electron micrographs revealed multilayered cell walls lacking an outer membrane that were more typical of gram-positive than gram-negative cell walls; nonetheless, the organisms tend to stain gram-variable in young culture and gram-negative in older culture. The name *Mobiluncus* was derived from the Latin words *mobilis* (meaning capable of movement) and *uncus* (which means hook).[456] The pathogenic potential of *Mobiluncus* spp., if any, is still not clear. It has been isolated from patients with extragenital infections on a few occasions, and there is a report of the isolation of this organism from a patient with severe sepsis.[185,218] The organism is one of many genera of anaerobes that colonizes the vagina in both health and disease.[499] *Mobiluncus,* along with other anaerobes, can be present in vaginal samples of women with bacterial vaginosis.[219,455]

Bacterial Vaginosis. Bacterial vaginosis involves an overgrowth of multiple bacteria, including *Gardnerella vaginalis, Mycoplasma hominis, Mobiluncus spp., Prevotella bivia, Prevotella disiens* and other *Prevotella* spp., *Peptostreptococcus anaerobius, P. asaccharolyticus, P. magnus* and other anaerobic cocci and anaerobic gram-positive rods including *Propionibacterium spp*.[219,455] Therefore, vaginosis is a synergistic infection in which many organisms play a role. As reviewed elsewhere,[445,531] the diagnosis of bacterial vaginosis is based on demonstrating consistent clinical, as well as, microbiologic criteria. In the laboratory, the scored Gram's stain has become the gold standard for diagnosis, evaluating the predominating presence and quantity of normal lactobacilli versus gram-negative and gram-variable short rods. The presence of typical "clue cells" continues to be utilized at point of care testing and can be visualized on the Gram's stain as well.[359] In the future, use of molecular methods to identify specific anaerobes that are responsible for syndrome and/or the presence or

absence of specific metabolic biomarkers may become the gold standard for diagnosis.[78,531] Newer molecular assays are being used for the assessment of specimens from patients suspected to have bacterial vaginosis. Among the more recently named genera of anaerobic gram-positive NSF, *Catabacter hongkongensis*, has been isolated from patients with bacteremia and is associated with high rates of mortality, especially in patients with underlying malignancies. The source of the bacteremia is usually the GI tract.[278,442] The clinical significance of other newer genera of anaerobic gram-positive NSF bacilli can be reviewed in the references given in Table 16-2, Box 16-6 and other tables throughout this chapter.

BOX 16-6

Selected Infections Caused by Anaerobic Gram-Positive Non–Spore-Forming Rods

Species	Clinical Relevance
***Actinomyces* spp.**	
A. israelii, A. meyeri, A. naeslundii, A. odontolyticus, A. viscous, and A. gerencseriae	Thoracic,[109,186] cervicofacial,[382] abdominal,[378] and pelvic actinomycosis[401]
A. meyeri, A. funkeii, and *A. cardiffensis*	Bacteremia[153,203,506]
A. europaeus	Breast absscess[435]
A. neui	Neonatal sepsis[310]; abscesses, endophthalmitis. Bacteremia and endocarditis[497]
A. turicensis	Breast abscess, bacteremia[402]
Actinobaculum spp.	Urinary tract infections[204]
***Propionibacterium* spp.**	
P. acnes	Shunt-associated infections[501]; endophthalmitis following cataract surgery[328]; prosthetic joint infections[290,324]; endocarditis[295]
P. propionicum	Actinomycosis-like infection[524]; lacrimal

P. propionicum	canaliculitis[238]
Propionimicrobium lymphophilum	Isolated from lymph node in patient with Hodgkin's disease[457]
P. granulosum	Arthroplasty infection[360]
***Bifidobacterium* spp. and related genera**	Oral/dental infections; mainly involved as normal flora in maintenance of health; used in probiotics; some species involved in urinary tract infections and isolated in blood cultures[307]
Alloscardovia spp., *Scardovia* spp.	Isolated in blood cultures,[307] dental caries[126]
***Eubacterium* spp. and related genera**	Bacteremia[287]; dental infections
Eggerthella spp. and *Paraeggerthella* spp.	Bacteremia[280,287,310,493,498]
Catenibacterium sp., *Colinsella* spp., *Dorea* spp., *Flavonifractor plautii*, *Holdemania*	Fecal flora
Cryptobacterium sp., *Bulleidia* sp., *Mogibacterium* spp.	Oral flora
Pseudoramibacter alactolyticus	Oral flora; involved in endodontic infections[439]
Slackia exigua	Oral flora; wound infections/abscesses[257]
Shuttleworthia satelles	Oral flora; endocarditis[430]
Solobacterium moorei	Fecal flora; bacteremia[369]
Turicibacter sanguinis	Fecal flora; bacteremia[46]
***Lactobacillus* spp. and related genera**	Normal vaginal flora; bacteremia[68,195]
Atopobium vaginae	Association with bacterial vaginosis[479]
Atopobium rimae	Oral infection[128]; bacteremia[13]
Olsenella spp.	Oral cavity; gingivitis and periodontitis[118]
Miscellaneous new genera	
Anaerofustis sp., *Blautia wexlerae*; *Rosburia* sp.	Fecal flora
Anaerotruncus colihominis	Fecal flora; bacteremia[499]

Catabacter hongkingensis	Bacteremia[278,442]
Mobiluncus sp.	Association with bacterial vaginosis[120]; bacteremia[185,218]
Slackia heliotrinireducens	Abscesses[381]

This is not a comprehensive list of all infections published by these organisms; refer to text and Table 16-2 for references to individual organisms or infections, if not given in table.
Adapted from reference 499 and other references as noted in table.

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Infections Involving Clostridium *spp.*

Clostridium spp. are responsible for long recognized exogenous, toxigenic infections, including tetanus (*C. tetani*), botulism (*C. botulinum*) and trauma-associated myonecrosis or gas gangrene (*C. perfringens, C. septicum, C. histolyticum* and occasionally *C. sordellii*). *C. tetani* and *C. botulinum* infections are described in a separate section below. *C. difficile* colitis is also a now well-described toxigenic infection, resulting after colonization of the GI tract, either in the hospital or community; it, too, will be described in detail below).[463] Species of *Clostridium* can also cause endogenously acquired infections from species that are part of the normal GI or GU tract microbiota, including such species as *C. perfringens,* members of the *C. clostridioforme* group, *C. ramosum, C. difficile, C. innocuum, C. septicum, C. sordellii,* and *C. bifermentans* (Tables 16-2 and 16-23). Many other *Clostridium* species are found in human feces; in most instances, the significance of any species varies with the clinical setting. Box 16-7provides information on clinical relevance of selected *Clostridium* species.

Isolation of a *Clostridium* species from a wound, blood culture or other body fluid does not necessarily have clinical significance. *C. perfringens*, the most frequently isolated *Clostridium* species, is a common inhabitant of the large bowel; it and other clostridia transiently contaminate the skin of the perianal area and other skin surfaces. However, when *C. perfringens* or other species of *Clostridium* are isolated from multiple blood cultures in patients with signs of bacteremia, they are likely clinical relevant. Most commonly, *C. septicum, C. tertium, C. sordellii* and members of the *C. clostridioforme* group (*C. bolteae, C. citroniae, C. clostridioforme, C. aldenense,* and *C. hathewayi*) have been associated with bacteremia, including septic shock in patients with malignancies or other underlying diseases.[163,463,464,502,518] In a study of

Clostridium from 138 patients with clostridial bacteremia were *Clostridium perfringens* (42%) followed by *Clostridium septicum* (14%), *Clostridium ramosum* (9%), *Clostridium clostridiiforme* (6%), and *Clostridium difficile* (5%).[284] Thirty percent of the patients died, and most were on hemodialysis or were patients with malignancy. It will be interesting if these findings change as more molecular methods, like 16S rRNA sequencing, are used for identification of bloodstream isolates.[436,522]

C. perfringens and Related _Clostridium_ spp. Involved in Skin and Soft Tissue Infections. *C. perfringens* is encountered in myonecrosis (gas gangrene), gangrenous cholecystitis, septicemia, and intravascular hemolysis following abortion and anaerobic pleuropulmonary infections. It is also a major cause of food poisoning in the United States. The other clostridia most often involved in gas gangrene are *C. sordelli, C. septicum,* and occasionally *C. histolyticum*.[463] Clostridial myonecrosis (gas gangrene) is a clinical entity that involves rapid invasion and liquefactive necrosis of muscle with gas formation and clinical signs of toxicity. It is a serious condition requiring close cooperation between the microbiology laboratory and clinical staff for confirmation of the clinical diagnosis. Gram-stained smears of aspirated material from myonecrosis reveal a necrotic background with a lack of inflammatory cells and presence of gram-positive bacilli with a morphology resembling *C. perfringens* or other clostridia. In other conditions, such as simple wound infection or anaerobic cellulitis, in which there may also be gas in tissue, muscle cell outlines and/or the presence of granulocytes and mixed morphologic forms of bacteria in Gram-stained smears of lesions would be evidence against clostridial myonecrosis.[463,464]

BOX 16-7

Clinical Relevance of Selected *Clostridium* spp.

Species	Clinical Syndromes/Infections
C. botulinum A,B,E,F	Botulism: Food-borne, wound, infant, adult colonization (toxemia), iatrogenic (inhalation)[79,464]
C. argentinense	Produces *C. botulinum* G Toxin and can cause botulism[181]
C. baratii and *C. butyricum*	Can produce botulism toxin and cause the disease[181]
C. clostridioforme group: *C. aldensense, C.*	Bacteremia, abscesses,

bolteae, C. citroniae, C. clostridioforme, C. hathewayi and *C. lavalense*	Bacteremia, abscesses, peritonitis[121,163,463,502]
C. perfringens	Gas gangrene, necrotizing fasciitis, food poisoning, many other skin and soft tissue infections[332,463,464]
C. ramosum	Bacteremia[167]
C. septicum	Bacteremia[253,311]; necrotizing fasciitis[416]
C. sordelli	Bacteremia[15]; postabortion infections[463,464] necrotizing fasciitis[258]
C. tertium	Bacteremia[330]
C. tetani	Tetanus[88,463,464]

Adapted from reference 464 and other references as shown in table.

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Myonecrosis due to *C. perfringens* usually results from a crushing type injury, with laceration of large arteries and open fractures of long bones that become contaminated with the spores of the bacterium. It can also occur in the abdomen or flanks after traumatic injury, for example from knife or gunshot wounds. There have also been patients with gas gangrene due to *C. perfringens, C. novyi* type A and *C. sordellii* following intravenous drug abuse among the users of black tar heroin.[258,463] *C. perfringens* produces an alpha toxin and theta toxin (perfringinolysin) that are responsible for the pathogenesis of myonecrosis. Alpha toxin has phospholipase C and sphingomyelinase activity and is a potent platelet agonist. The formation of thrombi occludes capillaries, venules, and arterioles, which decreases tissue perfusion and further enhances an anaerobic environment, resulting in rapid destruction of the tissue and producing the symptoms of myonecrosis. Theta toxin is a cytolysin that causes the lysis of human cells, and in addition modulates the inflammatory response to infection.[380,463,464]

C. septicum also produces alpha toxin, but the toxin it produces lacks phospholipase activity. In addition, three other toxins are produced: beta toxin (DNase), gamma toxin (hyaluronidae), and delta toxin (septicolysin, a hemolysin).[380] All of these may contribute to myonecrosis, which usually occurs without preceding trauma. *C.*

contribute to myonecrosis, which usually occurs without preceding trauma. *C. septicum* can proliferate in normal tissue, often following bacteremia. *C. sordellii* can produce up to seven exotoxins, of which lethal toxin and hemorrhagic toxin are the most virulent.[380] Gas gangrene can follow medically induced or self-induced abortions, as well as cesarean sections. The onset of shock and multi-organ failure in these gynecologic infections is very rapid and fatalities are high. Recognition of the infection early on is extremely important.[463]

Crepitant cellulitis (anaerobic cellulitis) seen in diabetic patients, involves subcutaneous tissue or retroperitoneal tissue and can progress to fulminant disease rapidly. Unlike myonecrosis, muscle and fascia are not involved. Several references are available for further details on the histotoxic clostridia and anaerobic cellulitis.[59,161,300,463]

Intestinal Diseases Involving *C. perfringens*. *C. perfringens* has become the second most common bacterial agent of food-borne disease in the United States causing one million illnesses each year. Most of the *C. perfringens* food-borne outbreaks in the United States have involved strains that produce toxin type A.[197,463] *C. perfringens* food poisoning results from the consumption of contaminated beef, turkey, chicken, pork, gravy, and other foods containing large numbers of the organism; outbreaks are often associated with consumption of these products at restaurants or catering facilities.[197] Crampy abdominal pain usually develops, within 7 to 15 hours after eating the suspected food. In most patients, there is foamy, "foul-smelling" diarrhea, usually without vomiting or fever. Illness occurs after viable vegetative *C. perfringens* cells reach the small intestine and undergo sporulation. A potent enterotoxin, produced in the gut while the spores are being formed, causes the diarrhea. The illness tends to be mild and self-limited, and patients usually recover in 2 to 3 days from the onset; at greater risk for more severe disease are the very young and the very old.[463,464] Laboratories can diagnose *C. perfringens* food poisoning by detecting the bacterial toxin in feces or by tests to determine the number of bacteria in the feces. Quantitative anaerobic cultures on selective media, demonstrating at least 10^5 *C. perfringens* organisms in the epidemiologically implicated food, or spore counts showing 10^6 or more *C. perfringens* spores per gram of feces collected within 48 hours of onset of symptoms, are done to confirm the diagnosis in an outbreak investigation.[81] In addition, serotyping of the isolates is done to determine if the same serotype of *C. perfringens* is present in the epidemiologically implicated food and in the feces of ill persons, but not in controls. Serologic typing must be done in established laboratories that are satisfactorily equipped to perform this service, such as at the Centers for Disease Control and Prevention (CDC).[464] The CDC website contains more information about *C. perfringens* and food safety

Necrotizing enteritis (enteritis necroticans, NEC), caused by *Clostridium perfringens*, is much more serious than the food-borne illness described above. NEC is characterized by the sudden onset of abdominal cramps and abdominal distention, vomiting, bloody diarrhea, shock related to fluid and electrolyte problems and acute inflammation with focal to widespread necrosis of the intestinal mucosa. The disease, recognized in post-war Germany, called "Darmbrand" (meaning "fire bowels") was a severe form of necrotizing enterocolitis with an associated mortality of approximately 40%. "Pig bel," a form of necrotizing enteritis seen mainly in children in the highlands of Papua New Guinea, has been associated with a mortality rate of about 30% to 60%.[463] Factors believed to be important predisposing conditions to NEC include the consumption of excessive amounts of rich food or foods containing trypsin inhibitors (e.g., sweet potatoes, peanuts) by malnourished individuals. As reviewed elsewhere, *C. perfringens* type C and type A have been implicated in this disease. *C. perfringens* NEC have been reported from many countries around the world, including Europe and the United States in adults who are malnourished or suffer from diabetes, alcoholic liver disease, or neutropenia.[463] In low birth rate infants who are in intensive care, NEC due to *C. perfringens* (alpha toxin A) can be very serious with high mortality rates, up to 44%.[119,172] There is both a clinical and pathologic spectrum in the severity of NEC. Some patients may survive with supportive care and decompression of the bowel; others may require resection of the involved intestinal segment; still others are inoperable and die with extensive gangrenous necrosis of the small and large bowel. The differential diagnosis includes PMC (whether or not associated with *Clostridium difficile*), acute shigellosis, food-borne illness caused by various agents (including *Escherichia coli*, *Campylobacter jejuni,* and others), acute ulcerative colitis, and obstruction of the bowel (due to adhesions, volvulus, etc.). In suspected cases, efforts to culture *C. perfringens* type A and C (e.g., blood cultures, peritoneal cultures if there is peritonitis, and intestinal contents of surgically removed or necropsy specimens) should be made. A reference laboratory (such as the state health laboratories or CDC), where typing of isolates can be accomplished, should be consulted.

As mentioned above, *C. perfringens* is the primary cause of clostridial bacteremia often in older patients and/or

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patients with liver disease.[284,463] Central nervous system infections are rare, but the organism has been responsible for meningitis, meningoencephalitis, and subdural empyema.[225]

Infections Involving Miscellaneous *Clostridium* spp.

Clostridium ramosum. *Clostridium ramosum* is a prominent member of the large

in particular, in intra-abdominal specimens following trauma. A review of clinical cases showed that although *C. ramosum* is a very common GI commensal, and is often isolated from clinical specimens, the association with human infections is still quite rare. It has caused ear infections in young children and bacteremia in the immunocompromised patients.[167] *C. ramosum* can, however, be easily be misidentified or overlooked, since it usually stains as a gram-negative rod and its terminal spores are frequently hard to demonstrate.

Clostridium septicum. Although *Clostridium septicum* is not nearly as commonly encountered as *C. perfringens*, it is especially important to recognize in the clinical laboratory. *C. septicum* can be isolated from serious, often fatal infections. It can be a cause of myonecrosis, with a 67% to 100% mortality. The predisposing factors for this condition include colonic carcinoma, diverticulitis, GI surgery, leukemia, cancer chemotherapy, radiation therapy, and AIDS.[463] *C. septicum* bacteremia is associated with underlying malignancy, particularly carcinoma of the colon or cecum, carcinoma of the breast, and hematologic malignancies (e.g., leukemia-lymphoma); laboratories should not dismiss even a single blood culture positive for *Clostridium* sp, and should always notify clinicians when *C. septicum* is identified in a blood culture, since aggressive diagnostic studies should be done to rule out GI abnormalities in these patients, if it has not already been performed.[265,311]

***C. clostridioforme* Group.** The members of the *C. clostridioforme* group are commonly isolated *Clostridium* spp. in the clinical laboratory. As mentioned in the section on taxonomy, there are at least 5 to 6 species included in the group Table 16-2, many of which require molecular methods for definitive identification. The species differ in their virulence and antimicrobial susceptibility patterns. These organisms can be found as part of the microbiota of the GI tract, and occasionally found in GU tract and as part of oral microbiota.[163] The most common clinical finding associated with this group is bacteremia and most of the species in the group have been described in the literature to cause BSI, along with intra-abdominal abscesses, necrotizing fasciitis and peritonitis.[11,34,48,163,451,463,502,518]

Other *Clostridium* spp. *C. inocuum* is a common clostridial isolate in clinical laboratories. It can cause bacteremia; patients in one review of clostridial bacteremia fared much worse when it was the infecting species, as compared to bacteremia with *C. septicum* or *C. perfringens*.[424] *C. sordellii* has been found in patients with bacteremia and, as mentioned above, can cause skin and soft tissue infections such as gas gangrene and necrotizing fasciitis; some infections have been associated with injection heroin use.[15,463,464] *C. tertium*, one of the aerotolerant *Clostridium* spp., is one of the *Clostridium* spp. found in blood cultures and has been implicated in bacteremia, especially in the neutropenic patient.[330] It has also been isolated in a patient with gas gangrene in which it had initially been identified as a *Lactobacillus,*

patient with gas gangrene in which it had initially been identified as a *Lactobacillus,* because of its good growth on aerobic plates.[173]

Infections With *C. botulinum* and Related *Clostridium* spp. Botulism is a life-threatening neuroparalytic disease caused by antigenically distinct, heat-labile, protein toxins of *C. botulinum*.[294] Although seven toxin types (A to G) are produced by different strains of *C. botulinum*, most cases of botulism in humans are caused by types A, B, E, and F. Of these, type F is the least common. Types C and D are associated with botulism in birds and mammals, but not in humans. *Clostridium* that produces the type G toxin, now reclassified *Clostridium argentinense*, has been isolated from autopsy samples of a few individuals who died suddenly; it is not clear to what extent type G organisms cause botulism in humans.[294,380,464] Some strains of *C. butyricum* and *C. baratii* also carry botulinum toxins and produce disease.[464] Regardless of which antigenic type, botulinum toxin acts by binding to synaptic vesicles of cholinergic nerves, thereby preventing the release of acetylcholine at the peripheral nerve endings, including neuromuscular junctions. Acute, flaccid, descending paralysis eventually develops without supportive treatment.[122] The paralysis begins with bilateral impairment of cranial nerves resulting in paralysis of muscles of the face, head, and throat. The paralysis then descends symmetrically to involve the muscles of the thorax, diaphragm, and extremities. Patients may die of respiratory paralysis unless they have proper respiratory intensive care, including mechanical ventilation; death may also result from secondary pneumonia, caused by nonbotulinum organisms.[159,294,464]

There are about 145 cases of botulism in the US each year according to the CDC, representing five different categories of infection. The first of these is classical food-borne botulism, typically seen in adults, resulting from the ingestion of preformed toxin in contaminated food; this form accounts for 13% of reported patients with botulism. The second category, wound botulism results from production of botulinum toxin in vivo after *C. botulinum* has multiplied in an infected wound; this accounts for about 20% of patients. The third category, infant botulism, is the most common, accounting for ~65% of reported cases in the United States. It results from in vivo multiplication of *C. botulinum* with the production of the neurotoxin within the infant gut. Adult intestinal colonization or adult toxemia is the fourth category and is very rare; it mimics infant botulinum. The fifth category as defined by the CDC is iatrogenic botulism, caused by an overdose of botulinum toxin and is also very rare.[80,464] Included in this latter category is "inhalation botulism" resulting from aerosolization and inhalation of botulinum toxin. This type can be considered as a bioterrorism threat and *C. botulinum* toxin is categorized as a potential bioterrorism agent.[80,464] Home-processed foods, rather than commercially processed foods, have been involved in

processed foods, rather than commercially processed foods, have been involved in most of the outbreaks of food-borne botulism. The foods most often implicated have been vegetables (e.g., home-canned tomatoes, tomato juice, green beans, greens, peppers, corn, beets, and spinach), fish (e.g., home-processed tuna fish, smoked salmon, fish eggs, commercially processed tuna, smoked whitefish, etc.), fruits (home-canned apple butter, blackberries, etc.), and miscellaneous foods (e.g., beef stew, chili, spaghetti sauce, luncheon

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meats, etc.). Symptoms of food-borne botulism include double and/or blurred vision, droopy eyelids, slurred speech, difficulty in swallowing, dry mouth, and muscle weakness.[80]

Infant botulism was recognized as a distinct clinical entity in 1976. From 1976 to 1988, 760 patients were diagnosed with this disease.[14,294] Infant botulism has been reported from many different states, with the greatest number of cases in California. Most cases reported west of the Mississippi River have been type A; the majority of those east of it have been type B. Affected infants have ranged from 6 days to 11.7 months in age, and both sexes have been affected equally. The infants ingest spores, not preformed toxin, from soil, household dust, honey, or another source. Within the gut, *C. botulinum* multiplies and elaborates toxin. Clinical features include constipation (usually the first sign), listlessness, difficulty in sucking and swallowing, an altered cry, hypotonia, and muscle weakness. Eventually the baby appears "floppy" and loses head control. Ptosis, ophthalmoplegia, flaccid facial expression, dysphagia, and other neurologic signs may develop. Respiratory arrest or respiratory insufficiency necessitating respiratory therapy occurs. A small number of infants with laboratory-confirmed infant botulism have died. Infant botulism has accounted for a small number of sudden infant death syndrome.[44]

The diagnosis of classical food-borne botulism is confirmed in reference laboratories, such as the CDC, by demonstrating botulinum toxin in serum, feces, gastric contents or vomitus. Also, the organism may be isolated from the feces.[80,294,464] The detection of botulinum toxin in epidemiologically implicated food, with or without isolating the organism, is useful for ascertaining what food was involved in the outbreak. About 15 to 20 mL of serum and 25 to 50 g of feces should be collected for shipment to the appropriate reference laboratory, as directed by the appropriate state or federal public health official who is capable of providing epidemiologic and laboratory aid in the investigation. In suspected wound botulism, serum, exudate or swab samples from the wound should be collected, along with tissue (e.g., at autopsy), and feces.

When infant botulism is suspected, serum (2 to 3 mL) and as much stool as possible

refrigerated or placed on ice for shipment. However, many, if not most, of these infants are constipated early in the illness, and stool may not be available. Therefore, the clinician must decide whether the risk of obtaining an anorectal swab specimen is clinically warranted for the laboratory to isolate *C. botulinum* from this source. Wound botulism is caused by the infection of a wound with *C. botulinum*. A specific type of wound botulism has developed due to injection of black tar heroin. Most of the patients with this type of disease have been seen in California.[532]

Some, but not all, state health department laboratories provide diagnostic services for botulism. With prior approval of the CDC and/or the local state health department laboratory, specimens may be submitted to the CDC for laboratory diagnosis. Confirmation of the clinical diagnosis of botulism requires demonstration of botulinum toxin (mouse neutralization test) in serum or feces, and/or *C. botulinum* in feces.[80,464] Isolation and identification of the organism is performed by conventional cultural biochemical procedures and the confirmed with the toxin neutralization test. Toxin has only rarely been detected in serum from an affected infant. It is recommended that culture and toxin testing for *C. botulinum* be done only by properly equipped reference laboratories for this specialized testing. During investigations of possible terrorism, sera, gastric aspirates, feces, environmental samples or nasal swabs could be useful and should be refrigerated until they can be transported to a reference laboratory for testing. For further details, the interested reader is referred to the following references.[80,464]

Infections With *C. tetani*. Tetanus is an infectious disease caused by *C. tetani*. There is an effective immunization available. In resource-rich countries with effective immunization strategies, disease is most commonly seen with injection drug users. The incidence of *C. tetani* in the United States was reported to be 233 patients from 2001 to 2008, averaging approximately 29 cases per year. The case fatality rate (in those with known outcomes) was 13.2%, but in those ≥65 years of age it rose to 31.3%.[82] Tetanus is a dramatic illness characterized by spastic contractions of voluntary muscles and hyperreflexia. It is caused by the actions of two toxins, tetanolysin and tetanospasmin elaborated by *C. tetani*.[88,159,464] Tetanus shares some similarity with diphtheria, in that the infection, and the organism, remains localized (usually a minor penetrating wound), whereas the toxin is absorbed, producing major systemic effects. The spores of *C. tetani*, like those of *C. botulinum*, are widely distributed in the soil, as well as in aquatic environments. Tetanus usually results from spore contamination of puncture wounds, lacerations, or even crush injuries.[15,88,464] Fecal contamination of the umbilical cord has been the source of *C. tetani* in some instances of neonatal tetanus.[90] The incubation period ranges from 3 to 21 days. Following a localized penetrating injury, there may be a deep-seated mixed infection involving anaerobes

providing favorable conditions for *C. tetani* spores to germinate, for the vegetative cells to multiply and release the tetanospasmin on autolysis of the bacterial cells.[15] Tetanospasmin attaches to peripheral motor nerve endings, and travels along nerves to the central nervous system (CNS). The toxin binds to gangliosides in the CNS, and blocks inhibitory impulses to the motor neurons. Patients have prolonged muscle spasms of both flexor and extensor muscles. Tetanospasmin attaches to binding sites at myoneural junctions, thus inhibiting the release of acetylcholine. This is like the attachment of botulinum toxin to myoneural junctions, except that the binding sites for tetanospasmin and for botulinum toxin are different.[15,122] As indicated previously, patients with tetanus have spastic muscle contractions, difficulty opening the jaw (called lockjaw, "trismus"), a characteristic smile called "risus sardonicus" and contractions of back muscles resulting in backward arching. Patients are extremely irritable, and tetanic seizures develop, brought about by violent, painful muscle contractions following some minor stimulus, such as a noise.[15]

The regular use of vaccines and tetanus antitoxin for wound management, and improved childbirth practices have greatly assisted in decreasing the incidence of disease, and improving outcomes when infection does occur. Today,

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50% of infections are secondary to contaminated wounds, traumatic or surgical, and abscesses, as well as the association with intravenous drug abuse.[59] *C. tetani* is a reportable disease in the United States, and there have been reports of infections following laparoscopic hernia repair surgery and a rare instance of bacteremia in an 87-year-old wherein an endogenous source of the bacterium was suspected to be responsible for the disease.[209]

Tetanus is a medical emergency requiring hospitalization, immediate treatment with human tetanus immune globulin (TIG) (or equine antitoxin), tetanus vaccination, and drugs to control muscle spasms. In addition, aggressive wound care, and antibiotics are part of the appropriate treatment plan. Diagnosis is based on clinical findings rather than laboratory studies.[88]

The antecedent wound is often minor or trivial. Direct Gram-stained smears and anaerobic cultures of the wound site often fail to reveal the organism. When it is recovered in culture, *C. tetani* forms round, terminal spores, produces spreading or swarming growth on anaerobe blood agar, produces major amounts of acetate and butyrate with only a minor amount of propionate, is lipase and lecithinase-negative on egg yolk agar (EYA), and is asaccharolytic (Image Plate 16-4A and 16-4B).

Infections Involving Anaerobic Gram-Positive and Gram-Negative Cocci. The anaerobic cocci most commonly encountered in clinical specimens include *Finegoldia*

anaerobic cocci most commonly encountered in clinical specimens include *Finegoldia magna* (formerly *Peptostreptococcus magnus*), *Peptostreptococcus anaerobius*,*Peptoniphilus asaccharolyticus* (formerly *Peptostreptococcus asaccharolyticus), Anaerococcus prevotii* (formerly *Peptostreptococcus prevotii*), *Parvimonas micra* (formerly *Peptostreptococcus micros and Micromonas micros)* and *Streptococcus intermedius.*[446]Nomenclature changes for the anaerobic cocci are listed in Table 16-2. There is little doubt that anaerobic cocci can be pathogenic for humans in certain clinical settings. They can be isolated from approximately 25% of all anaerobic infections.[348] In pulmonary diseases, up to 40% of the anaerobic infections may contain a gram-positive anaerobic coccus, especially *F. magna, P. micra* or *P. anaerobius*.[348,351,446] With the exception of *F. magna,* most infections involving anaerobic gram-positive cocci are polymicrobial[446] and associated with abscesses from common skin abscesses to more life-threatening brain abscesses. In addition they have been involved in patients with bacteremia, meningitis, necrotizing pneumonia septic abortion, and decubitus ulcer infections and other skin and soft tissue infections. *F. magna* is the single most common pathogenic anaerobic gram-positive coccus. It possesses many pathogenic factors including adhesion factors, a capsule and various enzymes; it has been reported to cause endocarditis, mediastinitis, septic joint infections, prosthetic implant infections, respiratory tract infections and diabetic foot infections.[170,252,487]*P. micra* is predominantly an oral pathogen, most commonly involved in periodontal disease. It has, however, been associated in polymicrobial infections of bone and joints, brain abscesses, septicemia and human bite wounds, among others.[348] *P. anaerobius* has been isolated in polymicrobial infections involving the abdominal, urogenital and respiratory tract. Like *P. micra,* it has also been associated with oral infections, including peritonsillar abscesses and periapical abscesses in root canals.[396]

Atopobium parvulum (formerly *Streptococcus parvulus*) has been isolated from oral cultures in patients with odontogenic infection, although the specific contribution of this organism to the overall infection is unknown.[128]

Staphylococcus saccharolyticus is an anaerobic coagulase-negative *Staphylococcus* species that is part of the normal microbiota of the skin. It has been found to be responsible for native and prosthetic valve endocarditis. In addition, the organism has been isolated from a patient with spondylodiscitis, and has been recovered from stored platelet concentrates.[8,187,267] *S. aureus* subsp. *anaerobius* is an anaerobic *S. aureus* that can cause disease in animals and humans. It has been isolated from abscesses and shown to cause bacteremia.[141,368]

Anaerobic gram-negative cocci are much less involved in infections than other anaerobic or microaerophilic cocci.[238,446] There are rare reports of meningitis, osteomyelitis, prosthetic joint infections, discitis, endocarditis, and bacteremia, mainly

osteomyelitis, prosthetic joint infections, discitis, endocarditis, and bacteremia, mainly involving *V. parvula*.[41,45,94,298,312,316] In many of these instances, the patient had an underlying disease, including a primary periodontal infection, or the infection followed surgery or other invasive procedures such as endoscopy.

Box 16-8 provides information on the clinical relevance of anaerobic cocci. Tables 16-27 and 16-28 describe the identification characteristics of anaerobic gram-positive cocci and gram-negative cocci, respectively.

Isolation of Anaerobic Bacteria

The steps involved in the laboratory diagnosis of anaerobic bacterial infections are similar in many ways to those for aerobic bacteria, as described in Chapters 1 and 2. Particularly attention must be paid to the proper selection, collection, and transport of clinical specimens for recovering anaerobic bacteria. The processing of specimens, selection of media, inoculation and incubation methods, and inspection of positive cultures are laboratory procedures that must be carefully quality-controlled. Performing any one step incorrectly may lead to erroneous results, thus creating the potential to supply misinformation to the physician.

Selection of Specimens for Culture

With few exceptions, all materials collected from sites not harboring an indigenous microbiota, such as body fluids other than urine, exudates from deep abscesses, fine-needle aspirates and tissue biopsies, should be cultured for anaerobic bacteria. However, since anaerobes normally inhabit the skin and mucous membranes as part of the normal indigenous microbiota, the specimens presented in Table 16-5 are unacceptable for anaerobic culture, because the results cannot be interpreted.

Collection and Transport of Specimens

When collecting specimens through the skin or mucous membranes, stringent precautions must be taken to decontaminate the surface properly. A surgical soap scrub should be used, followed by application of 70% ethyl or isopropyl alcohol, then tincture of iodine for 1 minute (or 10% povidone-iodine, Betadine for 2 minutes, or more recently, use of chlorhexidine). This should be applied in concentric circles starting from the center. Because some patients are sensitive or allergic to tincture of iodine, it is important to remove the iodine with alcohol after collecting the specimen.

BOX 16-8

Clinical Findings Associated With Selected Anaerobic Gram-Positive Cocci

Species	Clinical Infections
Finegoldia magna	Bacteremia and endocarditis[170,487];soft tissue infections[252]; prosthetic joint infections[291]
Parvimonas micra	Predominantly an oral pathogen[348,351,396]; human bite wounds[446]; Osteomyelitis and prosthetic joint infections[446]
Peptostreptococcus anaerobius	Bacteremia and endocarditis[108]; recovered from many other clinical specimens[446]
Peptoniphilus spp.	Varies among the species and often isolated in polymicrobial infections, and in many different clinical specimens.[351,446]
Anaerococcus spp.	Varies among the species and often isolated in polymicrobial infetctions, and in many different clinical specimens.[351,448]
A. prevotii	Septic arthritis[230]
Atopobium parvulum	Dental abscesses[128]
Staphylococcus saccharolyticus	Bacteremia; prosthetic valve endocarditis[267]; contaminated platelets[8]

Adapted from reference 243 and other references as noted in table.

TABLE 16-5 Specimens That Should Not Be Cultured for Anaerobic Bacteria

Throat or nasopharyngeal swabs
Gingival swabs
Sputum or bronchoscopic specimens[a]

Gastric contents, small-bowel contents, feces, rectal swabs, enterocutaneous fistulae, colostomy stomata[b]
Surfaces of decubitus ulcers, swab samples of encrusted walls of abscesses, mucosal linings, and eschars
Material adjacent to skin or mucous membranes other than the above that have not been properly decontaminated
Voided urine
Vaginal or cervical swabs

[a] Collection of bronchoscopic specimens using sterile fiberoptic bronchoscopic may be acceptable for anaerobe cultures.

[b] When indicated clinically, specimens from these sources can aid in the diagnosis of botulism and for intestinal disease caused by *Clostridium difficile* and *C. perfringens*.

In general, swabs other than those already listed in this table, should not be processed for anaerobes from wounds without discussion with the healthcare team as to the need for aspirates, body fluids or tissue samples if at all possible. Swabs sent from the operating room for anaerobes should not be processed.

A needle and syringe should be used whenever possible for collecting specimens for anaerobic culture. Collection of swab specimens should be discouraged because they do not allow for collection of large enough specimens and also because they expose anaerobes, if present, to ambient oxygen. Some studies have shown that if there is abundant purulent material in the specimen, survival on swabs is sufficient and recovery enhanced provided the swabs are transported in appropriate anaerobic transport devices. Regardless, the collection of fluids, aspirates or tissue biopsies are preferred for the optimal recovery of anaerobic bacteria.[96] Once any specimen is collected, particular precautions should be taken to protect them from oxygen exposure and most importantly to deliver them to the laboratory promptly.

Anaerobic Blood Cultures

Blood culture techniques should permit optimal recovery of obligate anaerobes, as well as, aerobes and facultative anaerobes. Before the 1980s, anaerobes were encountered in about 9% to 20% of all positive blood culture sets at a number of medical centers.[159] During the past two to three decades, because of surgical prophylaxis

protocols for GI and GU surgeries in particular, anaerobes have accounted for about ≤5% to 10% of bacteremias, dependent on varying patient populations.[58,155,229,354] A study at the Mayo clinic in 2007 suggested a possible reemergence of anaerobic bacteremia, making the point that blood cultures should always include inoculation into aerobic and anaerobic media. There may be differences between incidence of anaerobes in different hospital settings (e.g., community hospitals vs. tertiary care medical centers), but culturing for anaerobes should be performed with all blood cultures as a matter of routine; this will ensure the optimal assessment of the patient suspected to have bacteremia.[155,277] A recent report documented adverse outcomes in patients with antibiotic-resistant *Bacteroides* bacteremia, stimulating renewed interest in the laboratory diagnosis of anaerobic bacteremia and the need for antimicrobial susceptibility testing of these bacteria.[214,355] In a study of on bone marrow transplant recipients, 17% of all bloodstream infections were due to anaerobes, with *F. nucleatum* being the most common, followed by *Leptotrichia buccalis,*

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C. septicum and *C. tertium*. Risk factors among this group included neutropenia and mucositis.[274]

The decontamination of the skin at the venipuncture site cannot be overemphasized for optimal recovery of significant anaerobes. Chlorhexidine appears to be comparable or superior to alcohol and povidone-iodine or alcohol and tincture of iodine, and is now being used for skin antisepsis in a number of medical centers.[26,332] To optimize the recovery of microorganisms from patients with bloodstream infections, the blood specimen volume from adult patients should be 20 to 30 mL per venipuncture.[503] For neonates and older children, smaller volumes are usually collected (e.g., 0.5 mL for an infant; 2 to 5 mL for an older child), based on the weight of the child. Collection of more than three blood specimens per 24-hour period to diagnose a suspected episode of bacteremia is not required and is not recommended for any patient.[389]

For instrumented blood culture systems, bottles currently available that support growth of obligately anaerobic bacteria, as well as facultatively anaerobic bacteria, are listed below.

Bactec Plus Anaerobic/F (resin, anaerobic; up to 10 mL of blood)
Bactec Lytic/10 Anaerobic F (lytic agent, anaerobic; up to 10 mL of blood)
Bactec Anaerobic F (standard anaerobic; up to 7 mL of blood)
BacT/Alert FN (FAN, anaerobic with activated charcoal; up to 10 mL of blood)
BacT/Alert (standard anaerobic; up to 10 mL of blood)
VersaTrek REDOX 2 Anaerobic Broth (standard anaerobic; up to 10 mL of blood)

Instrumented blood culture systems have a special head space that contains an anaerobic atmosphere, containing nitrogen and carbon dioxide without oxygen. Both the aerobic and anaerobic bottles should be shaken during incubation and should be incubated at 35°C for 5 days. When either the aerobic or anaerobic bottles demonstrate evidence of growth, aerobic and anaerobic subcultures should be performed; anaerobe blood agar may be used for subculture, followed by anaerobic incubation. The use of selective plating media for anaerobic subculture (such as phenylethyl alcohol [PEA] anaerobe blood agar or kanamycin–vancomycin blood agar), in addition to nonselective anaerobe blood agar, is recommended to aid in recovery of anaerobes in the setting of polymicrobial bacteremia. Anaerobes can be isolated on occasion from the aerobic blood culture bottles. The lysis/centrifugation (Isolator; Wampole, Cranbury, NJ) system and the Septichek (Roche, Indianapolis, IN) blood culture system (discussed in Chapters 1 and 2) have performed suboptimally compared with the traditional and Bactec broth systems for anaerobic blood culture. It is recommended that laboratories using either of these systems also use a traditional method or an additional instrumented broth culture system for anaerobic blood cultures.[238]

Direct Examination of Clinical Material

Gross examination of specimens is particularly valuable in the assessment for the possible presence of anaerobes. Although unpleasant, a foul odor, the purulent appearance of fluid specimens, and the presence of necrotic tissue and gas or sulfur granules are all valuable clues (Image Plate 16-2C).

The importance of the microscopic examination of clinical specimens has been emphasized by several authors, and the information derived may give immediate presumptive evidence that anaerobes are present.[238,313] In preparing slides for Gram's staining, methanol fixation is much better than traditional heat fixation. The background and cellular characteristics of the smear should be observed; and the Gram reaction; the size, shape, and arrangement of bacteria; and the relative number of organisms present should be recorded. The presence of spores, their shape and position in the bacterial cell, and other distinctive morphologic features such as branching, filaments with spherical bodies, pointed ends, and granular forms should be noted. Although the Gram's stain is ordinarily satisfactory for determining cellular characteristics, acridine orange stains can be helpful for detecting bacteria in blood cultures, CSF, pleural fluid, joint fluid, and exudates.[269] The morphology of several anaerobes in stained preparations examined microscopically is illustrated in Image Plates 16-1, 16-2, 16-3 and 16-4, as well as in the Image Plates of Chapter 1.

The presence of numerous squamous epithelial cells without inflammatory cells in the Gram's stain preparations from skin wounds and in respiratory and urogenital tract specimens usually indicates poor quality, superficially collected specimens that usually

yield mixtures of insignificant organisms from the normal microbiota or surface contaminants. It is worthwhile to limit the extent of identifications when the direct microscopic examinations reveal samples of poor quality specimens, because the predictive value of such tests is limited. Of course, clinicians should be notified of the direct examination results and the problem of specimen quality in a timely manner, prior to discarding the primary culture plates. Microbiologists should work closely with clinicians in an effort to enhance the quality and the clinical relevance of the specimens processed and the results reported.

Selection and Use of Media

The media used for recovering anaerobes from specimens should include nonselective, selective and enrichment media, as illustrated in Tables 16-6 and 16-7. Other media may also be included or substituted for those listed in Table 16-6. For example, chopped-meat glucose medium is commonly used instead of enriched thioglycolate medium; colistin nalidixic acid (CNA) agar may be used instead of PEA agar. Either paromomycin–vancomycin blood agar or kanamycin–vancomycin blood agar can be used for the selective isolation of the gram-negative NSF anaerobes. *Bacteroides* bile esculin (BBE) agar is recommended for the selection and presumptive identification of the *Bacteroides fragilis* group and has also been found useful as a selective medium for *Bilophila wadsworthia*.[29,238] Results obtained with plating media formulations such as Schaedler, Columbia, Brucella, or other media may not be entirely comparable to the morphology and growth characteristics of anaerobes seen on the CDC anaerobe blood-agar–based media formulations (Image Plates 16-1 through 16-4).

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TABLE 16-6 Representative Media for Primary Isolation of Anaerobes From Clinical Specimens

Medium	Major Ingredients and Comments	Purpose
CDC anaerobe blood agar (AnBAP)	Trypticase soy agar base with 5% sheep blood; supplemented with yeast extract, hemin, vitamin K_1 and L-cystine for anaerobes requiring additional growth factors (e.g., *Prevotella melaninogenica, Fusobacterium necrophorum*, and others). Additional media bases, including Brucella, brain-heart infusion, Schaedler, and Columbia blood agar support excellent growth of many anaerobes, but morphology and other characteristics tend to differ on these media.	Nonselective blood agar-plating medium for the primary isolation of all types of anaerobes found in clinical materials (see text and Image Plates 16-1 through 16-4).
Anaerobe phenylethyl alcohol blood agar (PEA)	In addition to containing the same ingredients as the CDC anaerobe blood agar formulation above, this medium has phenylethyl alcohol (2.5 g/L). PEA inhibits the swarming of *Proteus* spp. and inhibits the growth of many other gram-negative facultatively anaerobic bacteria, including most *Enterobacteriaceae*. PEA is volatile. Plates should be tightly sealed in cellophane or plastic bags and stored at 4°C. A batch of plates that no longer inhibits the swarming of *Proteus* should be discarded, regardless of the expiration date.	PEA medium aids in selective isolation of anaerobes from infected materials containing a mixture of bacteria. It supports good growth of most gram-positive and gram-negative obligate anaerobes. Facultatively anaerobic gram-positive bacteria such as staphylococci, streptococci, *Bacillus* spp., and coryneform bacteria also grow well on this medium.
Anaerobe laked kanamycin-vancomycin blood agar (KV)	Contains the same CDC anaerobe blood agar formulation as AnBAP above, but in addition, contains 100 mg/L of kanamycin and 7.5 mg/L of vancomycin. The kanamycin inhibits many (but not all) facultatively anaerobic gram-negative rods and the vancomycin inhibits gram-positive bacteria in general (including most gram-positive anaerobes and aerobes). Vancomycin in this concentration can also inhibit the growth of *Porphyromonas* spp.	KV medium is useful for selective isolation of most *Bacteroides* spp., *Prevotella* spp., *Fusobacterium* spp. and *Veillonella* spp. from clinical specimens containing mixed aerobic and anaerobic bacteria.
Anaerobe paromomycin-vancomycin blood agar (PV)	Laked PV medium is similar to the formulation above, except that 100 mg/L of paromomycin is substituted for the kanamycin. Also in PV, the blood is laked before it is added (by freezing and thawing the blood). Performance is similar to KV, except that the paromomycin may inhibit some additional facultative anaerobes that are resistant to kanamycin, such as some strains of *Klebsiella* sp. Similar to KV agar, laked PV should inhibit growth of gram-positive organisms. The laked blood may aid in early recognition of pigmented *Prevotella*.	Laked PV is an excellent medium for selective primary isolation of organisms in the *Bacteroides fragilis* group, the pigmented and nonpigmented *Prevotella* spp., *Fusobacterium nucleatum, F. necrophorum, F. mortiferum, Veillonella*, and other obligately anaerobic gram-negative, non–spore-forming anaerobes. It is not necessary to use both KV and PV; rather, it is reasonable to select one or the other of these media, based on preferences of the microbiologist.
Cycloserine-cefoxitin fructose agar (CCFA)	Trypticase soy or proteose peptone base containing fructose and neutral red indicator. In addition, cycloserine (500 mg/L) and cefoxitin (16 mg/L) are added to inhibit intestinal microbiota. *C. difficile*, at 48 hours of incubation, forms 4 mm or larger yellowish rhizoid colonies that have birefringent crystalline internal structures ("speckled opalescence") (Image Plate 16-4F). *C. difficile* colonies show yellow-green fluorescence under long-wave UV light. In addition, their odor is reminiscent of horse manure. *C. difficile* is negative for both lipase and lecithinase activity.	CCFA is for selective isolation of *C. difficile* from stool specimens or other intestinal materials. However, growth on CCFA is not specific for only *C. difficile*; therefore, identification of pure culture isolates is still required. It is common to find breakthrough growth on the medium of *Enterobacteriaceae, Bacillus* spp., staphylococci, and other clostridia.
Bacteroides Bile Esculin Agar (BBE)	BBE agar contains digest of casein and soybeans. Hemin, vitamin K_1, oxgall and gentamicin are included in the agar as selective factors. The bile (oxgall) is inhibitory to most bacteria other than the *B. fragilis* group. Hydrolysis of esculin helps differentiate among the species; there will be a blackened zone around positive colonies.	BBE agar is used for the selection and presumptive identification of the members of the *Bacteroides fragilis* group, including *Parabacteroides* spp. and *Bilophila wadsworthia*. Organisms that grow will appear as gray, circular, raised colonies of >10 mm. There will be a blackening of the agar around colonies capable of hydrolyzing the esculin. Most gram-positive aerobes and anaerobes will be inhibited as will members of the *Enterobacteriaceae* and other gram-negative aerobic bacteria.
Enriched thioglycolate medium (THIO)	THIO is an enriched liquid medium prepared by supplementing the BBL-0135C formula thioglycolate medium (without indicator) with hemin and vitamin K_1	This is a noninhibitory broth that is especially useful for primary isolation of actinomycetes. THIO is also an excellent supplement to semisolid (i.e., agar) media for isolation of slow-growing or fastidious organisms. It supports good growth of essentially all anaerobes commonly found in clinical materials.

All the media in this table are available in prepared form from several manufacturers.

TABLE 16-7 Additional Media for Use in Cultivating or Selecting for Specific Anaerobes

Medium	Composition	Purpose
Anaerobe Blood Agar with neomycin	TSA supplemented with yeast extract, vitamin K_1, hemin, cystine, and 5% sheep blood agar plus neomycin	Inhibitory to most strains of *S. aureus* and many of the *Enterobacteriaceae*
Forget–Fredette Agar	Digest of casein and soybean milk, glucose, and sodium azide	Used for cultivation of *Clostridium* spp.
Hartley's digest broth	Ox heart, pancreatin, sodium carbonate, and HCl	Used for cultivation of *Actinomyces* spp.
Lombard Dowell Agar	Digest of casein, yeast extract, cystine, tryptophan, sodium sulfite, hemin, and vitamin K_1	Used for isolation of most anaerobes
McClung–Toabe agar base with egg yolk	Peptone, glucose, and egg yolk emulsion	For isolation and cultivation of *C. perfringens*
Shaidi–Ferguson Perfringens Agar	Tryptose, digest of soybean meal, yeast extract, ferric ammonium citrate, sodium sulfite, egg yolk emulsion, kanamycin, and polymyxin sulfate	For isolation and enumeration of *C. perfringens*; they appear as black colonies surrounded by a precipitate.
Tryptose–sulfite–cycloserine agar	Tryptose, beef extract, digest of soybean meal, yeast extract, ferric ammonium sulfate, ferric ammonium citrate, cycloserine, polymyxin B, and kanamycin	For isolation and enumeration of *C. perfringens*
Wilkens–Chalgren anaerobe Broth	Digest of casein, gelatin, peptone, yeast extract, glucose, sodium pyruvate, arginine, hemin, and vitamin K_1	For cultivation of most anaerobes; also used for antimicrobial susceptibility testing
Fusobacterium selective agar	Sheep blood agar with josamycin, neomycin, and vancomycin	Inhibit most gram-positive aerobes and anaerobes as well as most other gram-negative anaerobes, except

		Fusobacterium spp.
Fusobacterium necrophorum selective media[24]	Horse Blood agar with nalidixic acid and vancomycin	Inhibits most gram-positive bacteria and many gram-negative aerobes and anaerobes; β-hemolysis on horse blood helps to specifically identify *F. necrophorum* from throat swabs or other respiratory specimens when Lemierre's disease is suspected.

The use of a nonselective anaerobic blood agar such as Brucella or CDC blood agars that contain hemin, L-cystine and vitamin K_1 is recommended for the primary isolation and subculture of most anaerobes including certain thiol-dependent or sulfur-containing amino-acid–requiring bacteria such as *Fusobacterium necrophorum* and fastidious, thiol-dependent streptococci that have been isolated from patients with endocarditis. This medium also supports excellent growth of the strict anaerobes *Clostridium novyi* type B and *Clostridium haemolyticum*. These can be purchased from the major media vendors. The formula for CDC anaerobe blood agar can be found in Chart 16-1. Prereduced anaerobically sterilized (PRAS) media can be purchased from Anaerobe Systems, CA. Many of the commercially prepared anaerobe blood agar plates can be stored in the refrigerator in cellophane bags for at least 6 weeks provided precautions to prevent drying out are utilized. Prior to use, the plates can be prereduced by holding them for 4 to 16 hours in an anaerobic jar or an anaerobic glove box in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. The enriched thioglycolate medium (BBL-135C with hemin and vitamin K_1 supplement) is recommended as a backup to the plating media. This medium is particularly helpful for cultivating slow-growing species of *Actinomyces* and *P. acnes*. Chopped-meat glucose broth, a good alternative to enriched thioglycolate medium, is useful for the isolation of *Clostridium* species by the spore selection technique and as a holding medium for anaerobic cultures in general.

Other special purpose selective media for primary isolation of anaerobes of the normal microbiota studies have been described in many texts and references.[18,238] Many scientists have developed media specific for various fastidious anaerobes. Smith and Moore described selective media and a cold enrichment technique to aid in isolation of *Mobiluncus* species from vaginal specimens.[441] Eley et al. reported on a selective and differential medium for isolation of *Bacteroides ureolyticus*.[142] Lee and coworkers developed a new selective medium for *Bacteroides gracilis*.[285] Malnick and associates formulated a new selective medium for isolating *Anaerobiospirillum*

spp. from feces.[308] There have been a few selected media described for the isolation of *Fusobacterium necrophorum*, in particular from throat swabs and other respiratory specimens for the determination of pharyngitis and the more severe Lemeirre's syndrome. *F. necrophorum* can be a component of normal oral microbiota, so the mere isolation of this organism from the oropharynx does not always indicate clinical significance; a selective media can assist in enabling isolation of the organism from amid other normal microbiota in instances of suspected infection. The addition of horse blood for detection of hemolysis and the addition of vancomycin and nalidixic to inhibit the growth of many other gram-positive and gram-negative normal microbiota has been shown to enable detection of *F. necrophorum* from throat swabs.[24,84] Some of the newer anaerobic selective media are provided in Table 16-7.

The OxyPlate is a commercially available medium that can be used for isolation of anaerobes without the need for anaerobic jars, chambers, or pouches. It consists of plastic plates that are manufactured and used as seals so that

anaerobic conditions are maintained inside. An enzyme, Oxyrase, (Oxyrase, Inc., Mansfield, OH), which is in the media, removes oxygen from the PRAS blood agar and the space above the media as well. Once inoculated with specimens or colonies, the plates are stored in a routine aerobic incubator. There have been a few studies to date comparing the use of OxyPlate to conventional anaerobic incubation approaches. One evaluation with eye specimens found that it was equivalent to the recovery of anaerobes in a GasPak jar and although it did not necessarily recover more anaerobes, it could be an alternative in the laboratory so that chambers and jars might not be required.[117,512]

Systems for the Cultivation of Anaerobic Bacteria

Comparative studies have shown that the following systems, when used properly, are satisfactory for the cultivation of anaerobic bacteria commonly associated with human disease[255,400]: evacuation-replacement jars; anaerobic jars with disposable gas generators; anaerobic glove box techniques; and, roll tube and roll-streak tube with PRAS media. To ensure optimal results, the general principles listed in Box 16-9 must be followed. Bacic and Smith describe the proper methods for cultivation and storage of *Bacteroide*s spp. and in the article provide useful information about some of the various methods used for incubation of anaerobes and denote the advantages and disadvantages of these techniques.[20]

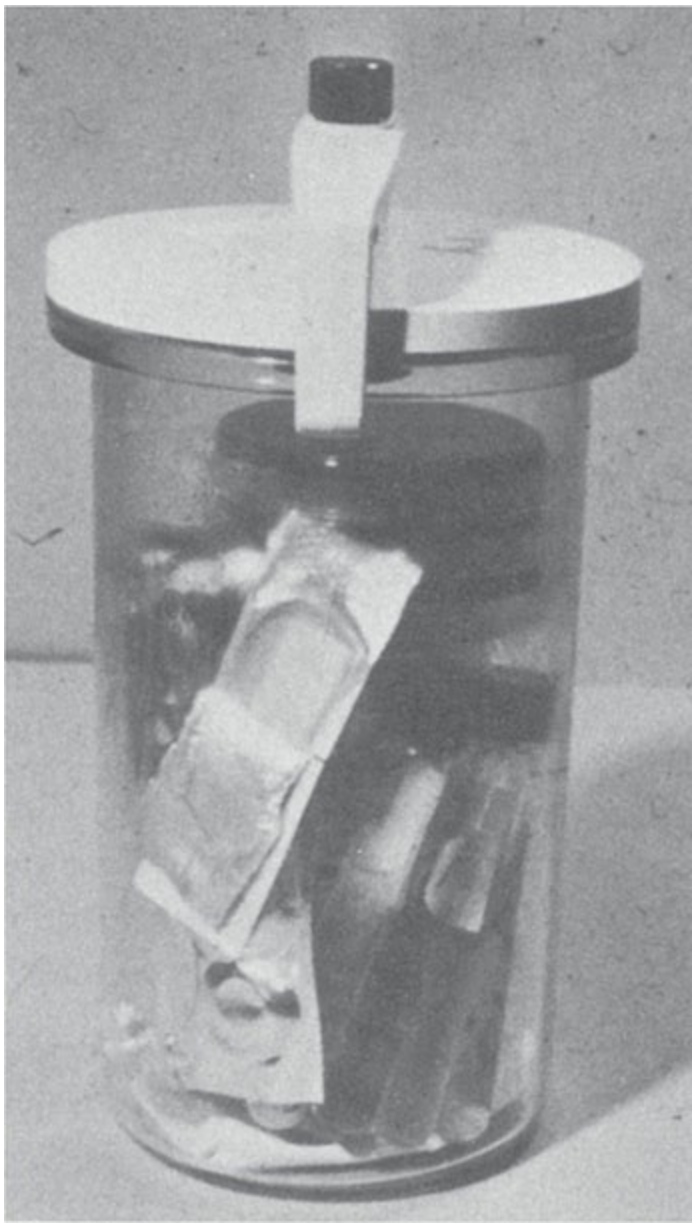
Use of the Anaerobic Jar

Anaerobic jars are commonly used for cultivating anaerobic bacteria on primary plating media or on subculture plates. The GasPak jar (Becton Dickinson), illustrated in Figure 16-2, has long been used in clinical laboratories in the United States.[317] The Oxoid anaerobic jar (Fig. 16-3) has a metal lid, Schrader valves, and a pressure gauge. Like the GasPak jar, the Oxoid jar can be used either as an evacuation-replacement jar or it can be used with a disposable gas generator. Other commercially available sources of anaerobic jars include Hardy Diagnostics, Santa Maria, CA and Thermo Fisher Scientific, Waltham, MA, among others. The basic principle of the jars that require catalyst (e.g., GasPak) is the removal of oxygen from the chamber by reaction with hydrogen added to the system in the presence of the catalyst. Oxygen is reduced to water as follows: $2H_2 + O_2 + \text{catalyst} = 2H_2O$.

BOX 16-9

Principles for Optimal Recovery of Anaerobes

1. Proper collection of the clinical specimens with guidelines provided by the laboratory
2. Appropriate and timely transfer of the clinical specimens to the laboratory
3. Appropriate labeling, including specimen type and site of collection
4. Published rejection criteria that are adhered to by the laboratory
5. Processing of specimens with minimal exposure to atmospheric oxygen
6. Use of fresh or prereduced media
7. Use of a combination of selective and nonselective media
8. Proper use of an anaerobic incubation system



■ **FIGURE 16-2** The GasPak (Becton Dickinson Microbiology Systems, Cockeysville, MD) anaerobic system: The jar contains inoculated plates, broth tubes, a GasPak hydrogen and carbon dioxide generator envelope, a disposable methylene blue indicator strip, and a catalyst basket in the lid.

Anaerobic conditions can be produced in jar systems with either the disposable hydrogen–carbon dioxide generator (GasPak, Oxoid) or by the evacuation/replacement procedure. The evacuation/replacement procedure, in which the air in the jar is removed and replaced with a mixture of 85% N_2, 10% H_2, and 5% CO_2 is more economical than gas generators and allows anaerobic conditions to be established more rapidly. Any airtight container can be used, including a GasPak jar with a vented lid, a

Brewer jar, an Oxoid jar, or even a modified pressure cooker.

Anaerobic conditions should always be monitored when using either of the two jar techniques by including an oxidation–reduction indicator. Methylene blue strips are available commercially for this purpose. Alternatively, a 13-mm × 100-mm test tube containing a few milliliters of methylene blue–$NaHCO_3$–glucose mixture can be placed in the jar. Methylene blue is blue when oxidized and clear when reduced. The color changes at about +11.0 mV. Thus, if anaerobic conditions are achieved, the methylene blue indicator solution will gradually turn colorless and will remain that way if there are no leaks that allow additional oxygen to enter the system. If the solution turns blue after being colorless, this indicates that anaerobic conditions were not maintained and that the culture results may not be valid.

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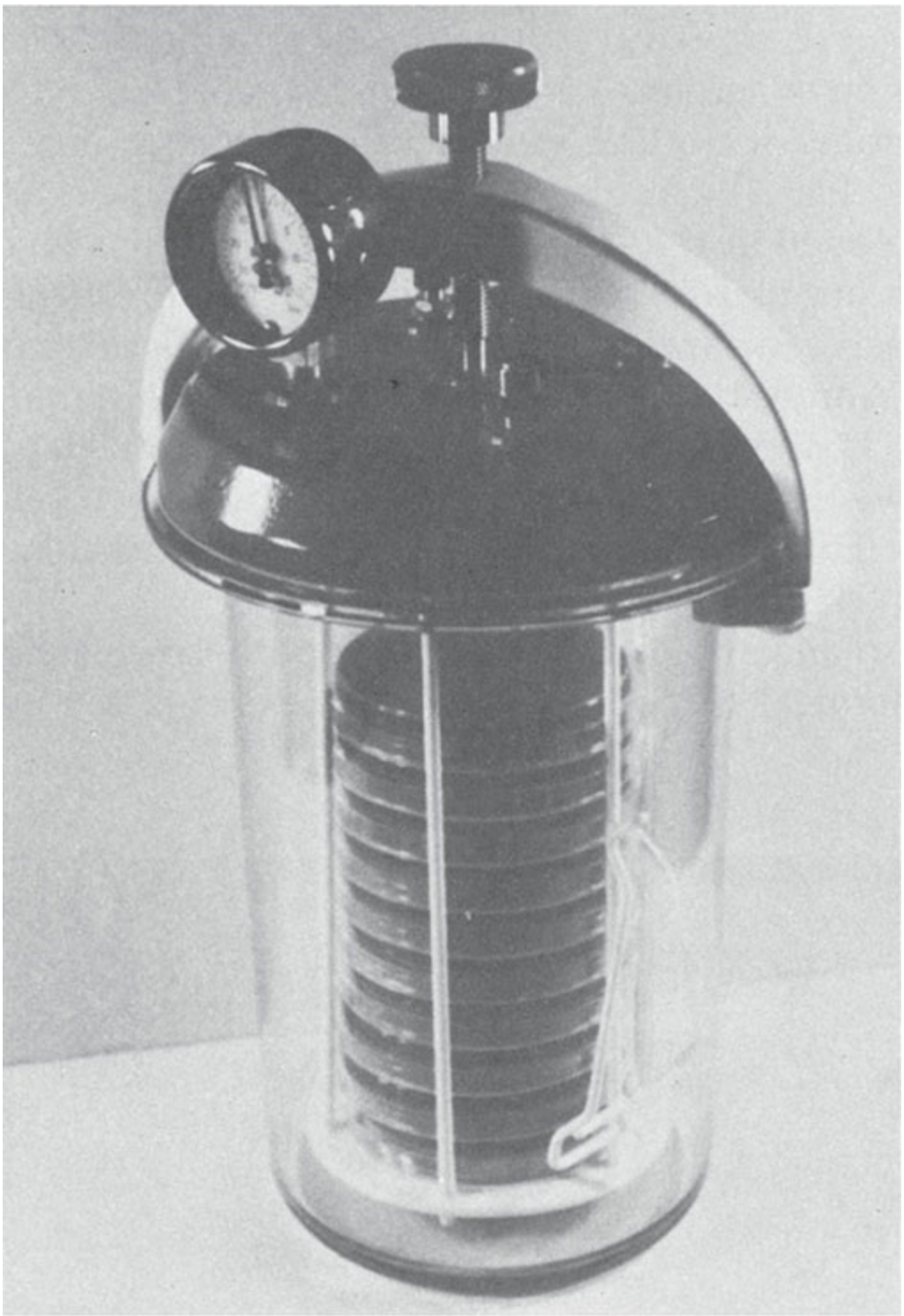
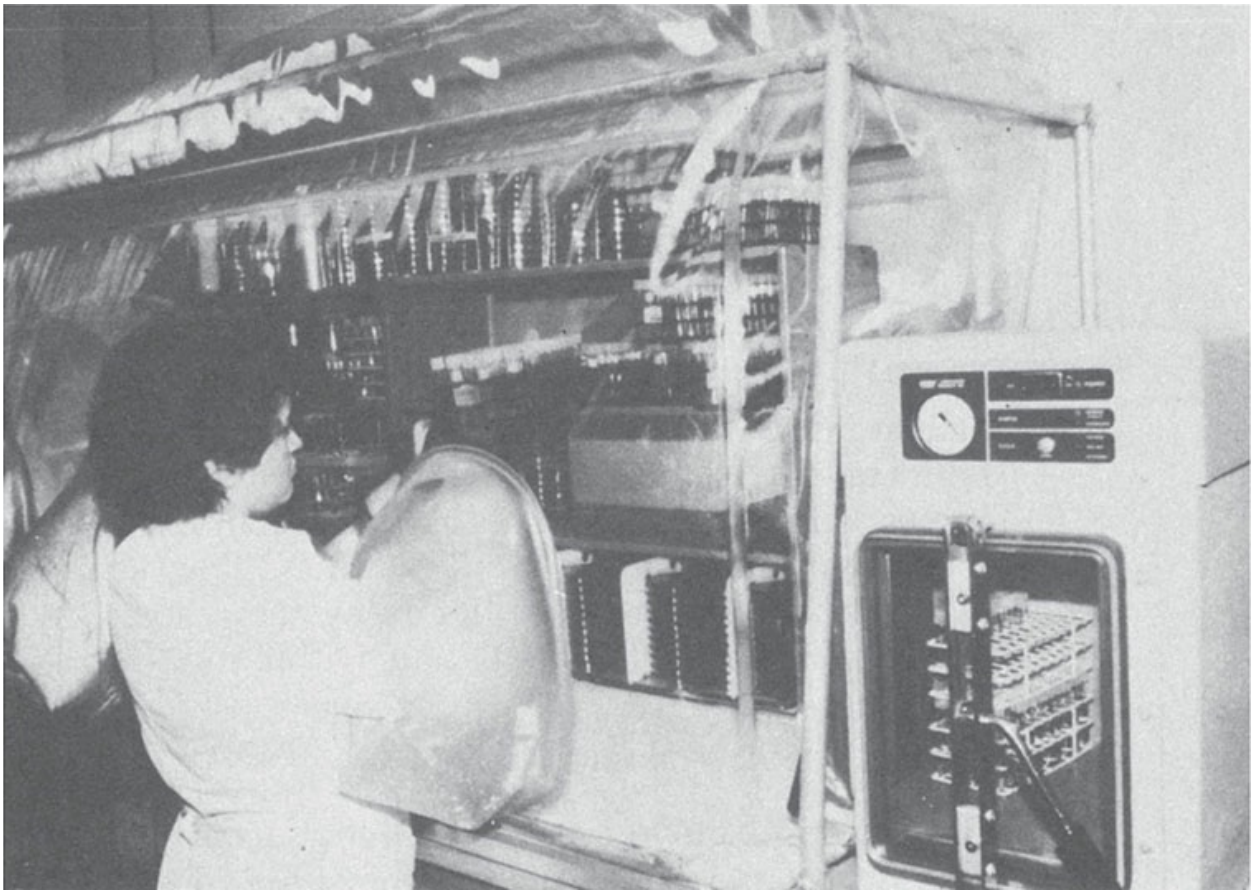


■ **FIGURE 16-3** The Oxoid anaerobic jar (Oxoid USA, Columbia, MD) contains a 3.5-L polycarbonate jar closed by a heavy-duty metal lid and metal clamp. The lid center has two Schrader valves and a plus-or-minus pressure gauge, with two valves to facilitate the evacuation/replacement (E/R) technique. There is also a safety valve in the lid to prevent extra gas pressure caused by incorrect use of the E/R technique. A sachet containing a low-

temperature catalyst is clipped to the undersurface of the lid. In lieu of using the E/R technique, the jar can be used with the Oxoid generating kit available from the manufacturer.



■ **FIGURE 16-4** The anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI). Materials are passed in and out of the large, flexible plastic chamber through an automatic entry lock. Anaerobic conditions are maintained by constant recirculation of the atmosphere within the plastic chamber (85% N_2, 10% H_2 and 5% CO_2) through a palladium catalyst. Cultures are incubated either within a separate incubator inside the glove box, or by maintaining the entire chamber at 35°C through use of heated catalyst boxes.

More recently, anaerobic gas-generating systems have been introduced that do not require either catalyst or the addition of water to activate these systems. The AnaeroPack (Mitsubishi Gas Chemical America, New York, NY), for example, absorbs oxygen and generates carbon dioxide, but does not generate hydrogen. It appears to be an excellent alternative to the GasPak and other established anaerobic incubation systems.[114] Becton Dickinson continues to distribute their traditional BBL GasPak system, but has also introduced the GasPak EZ gas-generating container and GasPak Generating Pouch Systems, both of which use a sachet or pouch,

respectively, without the need for addition of water to generate the anaerobic environment. Another type of commercially available catalyst-free system makes use of iron filings in a sachet to which water is added (Anaerocult, Merck, Darmstadt, Germany), producing an oxygen-free, carbon dioxide-rich atmosphere.[238] Manufacturers' websites can be used for more information on any of these products.

Use of the Anaerobic Glove Box

An anaerobic glove box (or anaerobic chamber) is a self-contained anaerobic system that allows the microbiologist to process specimens and perform most bacteriologic techniques for isolation and identification of anaerobic bacteria without exposure to air. Glove boxes suitable for cultivation of anaerobes can be constructed from various materials, including steel, acrylic plastic or fiberglass (Fig. 16-4). The flexible vinyl plastic anaerobic chamber and its modifications are available in varying sizes from many manufacturers including: Coy Manufacturing (Ann Arbor, MI), A Forma Scientific (Marietta, OH) and Toucan Technologies (Cincinnati, OH), among others. Rigid, "gloveless," glove boxes are marketed by Anaerobe Systems (Bactron Anaerobe Chamber and Sheldon Manufacturing (CorneOR), among others. An example of a glove box is shown in Figure 16-5.

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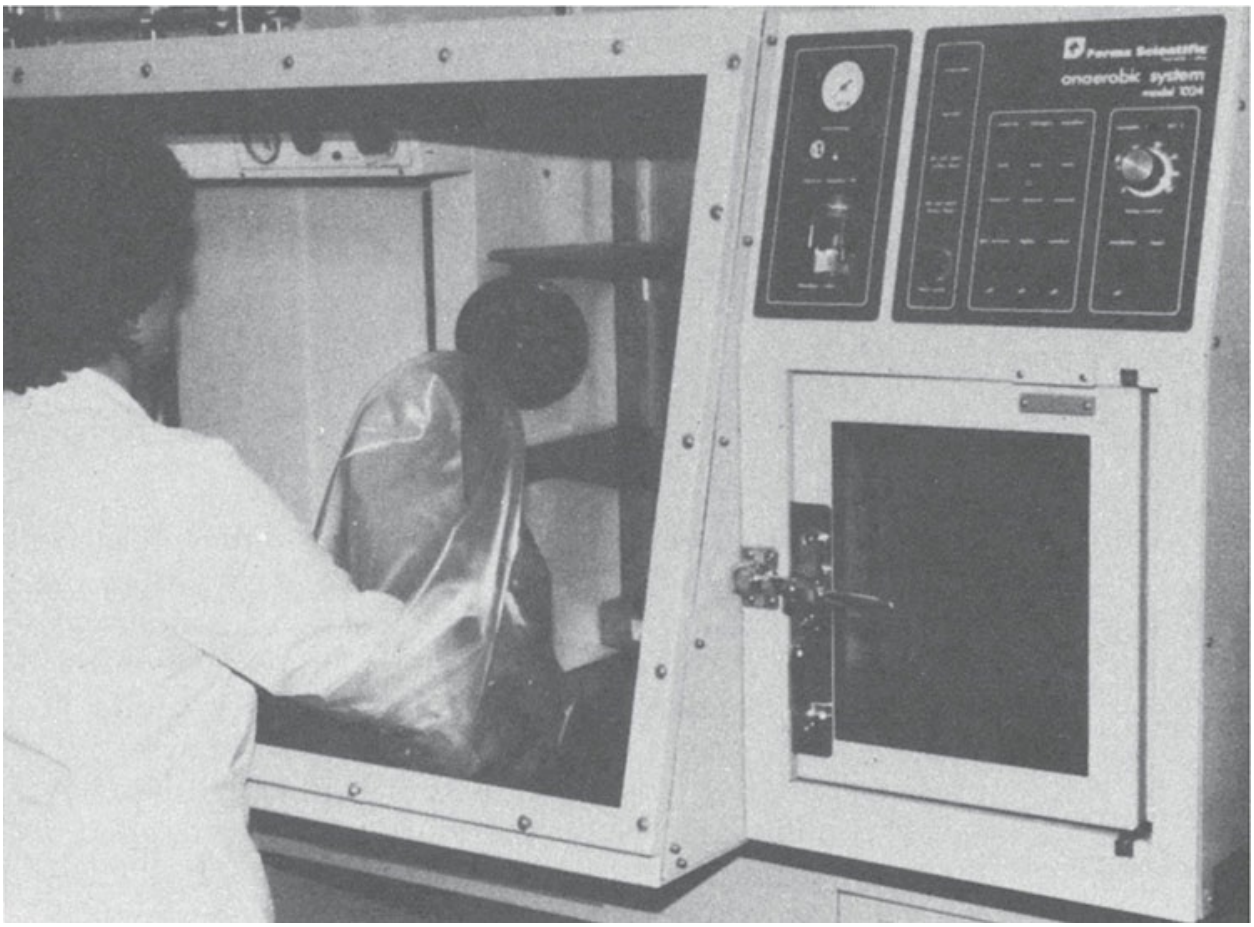

■ **FIGURE 16-5** The Forma Model 1024 anaerobic glove box (Forma Scientific, Marietta, OH). This system has an automatic entry lock. During routine daily operation, atmospheric air is bubbled through a methylene blue–glucose–HEPES buffer solution, which aids in monitoring O_2 leaks or determining if the catalyst is not working properly.

A certified anaerobic glove box is economical to operate because it permits the use of conventional plating media and the cost of gases for operation of the system is minimal. Once set up, the major expense is for the 85% nitrogen, 10% hydrogen, 5% carbon dioxide gas mixture used to replace the air in the entry lock when materials are passed into the glove box chamber. The referenced review on the principles behind and proper use of an anaerobic chamber is available, if more information is needed.[454]

Use of the Roll-Streak System

The roll-streak system, developed by W.E.C. Moore and associates of the Virginia Polytechnic Institute (VPI) Anaerobe Laboratory,[238] is a modification of the roll tube technique developed by Hungate and associates for culturing anaerobic bacteria from the rumen of cows and other herbivorous animals. Equipment for the VPI anaerobic

culture system, including the Hungate tubes, is available commercially from CEB Tech Services (Aberdeen, SD). The roll-streak system uses PRAS media prepared in tubes with rubber stoppers. This method is no longer widely used in clinical laboratories, and hence not discussed further; for those interested, more information is provided in earlier editions of this text and on the website for CEB Tech Services (www.CEBtechservices.com).

Use of Anaerobic Bags or Pouches

The Anaerobic Bag (BD Microbiology Systems, Sparks, MD) consists of a clear-plastic bag (sold in varying sizes capable of holding one to three, 100-mm-diameter Petri dishes), an H_2–CO_2 gas generator that generates an atmosphere when water is added (analogous to the generator used in a GasPak jar), cold palladium catalyst pellets, and a resazurin indicator. The bag is heat-sealed following activation of the generator to permit maintenance of anaerobic conditions according to the same principle as described for the GasPak jar system.

On the other hand, the AnaeroPouch (Mitsubishi, New York, NY) and the Anaerocult (Merck, Darmstadt, Germany) both achieve anaerobic conditions differently, without a catalyst, to remove oxygen from the atmosphere and generate CO_2. Oxygen is removed from within the sealed Anaerocult pouch by combining with iron powder to produce iron oxides. These anaerobic bag systems appear to be practical alternatives to anaerobic jar or glove box systems for the incubation of anaerobes when only a few plates are to be incubated. Additional bag systems are the Anaerogen (Oxoid) and the Anabag (Hardy Diagnostics). A study from 1990 that evaluated the pouches that were available at that time reported that although they performed well, the anaerobic chamber was superior for recovery of maximum number of anaerobes.[125] An example of an anaerobic pouch is shown in Figure 16-6.

Use of the Anaerobic Holding Jar

A modification of the Martin holding jar procedure is a convenient and inexpensive adjunct to the jar and glove box anaerobic systems that allows primary plating, inspection of cultures and subculture of colonies at the bench with only minimal exposure of anaerobic bacteria to atmospheric oxygen.[238,463,468] The holding jar assembly is illustrated in Figure 16-7, and its use is briefly described in Box 16-10.

Anoxomat. The Anoxomat (Mart Microbiology, Lichtenvoorde, the Netherlands) is an automated evacuation/replacement system. Although it is similar in principle to the procedure described by Whaley and Gorman, studies have shown somewhat better growth using the Anoxomat than with the other anaerobic systems with which it was compared.[54,429,464]

Incubation of Anaerobic Cultures

In most instances, 35°C to 37°C is the recommended temperature for primary isolation of anaerobic bacteria from clinical specimens. Plates inoculated at the bench and placed in anaerobic jars should be incubated for at least 48 hours, and reincubated for another 2 to 4 days to allow slow-growing organisms to form colonies; some anaerobes, such as certain species of NSF anaerobic gram-positive bacilli grow rather slowly, and colonies may not be detected if jars are opened sooner. Also, if the jar is opened too soon, some of the slow-growing organisms may be killed owing to oxygen exposure. In emergency situations, duplicate sets of plating media can be incubated in two different jars, one set incubated for 18 to 24 hours and the other for 3 to 5 days. This procedure allows rapid isolation of fast-growing anaerobes in the 18- to 24-hour jar and the later recovery of slow growers in the jars left for delayed incubation. If clostridial myonecrosis is suspected clinically, plates can be inspected as early as 6 to 12 hours after inoculation. This, however, is dependent on the volume of specimen received in the laboratory and the ability to plate more than one set of agar media.

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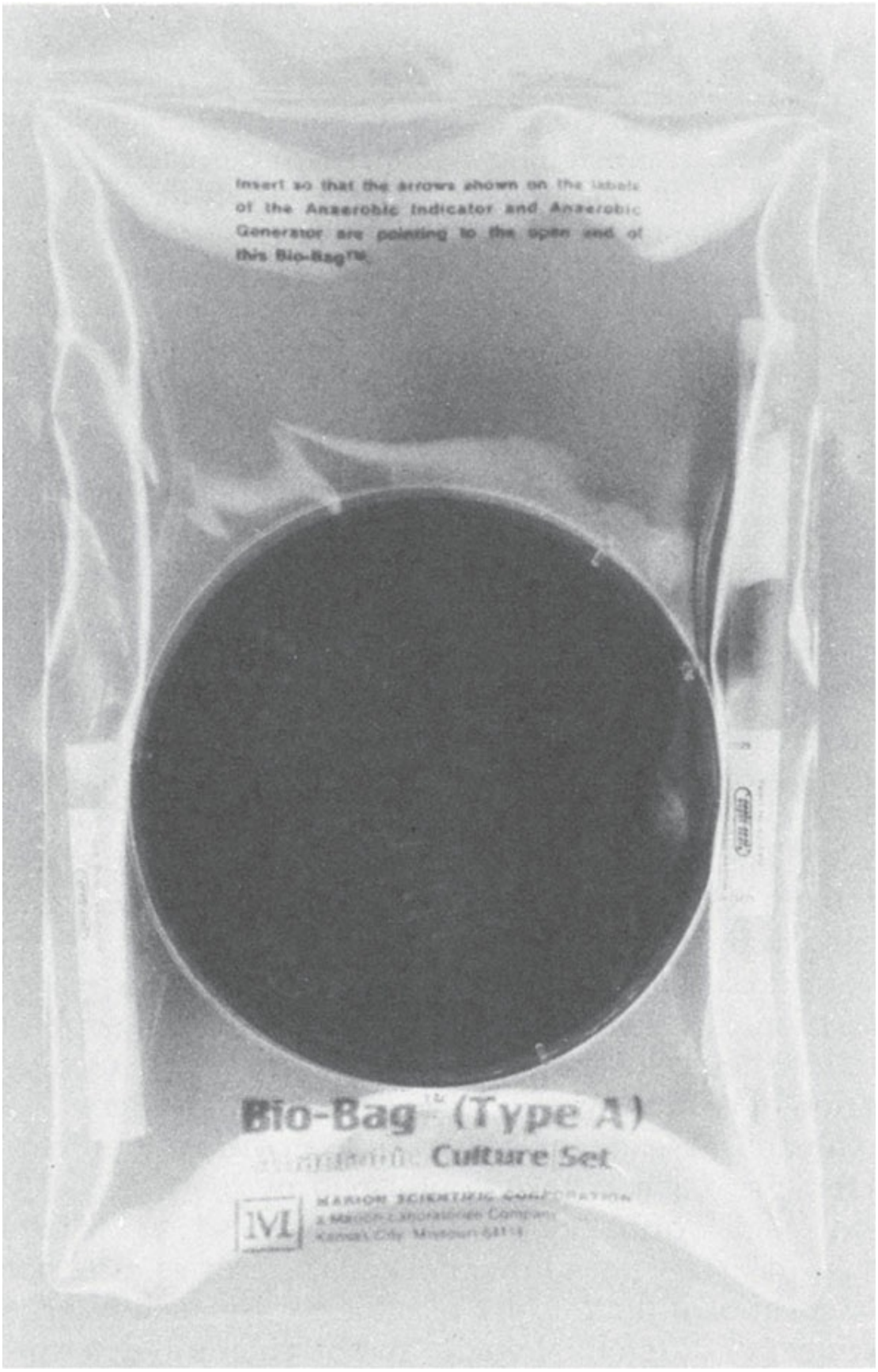
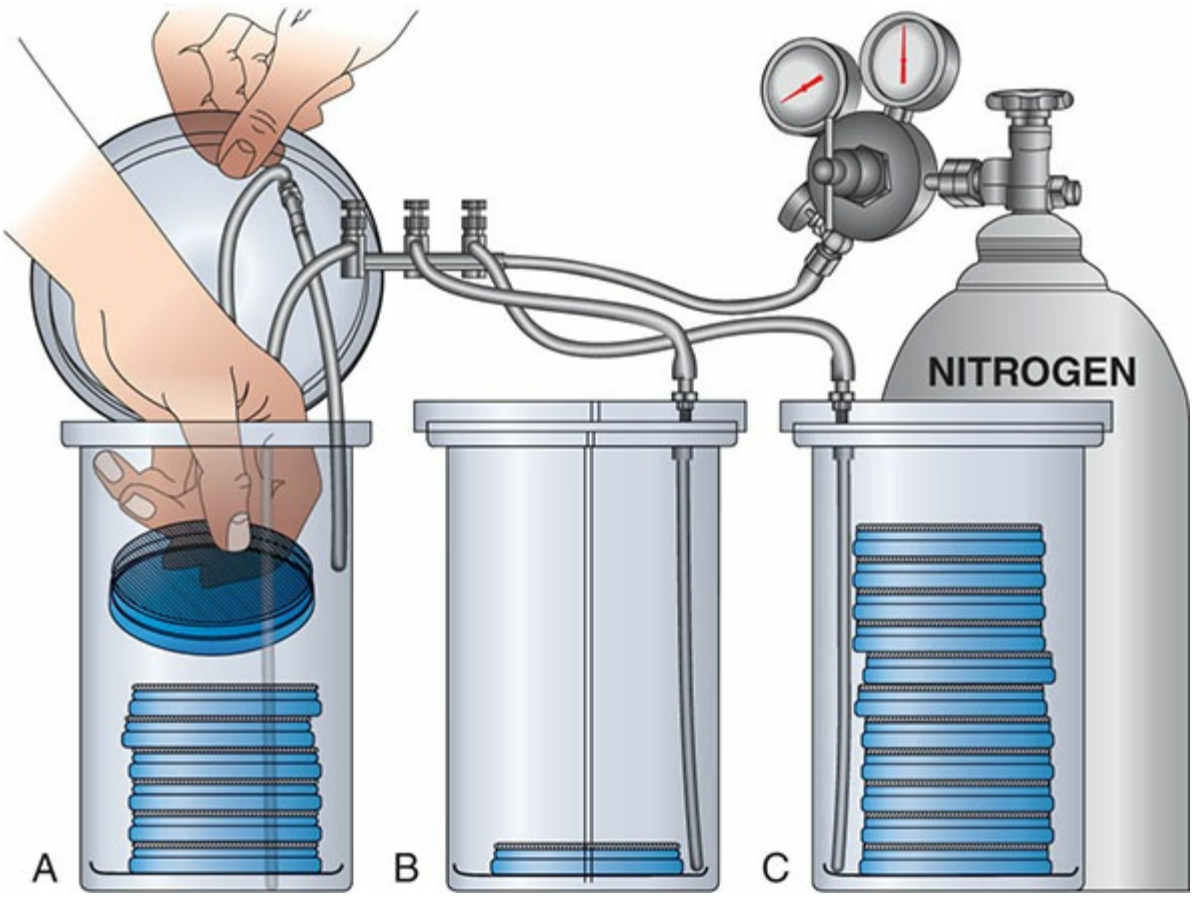

Insert so that the arrows shown on the labels of the Anaerobic Indicator and Anaerobic Generator are pointing to the open end of this Bio-Bag™.
Bio-Bag™ (Type A)
Culture Set

■ **FIGURE 16-6** The Anaerobic Bag Culture Set (Becton Dickinson Microbiology Systems, Sparks, MD). This culture set includes a plate of CDC anaerobic blood agar contained within an oxygen-impermeable bag. The system contains a gas-generating kit and cold catalyst.



■ **FIGURE 16-7** Schematic of the anaerobic holding jar system. The flow rate of nitrogen to each jar is regulated by the needle valves on the manifold (three-gang valve, available where aquarium supplies are sold). Jars A, B, and C contain uninoculated plates, plates with colonies to be subcultured, and freshly inoculated plates, respectively.

BOX 16-10

Anaerobic Holding Jar Procedure

1. Three holding jars are used, the first to hold uninoculated media, the second for plates on which colonies to be subcultured are growing, and the third to receive freshly inoculated plates of media.

2. Commercially prepared agar plates or agar media freshly prepared in the laboratory can be used. These may be held in a refrigerator for up to 6 weeks if bagged in cellophane.
3. The plates to be used on any given day should first be placed in an anaerobic glove box or an anaerobic jar for 4–15 hours before use to reduce the media.
4. As needed, the reduced media are placed in the first holding jar and continuously flushed with a gentle stream of N_2.
5. The plates of reduced media are surface-inoculated, one at a time, in ambient air and immediately placed in the third holding jar, which is also flushed with N_2. The second holding jar is used to hold any plates removed from the GasPak jar that require subculture.
6. After the jar, containing the newly inoculated plates, is filled, the plates can be transferred to a conventional anaerobic system, such as a GasPak jar, or into an anaerobic glove box for incubation at 35°C.

Prolonged exposure of freshly inoculated plates to ambient air must be avoided. Certain anaerobes commonly

encountered in clinical specimens, such as *Peptostreptococcus anaerobius*, may either fail to grow or may exhibit a prolonged lag in growth when freshly inoculated plates are held in ambient air for as short a time as 2 hours. Thus, if a holding jar procedure is not used, inoculated plates must be immediately placed in an anaerobic system (anaerobic jar or anaerobic glove box) to allow for effective cultivation of these anaerobes.

Enriched thioglycolate and chopped-meat glucose media should also be inoculated with clinical materials incubated in an anaerobic system to allow maximum recovery of anaerobes. It is not necessary to boil the tubes of enriched thioglycolate or chopped-meat glucose broth if they are prepared in tight-fitting screw-capped tubes and gassed in a glove box after autoclaving. Unless growth is apparent visually, broth cultures should be held a minimum of 5 to 7 days before discarding them as negative, with the exception of specimens for which *P. acnes* or *Actinomyces* spp. are common pathogens. These specimens would include prosthetic joint implant infections (PJI), specimens of aqueous humor or specimens from patients suspected to have endophthalmitis, cultures of postcataract specimens, CSF specimens from shunts, and specimens with request for *Actinomyces* spp. and/or those that demonstrate the presence of sulfur granules on Gram's stain.[65,66,224,324,431,499] There has been some controversy about the length of time needed for broths when *P. acnes* in particular is

being considered as a pathogen in infected prosthetic joints, especially when measures such as sonication of the joint are being used to prepare the inoculum. Some labs would incubate these broths for 10 days and others longer. Most recent articles have focused on incubation of at least 12 days before discarding these as negative. It is not certain whether this holds true for all specimens in which *P. acnes* is being suspected, but there will probably be continuing work and publications in this area.[65,66,275,419,431,480]

In regard to the processing of orthopedic specimens for culture in general, there is literature to support the sending of multiple specimens to the laboratory for aerobic and possibly anaerobic culture as well from the same site. The principle behind this practice is that many of the infecting organisms are normal skin microbiota organisms, in particular coagulase-negative staphylococci, and if the organism appears from three or more specimens, there is a higher likelihood that it is the pathogen in the infection; if it only grows from one or two of the specimens from the same site, then it is likely not the pathogen. This has at times led to the sending of >8 to 10 samples from one site, which is likely more than is necessary; there is evidence for sending 4 to 5 samples from a particular site.[17,115] Most of the work supporting these practices has been done with periprosthetic joint infections and revision arthroplasty. Each laboratory that receives such samples should work with their orthopedic colleagues to come to an agreement about the type and number of samples that is acceptable. There is no data to support the sending of multiple samples for fungal and mycobacterial cultures, however. Much work has been done with using molecular techniques for the identification of organisms directly in these orthopedic revision arthroplasty specimens and these techniques may be used more commonly in the future.[261,494]

Procedures for Handling Anaerobic Cultures

Inspection and Subculture of Colonies

After incubation, plates incubated in 5% to 10% CO_2 atmospheres should be examined with a hand lens or, preferably, a dissecting microscope. If anaerobic jars are used, a holding jar system should be used at the time of colony examination and subculture to minimize exposure of oxygen-sensitive isolates to air. The anaerobic glove box and the anaerobic disposable bag systems (Fig. 16-6) allow inspection of colonies in the absence of air. Use of the stereoscopic dissecting microscope during examination of colonies is extremely helpful because a number of anaerobes have distinctive colonial features. The dissecting microscope is also a valuable aid during the subculture of colonies to obtain pure cultures. During the inspection of colonies, any distinguishing characteristics on the medium, such as hemolysis of blood agar or clearing of EYA, as

well as the size and distinctive features of the colonies should be recorded. A number of characteristic colonies of anaerobes are illustrated in Image Plates 16-1, 16-2, 16-3, and 16-4. When recording colony characteristics, the following should be noted: the age of the culture and the type of medium, the diameter in millimeters of each colony in addition to the color, surface features (glistening, dull), density (opaque, translucent), consistency (butyrous, viscid, membranous, brittle), and other descriptive features (see Chapters 1 and 2).

Gram-stained smears of colonies from the anaerobic and CO_2-incubated plates should also be examined. Do not assume on the basis of colony and microscopic features only that colonies on plates that have been incubated in an anaerobic system are obligate anaerobes. Although the morphology and colony characteristics of certain anaerobes are distinctive, it is often impossible to distinguish some facultative anaerobes from obligate anaerobes without aerotolerance tests, even when the CO_2-incubated plates show no growth. Aerotolerance testing is very important to confirm that an isolate is an anaerobe.

The number of different colony types on the anaerobe plates should be determined and a semiquantitative estimate of the number of each type should be recorded (light, moderate, or heavy growth). Using a needle or a sterile Pasteur capillary pipette, transfer each different colony to another anaerobe blood agar plate to obtain a pure culture and an aerobic blood agar plate for aerotolerance testing (described below). If colonies are well separated on the primary isolation plate, a tube of enrichment broth, such as enriched thioglycolate or chopped-meat glucose medium, should be inoculated to provide a source of inoculum for differential tests. After incubation, the media should be inspected for turbidity or other evidence of growth. Once there is evidence of growth in the liquid media, Gram's stain should be performed. If the organisms appear to be in pure culture, they can be used to inoculate appropriate differential media for identification of isolates.

Examine the primary enriched thioglycolate and chopped-meat glucose cultures along with all primary isolation plates. If no growth is evident on the primary anaerobic plates, or if the colonies isolated fail to account for all the morphologic types found in the direct Gram-stained smear

of the specimen, the broth medium, if turbid, should be subcultured to two anaerobe blood agar plates, one for anaerobic incubation and the other incubated in a 5% to 10% CO_2–air incubator. Alternatively, a chocolate agar plate can be used for the subculture plate to be incubated in the 5% to 10% CO_2–air incubator. These subculture plates should then be examined as described above.

Aerotolerance Tests

Each colony type from the anaerobic isolation plate is subcultured to an aerobic (5% to 10% CO_2) and anaerobic blood agar plate for overnight incubation. An assessment is then made regarding the ability of the organism to grow in 5% to 10% CO_2, as well as the preference of the organism (i.e., if it grew in 5% to 10% CO_2, did it grow better than under anaerobic conditions or, conversely, were the anaerobic conditions preferential for growth.) (Figure. 16-8).

Preliminary Reporting of Results

Results of the direct Gram's stain and information about the probable presence of anaerobes should be reported to clinicians as quickly as possible so that appropriate therapy can be instituted. Unfortunately, a period of 3 days or longer is often required for these studies to be completed. Clinicians should be made aware that this extended time frame cannot be avoided with some slow-growing anaerobes (e.g., some species of *Actinomyces* and *Propionibacterium*). Fortunately, the colonial and microscopic morphology of certain anaerobic bacteria is often so distinctive that preliminary or presumptive reports of these isolates can be made prior to aerotolerance studies. Examples include *Clostridium perfringens*, members of the *Bacteroides fragilis* group and the pigmented *Prevotella* and *Porphyromonas* group, among others.

Identification of Anaerobic Isolates

Determination of Cultural and Biochemical Characteristics

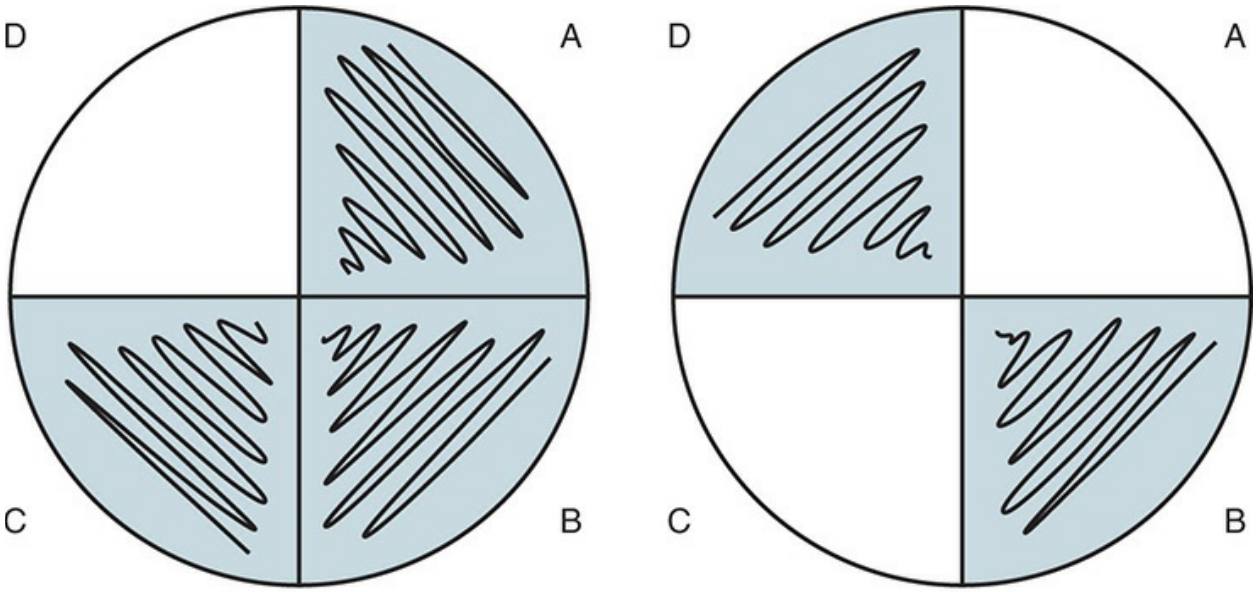
Once the presence of anaerobes has been confirmed by aerotolerance tests and a description of morphologic features has been reported, the next priority is to identify the pure culture isolates and to report the results to the clinician as rapidly and as accurately as possible. Although several hundred species of anaerobes are currently recognized by taxonomists, the task of identifying anaerobes for the clinical microbiologist is not nearly as formidable as it might seem because only a relatively small number are involved in anaerobic infections with any frequency.[160,238] Molecular methods and mass spectrometry (MALDI-TOF) are currently being employed to greatly reduce the time to identification.

Presumptive Identification

Nearly all clinically significant isolates are moderate obligate or aerotolerant anaerobes, and with practice are not particularly difficult to isolate and identify. In addition to organisms of the *Bacteroides fragilis* group, the pigmented *Prevotella-*

Porphyromonas group, *F. nucleatum,* and the anaerobic cocci that are quite frequently isolated, certain other, less common, anaerobes nonetheless have major pathogenic potential. Consequently, it is important to be familiar with and be able to recognize *Actinomyces israelii*, *A. naeslundii*, *A. meyeri, Propionibacterium acnes,* and *P. propionicum,* all of which may cause serious acute suppurative and chronic inflammatory disease; *Fusobacterium necrophorum*, which can be a highly virulent pathogen; *C. septicum*, an organism frequently associated with carcinoma of the colon or hematopoietic malignancy when isolated from blood cultures; the "histotoxic" clostridia in addition to *C. perfringens*, which can cause gas gangrene and various wound infections; *C. difficile*, a major cause of AAD and colitis; and *C. tetani* and *C. botulinum* because of the diseases they cause.[160,238,464]

Reference laboratories commonly use large batteries of tests in characterizing anaerobe isolates referred to them for identification or confirmation, similar to that listed in Box 16-11. In addition, new species are being recognized based on use of chemotaxonomic methods, nucleic acid sequencing and MALDI-TOF, which is becoming more common especially in reference laboratories. The data derived from the characterization of cultures with large numbers of tests has provided valuable databases for compiling tables of differential characteristics such as those published by the CDC, VPI, and State University and Wadsworth Anaerobe Laboratories. However, in most clinical diagnostic laboratories, it is not practical or economically feasible to use such a large number of differential media and biochemical determinations to identify isolates from clinical specimens.



Anaerobe blood agar incubated anaerobically

Aerobic blood agar plate incubated in candle jar

■ **FIGURE 16-8** Quadrant plating technique used for aerotolerance testing of four anaerobe isolates. The left plate has been incubated in an anaerobe jar for 18 to 24 hours, whereas the plate on the right was incubated in a candle jar. Isolates A and C are obligate anaerobes. Isolate B is facultatively anaerobic. Isolate D is possibly a microaerophile and should be tested further for its ability to grow in ambient air compared with an environment containing CO_2. A candle jar is not adequate for testing Campylobacter jejuni; this species grows optimally in 5% to 10% CO_2, 10% CO_2, and 85% N_2.

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BOX 16-11

Key Identification Characteristics of Anaerobic Bacteria

Relation to O_2 after aerotolerance testing is complete
Colony morphology: use of hand lens or dissecting microscope recommended
- Description of edges, internal structure, size
- Key characteristics: pigment, hemolysis, fluorescence, pitting of agar
- Time of incubation until appearance of colonies

Gram's stain characterization and morphology
- Include shape and arrangement of cells
- Comment on spores, filaments, branching, irregular staining

Type of growth in liquid media
Rapid or spot tests: growth in presence of 20% bile (BBE); catalase, indole, inhibition by sodium polyanetholsulfonate (SPS)
- EYA plate for lecithinase and lipase

Antibiotics potency disk tests: vancomycin, kanamycin, colistin; sometimes rifampin
Biochemical tests (often in kit format):
- Hydrolysis of starch, esculin, and gelatin
- Reduction of nitrate
- Fermentation of key carbohydrates: glucose, arabinose, cellibiose, sucrose, lactose, and others

Mass Spectrometry (MALDI-TOF)
16S rRNA Sequencing
Antibiotic susceptibility, if part of laboratory protocol or upon request
Optional:
- Metabolic products (GLC)
- Polyacrylamide gel electrophoresis of soluble proteins
- Cell wall long-chain fatty acids; menaquinones

Performed in reference laboratories only:

Toxicity, toxin neutralization, pathogenicity in animals

Fortunately, certain characteristics are especially useful in the identification of anaerobes (Chart 16-2). These characteristics form the basis of a practical approach for identifying anaerobic isolates that are commonly encountered in the clinical laboratory and additional species that either are less common or are potential major pathogens, even if they are rare.

Use of Differential Agar Media and Spot Tests

Several important characteristics for identifying anaerobic bacteria can be obtained with pure cultures on CDC anaerobic blood agar and in enriched thioglycolate medium. These characteristics, outlined in Chart 16-2, provide important clues for differentiating anaerobes in general. Additional characteristics are determined by use of differential antibiotic disks, which are added to freshly inoculated anaerobe blood agar plates. A 2-U penicillin disk, 1,000-mg kanamycin disk and 15-mg rifampin disk (available from Becton Dickinson Microbiology Systems) aid in the differentiation of anaerobic gram-negative bacilli and other anaerobes. The method for inoculation and use of these antibiotic potency disks is available in Chart 16-3. Colistin and vancomycin disks may also be extremely useful, as described elsewhere.[238] A sodium polyanetholsulfonate disk test is a practical way to separate *Peptostreptococcus anaerobius* from other anaerobic cocci. A nitrate disk test is a convenient method to demonstrate nitrate reduction during the workup of selected anaerobic bacteria. Three types of quadrant plates, labeled Presumpto Plate 1, Plate 2, and Plate 3, were developed by anaerobic microbiologists, Dowell and Lombard, and their associates in the 1980s to provide a system that would determine 20 phenotypic characteristics of anaerobes at a minimal cost. More information about the contents of, inoculation of and interpretation of these plates can be found in Image Plate 16-5, as well as in Charts16-2, 16-3, 16-4, and 16-5. Presumpto Plate 1 was available from Remel (now Thermoscientific); Anaerobe Systems (CA) can prepare EYA and at one time did make the other media upon request, but this author could not find anyone who sold all three-quadrant plates as of this writing.

Characteristics that can be determined with each of the three-quadrant plates are shown in Chart 16-5. The method for Inoculation of the Presumpto Quadrant Plates can be found in Chart 16-3.

Antibiotic Special Potency Disk Tests

Growth Inhibition on Anaerobe Blood Agar Using Antibiotic Disk Test. Zones of inhibition around the antibiotic disks are observed and recorded as follows:

Penicillin, 2-U disk: susceptible (S) if zone of growth inhibition is >12 mm in diameter; and resistant (R) if the zone is <12 mm.

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Rifampin, 15-mg disk: susceptible (S) if zone of growth inhibition is >15 mm; and resistant (R) if zone is <12 mm.
Kanamycin, 1,000-mg disk: susceptible (S) if zone of growth inhibition is >12 mm; and resistant (R) if zone is <12 mm.

The antibiotic disk potency tests are particular useful for differentiating between various gram-negative bacilli and gaining a presumptive identification. However, with the large number of anaerobes that are now being identified using molecular methods or MALDI-TOF, as well as the increasing resistance seen among many anaerobic bacteria, the utility of this assay may wane. Tables 16-11 and 16-27 provide information about how this can be used for the presumptive identification of anaerobic gram-negative bacilli and gram-positive cocci, respectively.

Use of SPS and Nitrate Disk Tests

(Inoculation of the sodium polyanethol sulfonate (SPS) and nitrate disks can be found in Chart 16-3).

SPS Disk Test. Measure the zone of inhibition around the 1-inch disk. A >12-mm zone of inhibition is recorded as susceptible (S). This test is specifically useful for the identification of *Peptostreptococcus anaerobius* and not for the determination of susceptibility testing for therapeutic guidance.

Nitrate Disk Test. Test for nitrate reduction by adding one drop of nitrate A reagent (sulfanilic acid) and one drop of nitrate B reagent (1,6 Cleave's acid) to the disk.[238] A pink or red color indicates that nitrate has been reduced to nitrite. If the disk was colorless after the addition of reagents A and B, then sprinkle zinc dust on the disk to confirm a negative reaction. The development of a red color after zinc dust is added confirms that nitrate is still present in the disk (a negative reaction).

The anaerobe blood agar (CDC anaerobe and other formulations), selective and nonselective media for primary plating, and PRAS media are available commercially from ThermoScientific Remel Microbiology products (www.remel.com), Hardy Diagnostics (www.hardydiagnostics.com), Anaerobe Systems (www.anaerobesystems.com), and BBL Microbiology Systems (www.bd.com) Remel. Details for preparation of the media and reagents may be found in previous editions of this textbook and elsewhere.[18,123] If prepared in one's own laboratory, there is the option of putting a single differential medium in a plate and not using quadrant Petri dishes. This approach increases the flexibility of the system for microbiologists who

would prefer to use other combinations of tests.

Identification of Anaerobes Using Phenotypic Characteristics

Conventional Approaches. It is not possible in this textbook to discuss all the procedures that are available for biochemical characterization of anaerobes. Tubed media can be purchased from some of the vendors mentioned above that also supply agar media for anaerobes. For further details on the use of conventional tube media, refer to the laboratory manuals on anaerobic bacteriology.[123,238]

Use of conventional media in Hungate or other large tubes is relatively time-consuming, and the media are costly to prepare or purchase. As alternatives to conventional media and methods, as well as to the Presumpto Quadrant Plate system, a variety of other procedures for characterizing and identifying isolates and alternative identification schemas have been described.[9,238] A number of these contain smaller volumes of media in containers that can be manipulated with reasonable speed at the bench. One of the more popular alternative approaches has been to test for certain key characteristics of isolates on differential agar media (e.g., BBE agar). Another has been to use disks impregnated with antibiotics or various other chemicals to test for growth inhibition or growth stimulation. Another excellent approach has been to use small tablets (e.g., WEE-TABS, Key Scientific Products, www.keyscientific.com) for determining certain enzyme-chromogenic substrate reactions. WEE-TABS have been used both as supplemental or stand-alone tests for differentiating various anaerobes. Details for their use and descriptions of several other tests for characterization of anaerobe isolates are given in the *Wadsworth Anaerobic Bacteriology Manual*,[238] on the Key scientific website and reviewed elsewhere.

Commercial Microsystems for the Identification of Anaerobes. Historically, two commercial packaged micromethod kits developed in the 1970s have continued to be used in clinical microbiology laboratories for identification of anaerobes, namely the API-20A (bioMérieux Vitek) and the Minitek (Becton Dickinson Microbiology Systems). The construction and use of these systems have been reviewed by Stargel et al.[460] These required overnight incubation and the use of manual or computerized databases for identification.

In addition, several manufacturers have marketed packaged kits that test for preformed enzymes, eliminating the need for overnight incubation. Most of these systems utilize a battery of chromogenic substrates to rapidly test for a number of amino peptidases and glycosidases. Each system requires the preparation of a heavy cell suspension from the surface of a purity plate culture. These packaged kits enable the microbiologist to determine multiple enzymatic characteristics of a pure culture isolate after 4 hours or less of aerobic incubation. Except for the API-ZYM kit,[27,314]

all of these packaged systems provide numerical codes, computerized databases and identification tables to aid in identification once an isolate has been characterized using the system. In a survey of laboratory methods being used in clinical laboratories in 2008, preformed enzyme kits were used by 66% of laboratories, whereas 30% used special potency disks (discussed above) for identification.[191] The list of products used for performing these tests includes the following:

API ZYM (bioMérieux Vitek)[314,460]
Crystal Anaerobe Identification System (Becton Dickinson Microbiology Systems)[43,79]
MicroScan Specialty Rapid Anaerobe Panel (Siemens Diagnostics)
RapID-ANA II (Thermoscientific Remel or Oxoid Products)[315]Rapid ID 32A (bioMérieux Vitek)
Vitek 2 ANC Card (bioMérieux Vitek).[43,286,391]

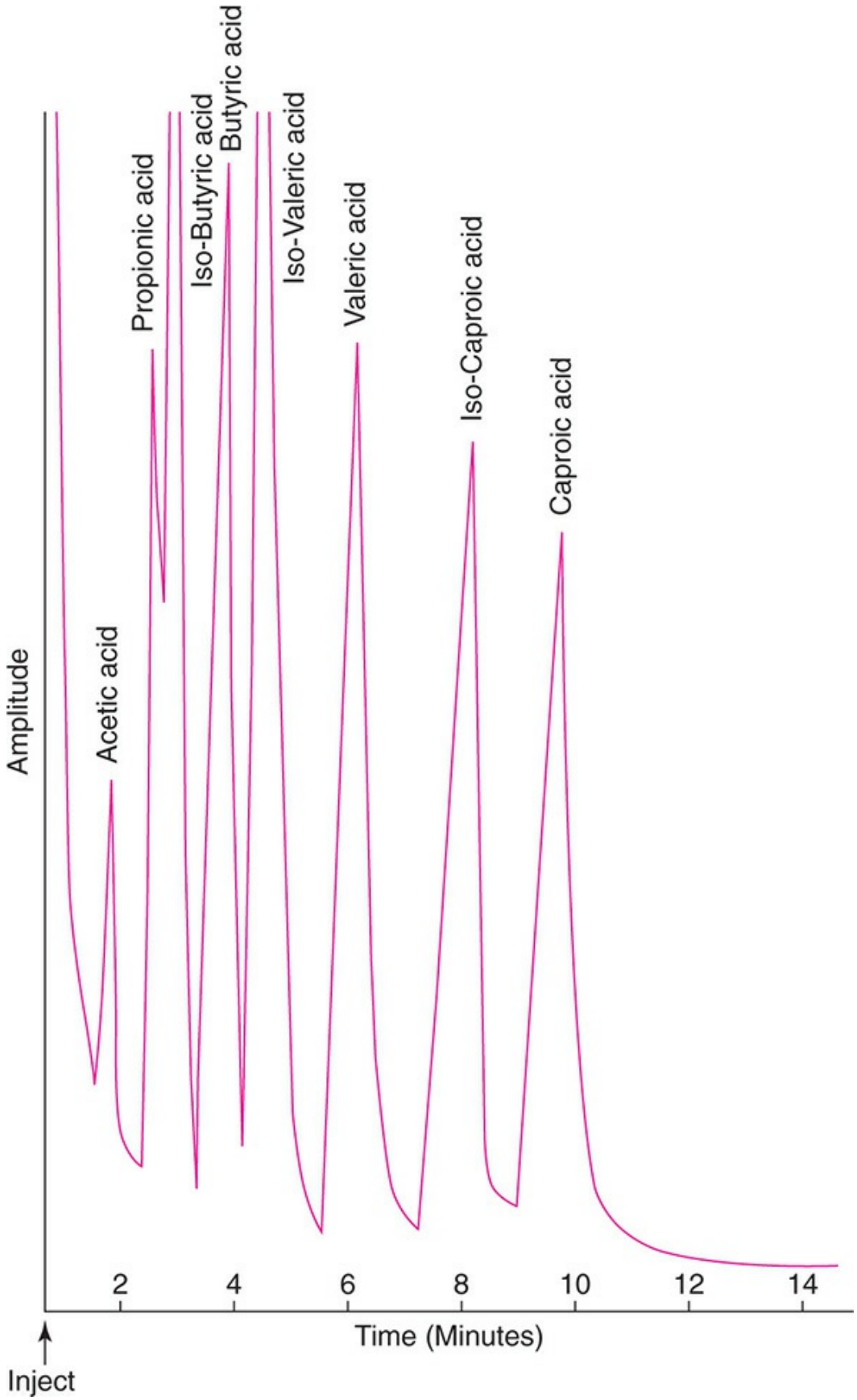
These kits are popular with microbiologists and are being used widely in diagnostic microbiology laboratories. In general, they are simple to use and less time-consuming than

conventional methods. A detailed description of each of the systems, including the substrates, procedures for use, interpretation of test results and a discussion of performance evaluations is available elsewhere or at each of the manufacturer's websites. Many of the packaged microsystems listed above must be supplemented with additional tests for accurate and definitive identification of selected groups of anaerobes (e.g., use of EYA for clostridia other than *C. perfringens*; the use of rapidity of growth and growth appearance in liquid as well as solid media, several additional supplemental tests, plus gas-liquid chromatography (GLC) for the complete identification of anaerobic gram-positive NSF rods; the use of 20% bile, esculin, selected conventional carbohydrate tests for *Bacteroides* spp., as well as additional tests for other anaerobes. Of course, it is still necessary to observe and correctly interpret Gram's stain reactions, consider microscopic and colony morphology and incorporate the results of aerotolerance tests. Pure cultures must always be used when attempting to differentiate anaerobes with the commercial packaged systems. When a kit produces an identification of a rare species, one should not accept this identification without repeat testing with alternative procedures, or perhaps confirmation from a reference laboratory when relevant clinically.

Determination of Metabolic Products by Gas-Liquid Chromatography. Metabolic products, released into broth culture media during anaerobic growth, are

key characteristics of anaerobic bacteria and many facultatively anaerobic bacteria. Together with determining the relationship to oxygen, most anaerobic bacteria can be identified to the genus level based on presence or lack of spores, Gram reaction, cellular morphology, and results of GLC analysis. This technique improves the speed and accuracy of identification; cost actually decreases because of the time saved.[338] Use of GLC in many clinical laboratories has, however, been greatly reduced and in many cases eliminated. It is still performed in reference and research laboratories and if needed, one could send isolates out for testing. Examples of GLC testing are included in Figures 16-9 and 16-10, as well as in Chart 16-6; additional information is also available in past editions of this textbook and anaerobe texts, if needed.[238,519] The definition of genera based on metabolic pathways remains scientifically valid, since the metabolic pathways of these organisms represent genetically stable or conserved traits of the bacteria. The schemas in Tables 16-9 and 16-10 illustrate the value of acid metabolic product analysis for differentiating genera of anaerobic bacteria. Although GLC analysis is not mandatory for presumptive identification of the *B. fragilis* group, the pigmenting anaerobic gram-negative bacilli, *F. nucleatum, C. perfringens, P. anaerobius,* and a few other anaerobes commonly recovered from clinical specimens, it is necessary for definitive identification of many species of *Bacteroides* and *Fusobacterium*, for most *Actinomyces, Bifidobacterium, Clostridium* species other than *C. perfringens, Eubacterium, Lactobacillus, Propionibacterium*, and for nearly all of the anaerobic cocci, unless molecular or mass spectrometry methods are utilized.[238]

Gas chromatography instruments are now relatively inexpensive, safe, simple to operate, reliable and are commercially available from various scientific instrument manufacturing companies. Equipment and procedures for determining metabolic products by GLC are described in more detail elsewhere.[238,338] Charts 16-6 and 16-7 provide procedures for inoculation of a gas chromatography instrument for detection of volatile and nonvolatile fatty acids, respectively.


Amplitude
Acetic acid
Propionic acid
Iso-Butyric acid
Butyric acid
Iso-Valeric acid
Valeric acid
Iso-Caproic acid
Caproic acid
2
4
6
8
10
12
14
Time (Minutes)
Inject

■ **FIGURE 16-9** A typical volatile acid standard chromatogram. The time elapsed between the injection of an ether extract of the standard solution and the peak for each acid (i.e., the retention time) is used to identify the acids. Note, for example, that the retention time for acetic acid is 1.8 minutes and for valeric acid is 6 minutes (instrument used: Dohrmann Anabac, Clinical Analysis Products, Sunnyvale, CA; Detector: thermal conductivity; column packing: 15%, SP–1220/1% H_3PO_4 on 100/120 Chromasorb W/AW from Supelco, Bellefonte, PA).

Identification of Volatile Fatty Acids. GLC is used for identifying short-chain, volatile fatty acids that are soluble in ether. The acids detected with this procedure include acetic, propionic, isobutyric, butyric isovaleric, valeric, isocaproic, and caproic. The procedure is explained in Chart 16-6.

Volatile fatty acids can be identified by comparing elution times of products in extracts with those of a known acid mixture (volatile fatty acid standard) separated under the same conditions on the same day. A representative standard tracing is shown in Figure 16-9.

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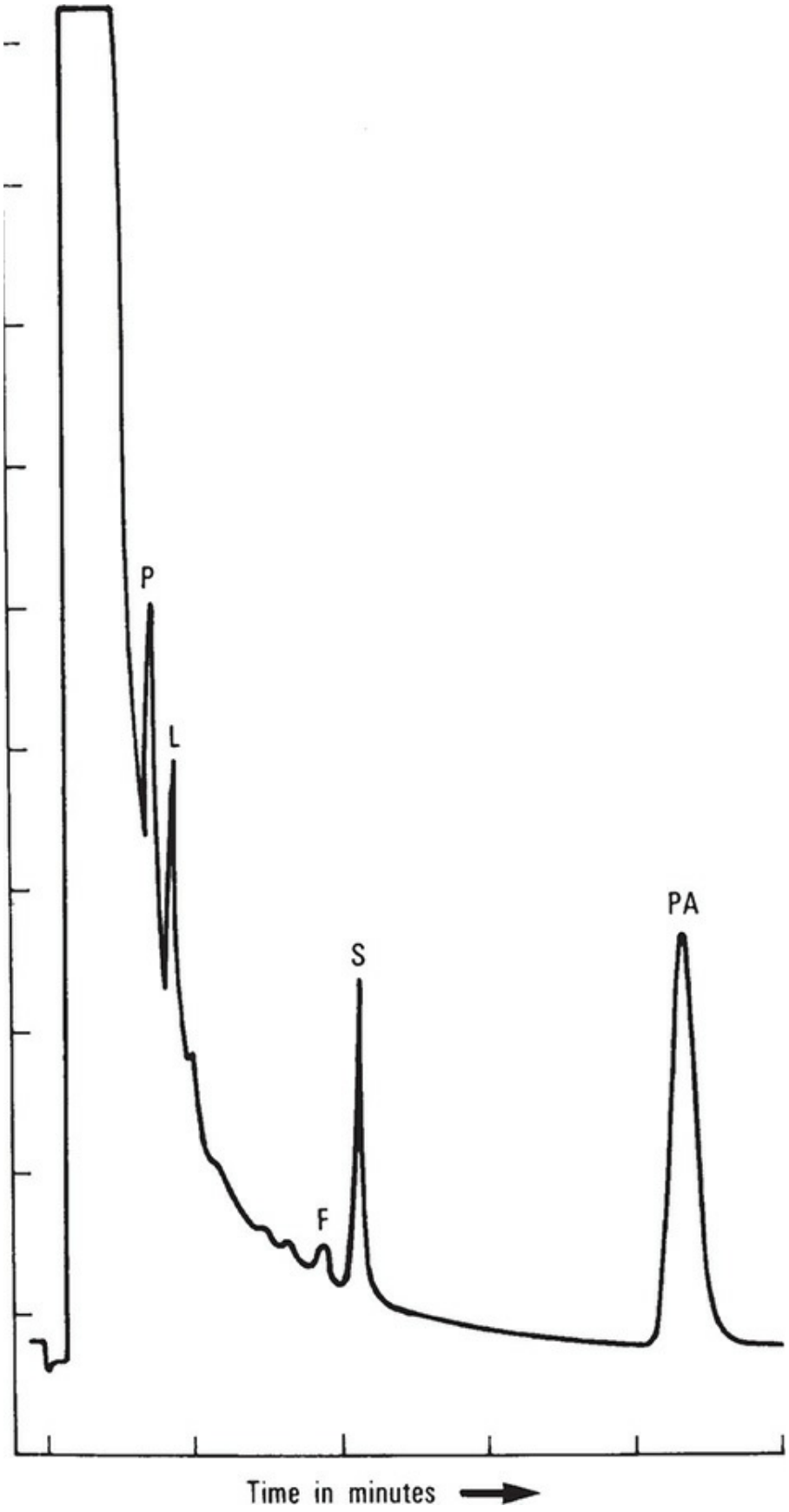
P
L
F
S
PA
Time in minutes

■ **FIGURE 16-10** A typical nonvolatile acid standard chromatogram. The column was packed with 10% SP. 1000/1% H_3PO_4 on 100/120 chromasorb W/AW (Supelco, Bellefonte, PA). P, pyruvic acid; L, lactic acid; F, fumaric acid; S, succinic acid; PA, phenylacetic acid.

Identification of Nonvolatile Acids. Pyruvic, lactic, fumaric, succinic, hydrocinnamic, and phenylacetic acids are not detected with the ether extraction procedure for volatile fatty acids (above). These nonvolatile acids are identified after preparation of methylated derivatives. The procedure is outlined in Chart 16-7. Analysis of chloroform extracts is performed using the same chromatographic conditions as for the volatile acids. The identification of nonvolatile or methylated acids is achieved by comparing elution times of products in extracts with those of known acids separated by chromatography on the same day.

The metabolic products of common species of anaerobic gram-negative rods are listed in Tables 16-16 and 16-21.

Use of Molecular and Mass Spectrometric Methods (MALDI-TOF) for Identification of Anaerobic Bacteria

Use of 16S rRNA Sequencing for the Identification of Anaerobes. Anaerobes are responsible as single pathogens or in polymicrobial infections in many clinical situations and yet there is often limited capability for their isolation and identification in many clinical laboratories. The various methods for recovery of anaerobes have been discussed in this chapter, as well as the use of specialized media and prolonged incubation in appropriate anaerobic conditions to enhance recovery. Most laboratories use phenotypic methods of identification and hence this often requires prolonged periods of time from isolation to identification, and at times, significant anaerobes may remain unrecognized, unidentified, or identified incorrectly. The use of molecular techniques, especially sequencing of the 16S rRNA, is rapidly changing these issues and may in time enhance the level of our understanding of the incidence and relevance of anaerobes in clinical disease. Whether or not all anaerobes in a culture should be characterized and reported because they may now be identified accurately and rapidly will remain an issue that warrants study, but use of sequencing can expand our knowledge of common and more fastidious organisms, which includes anaerobic bacteria. As with most molecular tools, the creation of a robust and consistent database is crucial to correct identification and this is still in its infancy in regard to anaerobes. However, numerous examples can be given in which use of sequencing has led to clarification of which anaerobes are involved in common and less common infections.[412,430,442,472,520,523] It has become the reference method used in studies of other methods for identification, especially the ongoing MALDI-TOF studies, and the

methods used to determine taxonomy and relatedness of anaerobic organisms.[106,179,279,491] Its use in determining the anaerobic normal microbiota of the GI and GU tract may change how we approach the identification of anaerobes in clinical infection, as well as, treatment practices in diseases such as bacterial vaginosis and recurrent *C. difficile* disease.[212,434,504] If only phenotypic characteristics are used to identify anaerobes in the GI tract, for example, only about 24% of the species in feces can be described as compared to the number of species detected when using molecular tools.[238] The same is probably true for the cause of clinical diseases in which traditional tools may not be adequate for definitive identification. Use of direct PCR and sequencing has been shown to have an advantage in patients presently on antimicrobial therapy and for the identification of pathogens, like anaerobes, that have specific growth requirements.[387]

Use of Mass Spectrometry for Identification of Anaerobes. There is a great deal of literature on the use of MALDI-TOF for the identification of bacteria and yeasts in clinical microbiology laboratories. This method is a soft ionization method which allows desorption of peptides and proteins from colonies of bacteria as well as crude bacterial extracts. Minimal preparation is required and the time to identification may be as little as minutes, as compared to the hours and sometime days needed for conventional biochemical identification of bacteria. Certainly, for many anaerobes, identification times can be very lengthy and in some cases still not definitive, especially if the isolate is not one commonly encountered in the laboratory. The initial capital cost of the mass spectrometer instrument may appear prohibitive for some laboratories. However, the low cost per identification compared to conventional methods can be used to readily cost-justify the instrument in most instances. There have already been many studies demonstrating the utility of MALDI-TOF for the identification of anaerobes (Table 16-8).

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TABLE 16-8 The Use of MALDI-TOF for the Identification of Anaerobes

Isolates Tested	MALDI-TOF System Used	Comparator	Results	Comments	Reference/Year
193 *Bacteroides* spp.	Bruker	Rapid 32A and 16S rRNA	MALDI: 87% correct ID and 93% agreed with PCR RAPID 32A: 52% species agreement with PCR	Easy and cost-effective and better identification than Rapid 32A; need to enhance databases	111; 2012
152 Clinical isolates: 45% GPB 35% GNB 20% Cocci	Bruker	Phenotypic and 16S rRNA	79% agreement to genus and species (using score of >2.0) 86% agreement to genus (>1.8) 92% *Actinomyces* spp. were correct	Most problems with *Fusobacterium* identification to species level; need expansion of database; Successfully used BBE medium for some isolates	154; 2012
238 Clinical isolates	Bruker	16S rRNA	**With extraction:** 78% genus and species, 14% genus only,8% with incorrect or no identification **No extraction:** 66% to genus and species 20%–13% incorrect or no ID	Direct smear not inferior to extraction	171; 2012
290 Clinical isolates: 73 *Clostridium* spp. 11 *Bacteroides* spp. 11 *Actinomyces* spp.	Both	16S rRNA	**Vitek:** 44%–49% for genus and species, 12% genus only, 1.4% incorrect identification, 13% no identification **Bruker:** 67% to genus and species, 8% incorrect identification	Bruker gave more correct identifications but also more incorrect identifications as compared to Vitek MS. Pretreatment with formic acid with the Bruker system enhanced identification of *C. ramosum*. Metronidazole-resistant gram-positive rods posed a problem with identification.	242; 2011
544 Isolates of 79 species 130 *P. acnes* 52 *Fusobacterium* spp. 117 *B. fragilis* group	Bruker	16S rRNA	MALDI identified 61% overall 16/212 isolates with no identification were not in the database Were able to identify 11 new organisms with MALDI and sequencing	*P. acnes* performed well Labs will be faced with "burden" of "new" anaerobes with MALDI and sequencing	276; 2011
283 Clinical isolates: 140 GNB 73 *Clostridium* spp. 8 *Actinomyces* spp.	Bruker	Conventional ID and then 16S rRNA for discordants	77% Identified to genus and species 11% to genus level 12% Nonreliable identification; after sequencing of discordants, 0.7% were incorrect MALDI results	Enhanced results using lower MS scores *P. acnes* did well Discussed need to enhance database Discovered many incorrect *B. fragilis* group identifications with phenotypic method	352; 2012
253 Clinical isolates: 40% GPB 27% GNB 32% Cocci	Bruker	16S rRNA	71% identified to genus and species and another 20% to genus level using manufacturer cut-offs 4% misidentifications at score of >2.0 and <1% at score of >1.7	Enhanced database useful; on-plate extraction worked well MALDI offered a rapid, inexpensive method for identification of anaerobes	417; 2013

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107 Isolates of GPC	Vitek	16S rRNA and FISH	90% good identification; only three isolates could not be identified	Excellent tool for identification of phylogenetically heterogeneous groups of anaerobes like the GPC	490; 2011
79 isolates	Both	16S rRNA	**Vitek:** 61% to genus and species; up to 71% to genus **Bruker:** 51% to genus and species; up to 61% to genus	Correct identifications for both were improved if organisms not in database were removed. Vitek performed better with GPCs and Bruker with the *B. fragilis* group.	492; 2011
102 *Prevotella* spp.	Bruker	16S rRNA	63% identified to species and 73.5% to genus level only Improvement to 83% to species and 89% to genus if database was extended	Identification of *Prevotella* is good, but database needs to be expanded	527; 2012
651 isolates of 11 genera	Vitek	16S rRNA	>90% correct ID to species level	Performed poorly (no identification) for some *B. uniformis*, *Fusobacterium* sp. and *Actinomyces* spp. Overall, the system provided excellent accuracy with rapid turnaround time	179; 2013
73 isolates (Part of large study of aerobes and anaerobes)	Both	16S rRNA	**Vitek:** 75% to genus and species 3% more to genus 18% no identification **Bruker:** 62% to genus and species 22% more to genus 15% no identification	Both performed very well with equivalent efficiency for all isolates. *Fusobacterium* spp. were poorly identified to species level with Bruker and Vitek misidentified more *Bacteroides* spp.	318; 2012
274 isolates	Both	API 20A; discrepant results with 16S rRNA	**Vitek:** 100% identified at the genus and species level **Bruker:** 89% identified at the genus and species level, 10% more at only genus level, and 0.72% misidentifications	Both platforms performed well and MALDI could be used as the first line of anaerobic identification to save time.	231; 2013

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Biswas and Rolain reviewed the literature that used both or either of the Bruker and Vitek instruments; these were able to demonstrate a >85% accuracy in 10 of 13 published papers, although the majority of the work was done with more common anaerobe species.[42] The need for a robust extraction method prior to testing remains

unresolved.[171,242] The importance of a robust database has been denoted by many researchers, not only for anaerobes, but for most organisms for which MALDI-TOF has been evaluated.[154,242,254,279,318,352,492] A multicenter study reported that >90% of the 651 anaerobes, which included 11 different genera, were correctly identified to species level using the Vitek MS system as compared to 16S rRNA sequencing; furthermore, these identifications were achieved without the need for extraction. There were some strains of gram-negative bacilli (*Fusobacterium* and *B. uniformis*) and *Actinomyces* that gave no identification results, but the authors felt the system overall provided excellent accuracy with rapid turnaround time.[179] Another large study in France evaluated MALDI-TOF for 1,325 anaerobes and found that 92.5% were correctly identified to the species level, including several uncommon or rare species. These authors felt that MALDIT-TOF was becoming the "gold standard" for the identification of anaerobes in the routine clinical laboratory.[31]

Use of the Bruker MALDI-TOF system for the identification of 484 anaerobes (>75% *Clostridium* spp. with >70% of these being *C. difficile*) resulted in >94% with correct identification versus 16S rRNA sequencing confirmation and 100% specificity for the *Clostridium* spp. tested. Performance was not good for *Fusobacterium* and *P. acnes* isolates; however, pretreatment of the colonies solved that problem in most instances.[106] Another study using a more diverse mixture of gram-positive and gram-negative anaerobes, analyzed 253 isolates of which 71% were correctly identified to species level and 92% to genus level (compared to phenotypic and molecular methods for comparison) using the Bruker system and on-plate extraction.[417] At least three comparative studies of both of the commercially available mass spectrometry instruments are in the literature analyzing the identification of anaerobes: one analyzed 290 anaerobes, demonstrating more species identifications with Bruker over Vitek MS (67% vs. 49%), but Bruker also had more incorrect identifications (8% vs. 1%). Sequencing was used for confirmation of discrepancies. The authors felt both systems showed real promise for anaerobic identification, but each database needed to be optimized for better performance, since many isolates were identified but were not found in the respective databases; in the case of Bruker, the manufacturer-suggested cut-offs for identification need to be adjusted for better results.[242] The second comparative study only analyzed 79 clinical isolates and found >61% correct identification for both systems and concluded, like the study above, that the databases needed to be further enhanced.[492] The third comparative study analyzed 274 clinically significant anaerobes, comparing results of MALD-TOF to API 20AN and found 100% correct identifications for the Vitek MS and 89% for Bruker after molecular confirmation was performed.[231] A large laboratory in France compared the identification of 1,506 isolates of anaerobes using conventional phenotypic methods during a specific time frame over 11 years versus MALDI-TOF in which 1,564

anaerobe isolates were identified. Forty distinct species were identified in the 1,506 isolates characterized by conventional methods, whereas 103 distinct species were identified within the 1,564 isolates characterized by MALDI, more than doubling the number that could be identified; the authors felt that MALDI might be used in place of molecular methods even for identification of rare human pathogens since the study also involved >280,000 isolates of aerobic isolates.[422] There are some anaerobic species (similar to aerobes) in which MALDI may not perform as well as molecular methods or even phenotypic methods for differentiation between species. For example, a recently described *Bacteroides fragilis* group member, *B. dorei* could not be differentiated from *B. ovatus, B. vulgatus,* and *B. xylanisolvens* with MALDI, but these could be distinguished using β-glucosidase and catalase tests; these could also be differentiated using sequencing-based methods.[370]

A potential advantage of the use of sequencing or MALDI-TOF is the direct identification of anaerobes in the clinical specimen by sequencing and more rapid isolate identification by MALDI-TOF to provide even more rapid results to the clinician. MALDI-TOF has been evaluated for the identification of positive BacT/Alert blood culture bottles and results were good; a >24-hour time-to-result was achieved when the MALDI-TOF was used in contrast to conventional methods.[168,327] The combination of sequencing and MALDI-TOF is certainly going to continue to be used to further clarify microorganism taxonomy, and will likely help to define the clinical relevance of many anaerobes not readily identified by phenotypic means. It may well be that MALDI-TOF, if improved as suggested, will become a routine tool for the identification of anaerobic bacteria, as it has for aerobes in many clinical laboratories. Individuals who are teaching the new generation of medical technology students need to incorporate the traditional methods of identification with these newer methods so that laboratorians retain expertise in Gram's stain interpretation and colonial morphology, which remain important skill sets. In many ways, the best method for identification is a combination of the "old" and the "new."

Identification of Specific Groups of Anaerobic Bacteria

Levels of Identification in Different Laboratories

An extensive update on the nomenclature (current names and former names) of the anaerobic bacteria is provided in Table 16-2. Fortunately, the anaerobes most important clinically and/or those most frequently isolated from properly selected and collected clinical specimens comprise a much smaller list (Table 16-3). Only about 12 to 15 groups or species account for about 75% or more of the isolates from properly

collected specimens, at least using phenotypic means for identification. These include the *Bacteroides fragilis* group, the pigmented *Prevotella–Porphyromonas* group, *Fusobacterium nucleatum, Peptostreptococcus anaerobius, Finegoldia magna* (and a few other species of anaerobic cocci), *Propionibacterium acnes, Clostridium perfringens, C. ramosum,* and *Veillonella* spp. Laboratory personnel should be familiar with

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these species, because they are so common and often clinically significant. It is also especially important that clinical microbiologists know about and are able to recognize *Fusobacterium necrophorum, Actinomyces israelii, Propionibacterium propionicum,* the *C. clostridioforme* group, *C. septicum, C. difficile, C. botulinum, C. tetani* and a few other species (discussed later in the text), because these organisms may be highly pathogenic for patients.

Beyond considerations of the need to be familiar with the common and/or medically significant anaerobes, it has been proposed that microbiologists in clinical laboratories may wish to limit the extent of anaerobe identification, based on levels of capability.[9] All laboratories, even those with limited capability should be able to isolate anaerobes in pure culture and to evaluate the microscopic and colony morphology. Together with results of aerotolerance testing, this information would then be reported to the clinician, as a preliminary report. In a laboratory with limited capability for identification of anaerobes, if the isolate(s) is (are) deemed clinically relevant, the identification should be completed either in the laboratory, and if this is technically not possible, then it should be sent to a reference laboratory that can complete the identification. The following factors are important considerations in determining clinical relevance: there is a strong clinical opinion by an experienced clinician that the isolate is an etiologic agent of disease; the direct Gram-stained smear of a wound or abscess, peritoneal fluid or other sterile specimen, demonstrates evidence of acute inflammation (i.e., numerous polymorphonuclear leukocytes, but a lack or limited number of squamous epithelial cells) or necrosis; the direct Gram's stain contains a microorganism that correlates with the culture findings (e.g., microscopic evidence of clostridial myonecrosis (Plate 16-3D); sulfur granules suggestive of actinomycetes are present (Image Plate 16-2C and D); there are additional cultures from another body site that demonstrates the same microorganism that was recovered from the primary site (e.g., positive blood cultures contain *C. perfringins* in a patient with clostridial myonecrosis). Even laboratories with full capability of definitive identification of anaerobic isolates may wish to limit the extent of processing when there is no clinical reason to take the identification further. For example, if the laboratory receives a swab from surgery labeled "fecal spillage into the abdomen"; the culture and identification is

unnecessary as this specimen predictably will have a predictable mixture of anaerobic and aerobic microbiota. Identification of any or all of these organisms would have no clinical usefulness and would be inordinately expensive to complete full identification of everything isolated.

The second level of anaerobic workup would require, in addition to what can be done in laboratories with limited capability, some identification capability. Specifically, the differentiation of the *B. fragilis* group from the other gram-negative anaerobes and identification of *C. perfringens* are skills that should be retained, even in laboratories with limited capabilities. Isolates, which cannot be further identified in the laboratory of limited capabilities, that are considered to be clinically relevant should be transported to a reference laboratory for definitive identification of isolates.

The third level of capability would require the ability to accomplish a full identification of the 12 or so common anaerobe groups and species mentioned previously, by use of growth and phenotypic characteristics obtained by rapid tests, kit systems, GLC, nucleic acid sequencing and/or MALDI-TOF. Details of how morphology, growth characteristics, aerotolerance, some rapid spot tests, the Presumpto plate system and other practical methods permit recognition and separation of these groups and species are given in the text that follows. Laboratories capable of definitive identification have the choice of limiting identifications to the preliminary or presumptive levels described previously depending upon the quality of the specimen and the medical necessity for full identification. Laboratories capable of performing definitive identification might include those at large university medical centers, reference laboratories and state and federal public health laboratories.

There is little doubt that definitive identification is required to better define the role of anaerobic bacteria in diseases, to provide an accurate microbiologic diagnosis that can aid in the optimal choice of antibiotics and clinical treatment of the patient, for public health purposes (e.g., nosocomial diarrhea caused by *C. difficile*) and to help educate both clinicians and clinical microbiologists. The level of anaerobe identification depends on several considerations that will differ among laboratories; not all laboratories can be expected to perform the same levels of anaerobic bacteriologic studies. Factors that influence decision-making for the extent of identification include the technical competence and experience of the technologists, the number of personnel available, supplies and capital equipment, the patient population being served and the needs of the clinicians who have responsibility for their patients. Clinical microbiologists must be competent in the work that they do and should not hesitate to use the services of reference laboratories for aid in definitive identification or for reference confirmation of isolates, as well as antimicrobial susceptibility testing, toxigenicity testing, nucleic acid studies, or other procedures, when relevant clinically. Additional approaches and current information on anaerobe identification, based on various combinations of tests at different levels, is given elsewhere.[238] It is expected

that in the future many laboratories will have the capability for MALDI-TOF and/or molecular methods for identification, which would reduce even further the routine need for many of the above-mentioned biochemical tests and GLC. However, when working with anaerobes, it continues to be vital to always include information about the Gram's stain and colonial morphology of the organism, and to perform necessary aerotolerance testing to assist in confirmation of the newer identifications.

Tables 16-9 and 16-10 provide a key to the genus-level identification of anaerobic gram-negative rods and anaerobic gram-positive NSF rods. The discussion below will give information on the laboratory identification of each group of anaerobes.

Identification of Anaerobic Gram-Negative Bacilli

The anaerobic, gram-negative, NSF bacteria are now classified in the genera listed in Table 16-1. Key characteristics for presumptive or level 1 differentiation of these are given in Table 16-11. In spite of many exciting new taxonomic breakthroughs based on molecular genetic research, phenotypic tests are still important for clinical diagnostic purposes, and must be relied on in most hospital laboratories to identify anaerobes until simple, user-friendly, low cost molecular identification procedures or MALDI-TOF become widely available for these bacteria. Thus, for identification of isolates in diagnostic laboratories, clinical microbiologists continue to depend on key morphologic features, biochemical and physiologic characteristics, and in many instances, susceptibility or resistance to certain antimicrobial agents by use of antibiotic disk tests.

TABLE 16-9 Differentiation of Anaerobic, Gram-Negative, Non–Spore-Forming Rods to the Genus Level

I. Nonmotile or motile with peritrichous flagella; straight or coccobacillary rods	
A. Proposal by Shah and Collins[227] to include only the highly fermentative and bile-resistant species resembling *B. fragilis* (i.e., *B. distasonis, B. caccae, B. ovatus, B. thetaiotaomicron, B. merdae, B. vulgatus, B. uniformis, B. eggerthii, and B. stercoris*). Several other species remain in this genus; further studies are needed to determine their correct taxonomic location (e.g., *B. capillosus, B. coagulans, B. cellulosolvens, B. pectinophilus, B. tectum, B. ureolyticus*, and others). Until the latter group is reassigned, this genus remains heterogeneous.	*Bacteroides*[a]
B. Most often black-pigmented colonies; produces acetic, butyric, and succinic acids, with minor amounts of propionic, isobutyric, and	*Porphyromonas*[b]

isovaleric acids. All are asaccharolytic and indole-positive.	
C. Nonpigmented to pigmented colonies; bile-inhibited and saccharolytic; usually oral microbiota	*Prevotella*[a]
D. Bile-resistant, catalase-positive, nitrate-reduced, and urease-positive	*Bilophila*[b]
E. Found as a part of the human normal microbiota, but rarely encountered in properly selected clinical specimens	*Alistipes*[c] *Anaerostipes*[c] *Anaerorhabdus*[c] *Catonella*[c] *Cetobacterium*[c] *Dialister*[c] *Faecalibacter*[c] *Fibrobacter*[c] *Johnsonella* *Megamonas*[c] *Mitsuokella*[c] *Oxalobacter*[c] *Sneathia*[c] *Sutterella*[c] *Tannerella*[c] *Tissierella*[c]
F. Found in animals and nature.	*Dichelobacter*[c] *Pectinatus*[c] *Rikenella*[c] *Roseburia*[c] *Ruminobacter*[c] *Sebaldella*[c]
G. Produces major amounts of butyric acid (but little or no isoacids) as the major metabolic product; succinic acid is not produced; all species are nonmotile	*Fusobacterium*[a]
H. Lactic acid is the only major product; *L. buccalis*, the only species, is nonmotile.	*Leptotrichia*[b]
I. Acetic acid is the major acid metabolic product; produces hydrogen sulfide; reduces sulfate; *D. pigra*, the only species, is nonmotile.	*Desulfomonas*[c]
II. Motile, peritrichous flagella not produced	

A. Curved rods with monotrichous or lophotrichous polar flagella or subpolar flagella; butyric acid is a major product of fermentation.	*Butyrivibrio*[b]
B. Succinic and acetic acids are major products of fermentation.	*Succinimonas*[c]
1. Short, straight rods to coccobacilli; a single polar flagellum; found only in bovine rumen.	*Succinivibrio*[b]
2. Curved, helically twisted rods with pointed ends; vibrating-type motility by a single polar flagellum	*Anaerobiospirillum*[b]
3. Helical, curved rods; bipolar tufts of flagella	*Campylobacter*[d]
C. Microaerophilic; oxidase-positive, curved and spiral rods; single polar flagellum, with absence of a flagellar sheath; carbohydrates not fermented; produce succinic acid from fumaric acid	*Wollinella*[e]
D. Propionic and acetic acids are major fermentation products	
1. Tufts of flagella on concave side of crescent-shaped cells	*Selenomonas*[b]
2. Single polar flagellum; lipolytic; curved rods	*Anaerovibrio*[b]
3. Flagella are inserted in a spiral along the cell	*Centipeda*[c]

[a] Commonly found in clinical specimens.
[b] Rarely found in clinical specimens.
[c] Normal flora of humans or other animals only.
[d] *Campylobacter gracilis* is oxidase-negative and lacks flagella, but has "twitching motility"; it differs from other *Campylobacter* species in these two respects.
[e] *Wolinella succinogenes* has been isolated from the bovine rumen and is now the only species remaining in the genus *Wolinella*. It is difficult to differentiate from organism from the genus *Campylobacter*.
Modified from Winn et al.[519] and other references cited elsewhere in the text.
Adapted from Table 16-8 in Winn et al.[519]

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TABLE 16-10 Differentiation of Anaerobic, Gram-Positive Bacilli to the Genus Level

I. Bacterial endospores not produced

A. Produce major amounts of propionic and acetic acids; catalase usually produced; irregular or regular-shaped rods, coccoid cells; occasional branching	*Propionibacterium*
B. Produce acetic and lactic acids (>1:1 ratio); very irregular rods with bifid forms and branching	*Bifidobacterium*
C. Produce lactic acid as sole major product	
1. Short to long and slender rods; chain formation common; irregular rods uncommon; usually grows on tomato juice agar at pH ≤4.5 (see Chapter 14)	*Lactobacillus*
2. Small, elongated cocci that occur singly, in pairs, or in short chains	*Atopobium*
D. Produce moderate acetic, lactic and major succinic acids; irregular rods predominant; filamentous forms with branching	*Actinomyces*
E. Produce mixtures of acetic, butyric, lactic and sometimes formic acids; pleomorphic diphtheroid rods; saccharolytic	*Eubacterium*
F. Produces minor amounts of acetic acid and traces of lactic and succinic acids; asaccharolytic	*Eggerthella*
G. Produces major amounts of acetic acid and a small amount of succinate; straight to curved rods with some branching ("*Actinomyces*-like")	*Actinobaculum*
H. Acetate and butyrate are the only acid metabolic products; saccharolytic; thin rods	*Anaerotruncus*
I. Produces major amount of lactate with variable amounts of formic, acetic and succinic acids; coccobacilli, pleomorphic, short diphtheroidal rods; facultatively anaerobic; saccharolytic; *A. pyogenes* is β-hemolytic on sheep blood agar	*Arcanobacterium*
J. Produces moderate amount of acetate and lactate, and trace amount of succinic acid; saccharolytic; short rods	*Bulleidia*
K. Acetic, butyric, isobutyric, and lactic acids produced; saccharolytic; occur as short rods in tangled chains	*Catenibacterium*
L. No metabolic products detected in peptone yeast extract glucose broth; asaccharolytic; very short rods	*Cryptobacterium*
M. Produces acetic, formic, and lactic acids, sometimes with a trace of succinic acid; abundant ethanol; saccharolytic; often in the form of	*Collinsella*

pleomorphic cocci to short rods; may be confused with streptococci or lactobacilli, but is distinguished by producing abundant H_2	*Collinsella*
N. Acetate and lactate are major products with a small amount of succinate; saccharolytic (weakly); short to long rods occur in pairs and short chains	*Holdemania*
O. Phenylacetic acid produced as sole metabolic product in peptone yeast extract glucose broth; asaccharolytic; short rods occur singly, in short chains, or clumps	*Mogibacterium*
P. Succinic and acetic acids are major fermentation products, with or without lactic acid; oxidase-negative, curved rods, motile with multiple subterminal flagella; fermentative in peptone yeast glycogen broth, supplemented with rabbit serum	*Mobiluncus*
Q. Produces formate, acetate, butyrate, caproate, and H_2; pleomorphic rods occur in pairs resembling birds in flight, clumps, or Chinese letters (formerly *Eubacterium alactolyticum*)	*Pseudoramibacter*
R. Produce only acetic acid or no acid metabolic products; occur in rods, cocci or coccobacilli in chains and clumps	*Slackia*
II. Bacterial endospores produced	*Clostridium*

Adapted from Winn et al.[519] and other references cited elsewhere in the text.

As previously indicated, the first major goal of identification should be to determine whether anaerobic bacteria are present and to isolate them in pure culture. The presence of anaerobic gram-negative rods suggestive of *Bacteroides, Prevotella, Porphyromonas,* or *Fusobacterium* spp., based on determining relationships to oxygen, together with the Gram's stain results and colony observations, should be promptly reported to the clinician. Presumptive or preliminary identification of the *B. fragilis* group, and the *Prevotella-Porphyromonas* group, along with other obligately anaerobic, gram-negative rods, can be done using differential characteristics obtained with growth characteristics (e.g., growth on blood agar, BBE, and EYA), fluorescence and a few simple disk/spot tests (Table 16-11). Additional clues for group identification along with key characteristics for various species are provided in Tables 16-12 through 16-16.

The *Bacteroides fragilis* Group. Organisms of the *B. fragilis* group are nonmotile, gram-negative rods with rounded ends, and are 0.5 to 0.8 mm in diameter × 1.5 to 9

mm long (Image Plate 16-1A).[238,464] Cells from broth culture tend to be pleomorphic, often with vacuoles; many strains are encapsulated which, as was described above in the section on human infections, contributes to the virulence of the species (Chart 16-10). Colonies of *B. fragilis* on CDC anaerobe blood agar are 1 to 4 mm in diameter, nonhemolytic, gray, entire, and semiopaque with concentric whorls or ring-like structures inside the colonies (Image Plate 16-1B). Colonies of other species of the group are similar in size and shape, but some of them differ with regard to their internal structures. A key characteristic of all species of the *B. fragilis* group is their resistance to 20% bile and ability to hydrolyze esculin, hence the utility of primary plating onto BBE agar along with nonselective blood agar. Colonies of the *B. fragilis* group on BBE after 18 to 24 hours will be large and black with blackening around the colony; they are nonhemolytic on blood agar. They are all resistant to penicillin, kanamycin, colistin, and vancomycin, but susceptible to the rifampin 15 µg disk (Table 16-11) All are saccharolytic and their carbohydrate fermentation patterns, along with indole, help to differentiate between the species. Detailed characteristics of the more common species of the *B. fragilis* group are given in Table 16-12. There are many of the 23 members of the *Bacteroides fragilis* group not included in Table 16-12; these have been isolated from feces and rarely if at all identified in clinical infections using traditional methods. These may be recognized, however, in clinical specimens if molecular methods are used for full identification. In many infections, it is not important to identify past the *B. fragilis* group level; however, this too may change as MALDI-TOF methods can provide species-level identification quite quickly.

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TABLE 16-11 Presumptive or Preliminary Identification of Anaerobic Gram-Negative Bacilli

Groups or Species	Grow on 20% Bile Agar (BBE)	Red Fluorescence (UV Light)	Brown or Black Colonies	PEN (2 U Disk)	RIF (15 μg Disk)	KANA (1 μg Disk)	COL (10 μg Disk)	Indole	H_2S	Lipase
B. fragilis group	+	–	–	R	S	R	R	V	–	–
Pigmenting *Prevotella/ Porphyromonas*	–$^{+}$	+	+	S or R[a]	S	R	R	+$^{-}$	–	–$^{+}$
B. ureolyticus[b]	–	–	–	S	S	S		–	–	–
F. nucleatum	–	–	–	S	S	S	S	+	–	–
F. necrophorum	V	–	–	S	S	S	S	+	+	+
F. mortiferum	+	–	–	R or S	R	S	S	–	+	–
F. varium	+	–	–	R or S	R	S	S	V	+	V
Bilophila wadsworthia	+[c]	–	–	R	S	S	S	–	+	–

[a] In the group, there is variability to the antibiotics and some strains may be resistant.
[b] *B. ureolyticus* is urease-positive and will corrode or "pit" the agar around their colonies; colonies are also small as compared to others in the table, <1 mm in diameter.
[c] *B. wadsworthia* on BBE agar will appear as a "black-eyed" colonies; it is strongly catalase-positive and urease-positive.
PEN, Penicillin; RIF, Rifampin; KANA, Kanamycin; COL, Colistin; R, Resistant; S, Susceptible; +, positive; –, negative; +$^{-}$, most strains are positive, but some strains negative; –+, most strains negative, but some positive; V, variable reactions among the strains.
Adapted from references 178, 238, and 263.

TABLE 16-12 Characteristics of Common Members of the *Bacteroides fragilis* Group and *Parabacteroides* spp.

				Fermentation of					
Species	Indole	Catalase	α-Fucosidase	Arabinose	Cellobiose	Rhamnose	Salicin	Trehalose	Xylan
B. caccae	–	–	+	+	+$^{-}$	+$^{-}$	–$^{+}$	+	–
B. dorei	–	NA	+	+	–	+	–	–	NA
B. eggerthii[a]	+	–	–	+	–$^{+}$	+$^{-}$	–	–	+
B. finegoldii	–	NA	–	+	+	+	+	–	NA
B. fragilis	–	+	+	–	+	–$^{+}$	–	–	–
B. massiliensis	–		+	–	–	–	–	–	NA
B. ovatus	+	+	+	+	+	+	+	+	+
B. stercoris	+		V	–$^{+}$	+	+	–$^{+}$	–	V
B. thetaiotaomicron	+	+	+	+	+	+	–$^{+}$	–	+
B. uniformis	+^{w}	–$^{+}$	+$^{-}$	+	+	–$^{+}$	+	–	V
B. vulgatus	+	–$^{+}$	+	+	–	+	–	–	–$^{+}$
Parabacteroides distasonis	–	+	–	–$^{+}$	+	V	+	+	–$^{+}$
P. goldsteinii	–	V	–	–	+	+	+	+	–
P. gordonii	–	V	–	+	–	–	–	–	NA
P. johnsonii	–	+	–	+	–	+	–	+	NA
P. merdae	–	–$^{+}$	–	–$^{+}$	V	+	+	+	–

+, positive; –, negative; +$^{-}$, most strains are positive but a few may be negative; –$^{+}$, most strains are negative, but a few strains may be positive; +^{w}, weak positive; V, variable reactions; NA, data not available.
[a] *B. eggerthii* does not ferment sucrose; all other species in Table ferment sucrose.
All isolates grow on agar with 20% bile (BBE agar).
Adapted from references 238, 263, and 519.

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TABLE 16-13 Phenotypic Characteristics of *Porphyromonas* spp.

Species	Fluorescence	Indole	β-gal	NAG	Chymo	Clinical Relevance
P. asaccharolytica[a]	+	+	–	–	–	Diabetic foot infections
P. bennonis	–	–	V	+	+	SSTI; animal bite wounds
P. catoniae	+	–	+	+	+	Oral infections
P. endodontalis	+	+	–	–	–	Chronic periodontitis
P. gingivalis	–	+	–	+	–	Oral and nonoral infections
P. somerae	W	–	+	+	+	Diabetic foot infections and other
P. uenonis	+	+	–	–	–	Oral infections; appendicitis, peritonitis, BV-assoc.

β-gal, β-galactosidase; NAG, *N*-acetyl-β-glucosaminidase; Chymo, chymotrypsin production; +, positive reaction; −, negative reaction; V, Variable; W, weak reaction; SSTI, skin and soft tissue infections; BV-assoc, associated with bacterial vaginosis.
[a] *P. asaccharolytica* may be lipase-positive; all others in table are lipase negative.
All of *Porphyromonas* spp. above are pigmented except *P. catoniae*; all are catalase-negative except for some strains of *P. bennonis*. Both of these species can variably produce α-galactosidase. *P. catoniae* and *P. somerae* may be weak fermenters; others are not fermenters.
P. gingivalis and some strains of *P. catoniae* produce trypsin.
Adapted from references 178, 263, 238, and 519.

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TABLE 16-14 Phenotypic Characteristics of *Prevotella* spp.

Species	ESC	IND	LIP	GEL	α-Fuc	NAG	Fermentation of					Clinical Relevance
							Arab	Cell	Lac	Sal	Suc	
Pigmented												
P. corporis	–	–	–	+	–	–	–	–	–	–	–	GU Tract abscesses
P. denticola	+	–	–	+	+	+	–	–	+	–	+	Carious lesions; BSI
P. intermedia	–	+	+	+	+	–	–	–	–	–	+	Periodontitis; GU abscesses; SSTI, BSI; intra-abdominal abscesses
P. loeschii	+	–	V	+	+	+	–	+	+	–	+	Oral cavity
P. melaninogenica	V	–	–	+	–/+	+	–	–	+	–	+	Oral cavity; human bite wounds; diabetic foot infections; retropharyngeal and peritonsillar abscesses
P. micans	–		–				–	+	+	+	+	Oral cavity
P. nigrescens	–	+	+	+	+	–	–	–	–	–	+	Periodontitis; GU abscesses; SSTI, BSI; intra-abdominal abscesses
P. pallens	–	+	–	+	+	–	–	–	–	–	+	Oral cavity
P. shahii	–	–	–	+	W		–	–	+	–	+	Oral cavity
P. tannerae	–	–	–	+	+	+	–	–	v	–	V	Oral cavity; carious lesions
Nonpigmented												
P. amnii	+	–	–	–	–	+	NA	–	+	–	–	Female GU tract abscesses
P. baroniae	+	–	–	W	+	+	–	+	+	+	+	Oral infections
P. bergensis	+	–	–	–	–	V	+	+	+	+	–	SSTI
P. bivia	–	–	–	+	+	+	–	–	+	–	–	GU Tract abscesses; BSI; diabetic foot infections
P. buccae	+	–	–	+	–	–	+	+	+	+	+	Odontogenic infections; BSI
P. buccalis	+	–	–	–	+	+	+	+	+	+	–	Oral cavity; has been isolated from urine
P. dentalis	+	–	–	–	–	+	+	+	+	–	w	Oral cavity
P. disiens	–	–	–	+	–	–	–	–	–	–	–	GU tract abscesses; BSI
P. enoeca	V	–	–	+	+	+	–	–	+	–	–	
P. heparinolytica	+	+	–	–	+	+	+	+	+	+	+	Animal bite wound infections
P. marshii	–	–	–	+	–	–	–	–	–	–	–	
P. multiformis	–	–	–	+	V	+	–	+	+	–	+	
P. multisaccharivorax	+	–	–	+	–	+	V	+	+	V	+	
P. nanceiensis	+	–	–	–	+	+	–	V	+	–	+	Rarely isolated from a variety of specimens
P. oralis	+	–	–	V	+	+	–	+	+	+	+	
P. oris	+	–	–	V	+	+	+	+	+	+	+	Odontogenic lesions
P. oulorum	–	–	–	–	+	+	–	–	+	–	–	
P. pleuritidis	–	–	–	+	+	+	–	–	+	–	–	One strain from pleural fluid
P. salivae	+	–	–	–	W	+	+	+	+	+	+	Isolated from saliva
P. timonensis	–	–	–	+	+	+	–	+	+	–	–	Breast abscesses
P. verroralis	+	–	–	V	+	+	–	+	+	–	+	Oral cavity
P. zoogleoformans	+	V	–	+	+	+	+	+	+	V	+	Oral cavity

+, positive reaction; −, negative reaction; V, variable; w, weak reaction; ESC, esculin hydrolysis; IND, indole production; LIP, lipase production; Gel, gelatin hydrolysis; α-Fuc, α-Fucosidase; NAG, *N*-acetyl-β-glucosaminidase; Arab, arabinose; Cell, cellobiose; Lac, lactose; Sal, salicin; Suc, sucrose; BSI, bloodstream infections; SSTI, Skin and soft tissue infections.

There are a few recently named species of pigmented *Prevotella* group. These have recently been isolated from the oral cavity and are not included in table: *P. maculosa, P. saccharolytica,* and *P. histicola. P. copri* and *P. stercorea* have been isolated from human feces but are not included above.

Adapted from references 178, 238, and 263.

TABLE 16-15 Phenotypic Characteristics of *Fusobacterium* spp.

Species	Bile	IND	LIP	ESC	Conversion to Propionate From: Lactate	Conversion to Propionate From: Threonine	Gram's Stain Morphology	Colonial Morphology
F. gonidiaformans	−	+	−	−	−	+	Gonidial	
F. mortiferum	+	−	−	+	−	+	Round bodies; pleomorphic cells; often described as "bizarre"; irregular staining	Fried-egg appearing
F. naviforme		+	−	−	−	−	Boat-shaped	Mottled internal structure
F. necrophorumam[a]								
subsp. *fundiliforme*	−+	+	−+	−	+	+	Coccobacillary; no tapered ends; some filaments; bizarre forms	Yellow to cream color; smooth, round and entire; may fluoresce chartreuse under UV; greening of agar
subsp. *necrophorum*	−+	+	+	−	+	+	Pleomorphic; rod-shaped with round ends and bizarre forms	Dull, umbonate; may be β-hemolytic; may fluoresce chartreuse under UV light
F. nucleatum	−	+	−	−	−	+	Thin, tapered, pointed or fusiform ends	Speckled or opalescent colonies; bread-crumb-like; may cause greening of blood agar; fluoresce chartreuse under UV
F. periodonticum[b]	−	+	−	−	−	+	Slender. Long cells	Resembles *F. nucleatum*
F. russii	−	−	−	−	−	−	Large with rounded ends	Smooth colonies
F. ulcerans[c]	+	−	−	−	−	+	Large, rounded ends	Smooth colonies
F. varium[c]	+	+−	−+	−	−	+	Large with round, not tapered ends	Fried-egg, but smooth colonies

[a] *F. necrophorum* has two subspecies that can be found in humans: *F. necrophorum* subsp. *fundiliforme* is the more common human isolate and is the virulent subspecies, responsible for Lemierre's syndrome, postanginal sepsis and many other severs infections. *F. necrophorum* subsp. *necrophorum* is more commonly isolated from animals, like cattle.
[b] *F. periodonticum* cannot be distinguished from *F. nucleatum* with biochemicals; both are found in oral cavity as normal microbiota.
[c] *F. ulcerans* and *F. varium* are very similar biochemically. However *F. ulcerans* is indole-negative, whereas *F. varium* is usually positive. Additionally, *F. ulcerans* does not ferment fructose, whereas *F. varium* is a weak fructose fermenter. *F. ulcerans* is responsible for causing tropical ulcers.[41c]
+, positive; −, negative; +−, most strains are positive but a few may give a negative reaction; −+, most strains are negative but a few strains may give positive reaction.
Adapted from references 178, 238, and 263.

TABLE 16-16 Characteristics of Other Anaerobic Gram-Negative Bacilli

Species	Bile	URE	PIT	FERM	MOT	NIT	Metabolic End Products	Disks	Morp
Allistipes spp.	V	+	–	+	–	–	S	R,R,R	Short Rod
Anaerobiospirillum spp.	V	NA	–	+	+	–	A,S	R,S,V	Long rods or filaments; spiral
Bacteroides ureolyticus[a]	–	+	+	–	–	+	A,S	R,S,S	Short rod
Bilophila wadsworthia[b]	+	+⁻	–	–	+	+	A	R,S,S	Straight rods
Desulfovibrio spp.[c]	V	V		–	+	V	A	R,S,R	Curved rods
Dialister spp.[d]	–		–	–	–	–	A,P	R,S,V	Coccoid
Leptotrichia spp.	–	–[e]	–	+	–	–	L	R,S,S	Pleomorphic; some longer rods; variability between species
Odoribacter splanchnicus	+⁻	–	–	+		–	A,P,S	R,R,R	Pleomorphic, but rods
Phoaceicola abscessus[f]	–	–	–	–	+	–			Coccoid and rods
Pseudoflavonifractor capillosus	–⁺	–	–	+[w]	–	–	a,s	R.R,R	Short rods
Selenomonas spp.	–	–	–	+	+	+⁻	A,P	R,S,V	Curved rods
Sutterella wadsworthensis[a,d,g]	V	–	V	–	–	V	S	R,S,S	Straight rods
Tannerella forsythensis	–	Unk	–	–	–	–	A,B,IV,P	R,S,S	Pleomorphic rods
Tissierella praecutus	+	–	–	–	+	V	A,B,IV	R,S,S	Rods

[a] These species require formate and fumarate in media for growth.
[b] *B. wadsworthia* produces "black-eyed" looking colonies on BBE agar.
[c] *Desulfovibrio desulfuricans* is urease-positive; other species are negative.
[d] These species may grow under microaerophilic conditions.
[e] Rare positive strains have been reported.
[f] Although *Sutterella wadsworthensis* will be resistant to a 20% bile disk, it does not usually grow on BE agar.
[g] Data taken from results of one strain. Required >7 days for initial growth.
+, positive; –, negative; +⁻, most strains positive, but a few may be negative; –⁺, most strains negative, but a few may have positive reactions; +[w], positive but weak reaction; A, major acetic acid peak; a, minor acetic acid peak; Bile, growth in presence of 20% bile; FERM, fermentation, if +, the organism ferments one or more carbohydrates; IV, major isovaleric acid peak; L, major lactic acid peak; MOT, motility; NIT, nitrate reduction; P, major proprionic acid peak; PIT, colonies corrode or pit the agar; R, Resistant; S, Susceptible; s, minor succinic acid peak; Unk, Unknown; URE, urease production; V, variable reactions among strains.
Antibiotic potency disks: Vancomycin, Kanamycin, Colistin.
Adapted from references 178, 238, and 263.

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A recently described genus of anaerobic gram-negative rods, *Parabacteroides,* includes *P. distasonis, P. goldsteinii, P. johnsonii*, *P. gordonii,* and *P. merdae*; all of these are indole- and α-fucosidase-negative. These are still considered part of the *B. fragilis* group, at least for clinical relevance purposes, and will grow on the BBE plate and hydrolyze esculin.[238,464] These have been included in Table 16-12 along with other *B. fragilis* group members.

Pigmented Anaerobic Gram-Negative Bacilli. Shah and Collins proposed that the genus *Bacteroides* be restricted to the *Bacteroides fragilis* group and related species, which actively ferment glucose (pH 5.4) (highly saccharolytic), grow in the presence of 20% bile and hydrolyze esculin.[426] Their major acid metabolic products from

glucose metabolism are acetic and succinic acids, although smaller amounts of other short-chain fatty acids may be produced.[426] Thus, the pigmenting anaerobic gram-negative bacilli are no longer classified in the genus *Bacteroides*. The moderately saccharolytic, pigmenting species were placed in the genus *Prevotella*. In 1988, the asaccharolytic, pigmented *Bacteroides*, all isolated from humans, were reclassified in the genus *Porphyromonas* as *P. asaccharolytica, P. gingivalis* and *P. endodontalis* (Chart 16-10) (Image Plate 16-1C and 16-1D).[425] All are susceptible to 20% bile. Additional *Porphyromonas* species of human origin now include *P. uenonis, P. bennonis, P. somerae,* and *P. catoniae* (nonpigmented).[263] All except *P. bennonis* and *P. gingivalis* will exhibit some fluorescence before pigmentation and all except *P. bennonis* are catalase-negative. There are many *Porphyromonas* spp. that are only isolated from animals; these are pigmented and indole-positive. They are usually catalase-positive in contrast to the catalase-negative species of human origin. The animal species of *Porphyromonas* that are not included in Table 16-13 include *P. cangingivalis, P. canoris, P. cansulci, P. macacae,* and *P. levii*. Some of these have been isolated from animal bite wounds.[1]

Clues for the presumptive recognition of anaerobic gram-negative bacilli that belong to the genus *Porphyromonas* include the formation of tan to buff colonies that fluoresce brick-red under long-wave ultraviolet light or brown-black colonies (most species), inhibition of growth in the presence of vancomycin (i.e., failure to grow on kanamycin–vancomycin blood agar), inhibition by bile, inhibition by penicillin and rifampin, but resistance to kanamycin, formation of indole and failure of most species to ferment glucose or other carbohydrates. Definitive identification of *Porphyromonas* to the species level based on phenotypic characteristics is difficult. Some key differential characteristics of the asaccharolytic, pigmented, and catalase-negative species encountered in human illnesses are listed in Table 16-13. The determination of enzyme activities through the use of a rapid, 4-hour test system (i.e., the Rapid ID 32A system or RapID-ANA II) is a practical aid to identify *Porphyromonas* species encountered in humans.[116,238,263]

The genus *Prevotella* consists of pigmented and nonpigmented species as is designated in Table 16-2. In contrast to *Porphyromonas*, all of the pigmented species of *Prevotella* ferment glucose and other carbohydrates (Table 16-14). It is emphasized that the "pigmenting" *Prevotella* group described in Table 16-14 may take 2 days to 3 weeks to form pigment on CDC anaerobic blood agar or may even fail to produce pigmented colonies. All of the pigmented *Prevotella* spp. are gelatin-positive and do not ferment arabinose; all but *P. corporis* are fermenters of sucrose and produce α-fucosidase. *Prevotella intermedia* is distinctive in that it forms black colonies on blood agar, produces indole, is lipase-positive on EYA and ferments sucrose. *Prevotella nigrescens* is a species derived from a genetically distinct group of strains formerly included in *Prevotella intermedia*.[427] Some of these strains were from patients with

periodontitis; others were from healthy individuals. As can be seen in Table 16-14, there are no phenotypic characteristics that can distinguish these two species. *P. denticola, P. loeschii* and some strains of *P. melaninogenica* are the only esculin-positive, pigmented *Prevotella* sp.; like *P. intermedia/nigrescens*, *P. loeschii* may be lipase-positive and these are the only lipase-positive strains of pigmented *Prevotella*. *P. denticola* is slow to produce pigment; some strains may not produce pigment even after 3 weeks of incubation. There are four more recently described species of saccharolytic, pigmenting *Prevotella* that may be isolated from humans: *P. micans, P. pallens, P. shahii,* and *P. tannerae*. They are all esculin-negative, and α-fucosidase-positive. *P. micans* is very active metabolically among these four, fermenting cellibiose, lactose, salicin and sucrose. *P. shahii* and *P. tannerae* are almost indistinguishable from each other phenotypically. *P. pallens* is indole-positive and does not ferment any sugars, except sucrose as shown in Table 16-14.

A major problem in identifying members of the pigmented *Prevotella* group to species is that they are often fastidious and slow-growing. These bacteria may require anywhere from 2 days to a full 3 weeks to form pigment (on laked rabbit blood or on sheep blood agar). Certain species, in particular *Prevotella intermedia* (and possibly *P. nigrescens*), are of special clinical interest because they commonly produce β-lactamase and may be resistant in vitro to

penicillin G and other antibiotics to which other pigmented gram-negative rods are susceptible.[160,238] Prior to pigmentation, young colonies often exhibit a brick-red fluorescence when examined under long-wave (365 nm) ultraviolet light. In Gram-stained preparations, the cells are short, coccoid, gram-negative rods, usually 0.3 to 0.4 mm in diameter × 0.6 to 1 mm long (Image Plate 16-1). For a presumptive identification of the *Porphyromonas* spp. (Image Plate 16-2A) and the pigmented *Prevotella* spp, all are inhibited by bile, usually (but not always) sensitive by the 2-U penicillin disk test, susceptible to rifampin, and resistant to kanamycin (Table 16-11) Further characteristics for differentiation include those given in Tables 16-13 and 16-14, or may be determined using kit systems for enzymatic activities.[116]

The Nonpigmented *Prevotella* spp. There are over 20 species of nonpigmented *Prevotella,* many of which have only recently been identified, and in some instances, only by using molecular methods. None of these are lipase-positive and only *P. heparinolytica* and some strains of *P. zoogleoformans* are indole-positive. All but *P. disiens* and *P. marshii* ferment lactose. *P. disiens* and *P. bivia* are two very common members of the nonpigmented *Prevotella*. These are similar phenotypically, except *P. bivia* ferments lactose and is esculin, whereas *P. disiens* does not. Similarly, α-

fucosidase and NAG (*N*-acetyl-β-glucosaminidase) are positive for *P. bivia,* in contrast to *P. disiens*. *P. bivia* is found in the urogenital tract and mouth, and *P. disiens* is commonly isolated from the urogenital tract (Table 16-14). Characteristics useful for separating *P. bivia* and *P. disiens* from other *Prevotella* species are given in Table 16-14. In addition to *Prevotella tannerae*, Moore and associates described another species that inhabits the human gingival crevice, *Prevotella enoeca*, and emended the description of *Prevotella zoogleoformans*.[337] Both species have been isolated from patients with periodontitis. Key features that aid in distinguishing *P. enoeca* from other species of *Prevotella* are its inability to digest gelatin, its failure to ferment sucrose and its cellular fatty acid profile. Fermentation of cellobiose and lactose, lack of pigment, and its cellular fatty acid profile, are key characteristics that aid in differentiating *P. zoogleoformans* from other indole-positive *Prevotella* species, especially *P. intermedia* and *P. nigrescens*, which share many phenotypic characteristics, although it lacks pigment.[337] An additional taxonomic change in 1995 was the reclassification of *Hallella seregens* and *Mitsuokella dentalis* as *Prevotella dentalis*.[514] Isolated from dental root canals, the colonies of *P. dentalis* are not pigmented; they differ from those of other *Prevotella* species by having a characteristic "water drop appearance."

Prevotella buccalis, *P. veroralis,* and *B. oralis* were all previously called *B. oralis*. They are all inhibited by 20% bile, are indole-negative, are esculin hydrolysis–positive, and ferment several carbohydrates. If they fail to produce pigment, *P. denticola* and *P. melaninogenica* can be easily confused with *P. buccalis*, *P. veroralis,* and *P. oralis*. However, the latter three species ferment cellobiose; *P. denticola* and *P. melaninogenica* are cellobiose-negative. *P. oris* and *P. buccae* have been isolated from periodontal infections and various other sites of infection in humans. The production of acid from either arabinose separates *P. oris* and *P. buccae* from *P. oralis.* The fermentation of salicin helps to separate *P. buccae* and *P. oris* from *P. buccalis* and *P. veroralis*, which are salicin-negative. *P. buccae* has β-glucosidase activity, whereas *P. oris* does not, which is a feature that aids in separating these species. Reactions obtained using the RapID-ANA II system may also be useful as a supplement to the tests in Table 16-13, to help differentiate these species.[116]

Bacteroides ureolyticus. *Bacteroides ureolyticus* (formerly *B. corrodens*), is a microaerophilic, fastidious, relatively small gram-negative rod (about 0.5 mm in diameter × 1.5 to 2 mm in length) that produces pitting of anaerobic blood agar. The cells do not produce flagella and are nonmotile. Characteristically, the colonies may be of two types: (1) 0.5 to 1 mm diameter after 2 or more days incubation, circular, convex and translucent with entire or erose margins, or (2) thin, flat, spreading, irregular translucent colonies that extend out from a slightly raised central area. These latter colony types produce depressions or "pits" within the surface of agar, resembling to some the "corrosion" of pitted sheet metal. *B. ureolyticus* is inhibited by penicillin

(2-U disk), rifampin (15-mg disk) and kanamycin (1-mg disk), does not grow in the presence of 20% bile and is asaccharolytic. *Campylobacter gracilis*, formerly called *Bacteroides gracilis*, is also microaerophilic, and has growth characteristics similar to those of *B. ureolyticus*. A positive test for urease activity and gelatin and casein hydrolysis separates *B. ureolyticus* from *C. gracilis* and phenotypically similar species of *Campylobacter* and *Wollinella succinogenes* which are all urease-negative. There is presently a proposal to move place *B. ureolyticus* in the genus *Campylobacter* as *C. ureolyticus*.[485] If this occurs, the only remaining *Bacteroides* spp. of clinical relevance would be members of the B. *fragilis* group. The 10th edition of the Manual of Clinical Microbiology now includes *B. ureolyticus* in chapter on *Campylobacter* instead of the anaerobe chapter.[263] *S. wadsworthensis* is an organism similar to *B. ureolyticus* and *Campylobacter gracilis*. It may pit the agar like *B. ureolyticus*, but is urease-negative and may grow in presence of 20% bile. The other *Bacteroides* spp., non-*B. fragilis* group, are encountered only rarely in human clinical specimens. For excellent reviews of their role in infections and their susceptibility to antimicrobial agents, see Kirby et al. and Wexler.[259,507]

***Bilophila* Species.** During a study of bacteria isolated from appendicitis specimens and human feces, a unique new anaerobic gram-negative bacillus was isolated on BBE agar by Baron and associates.[30] This new species was reported to show the characteristics listed in Box 16-4. Although *Bilophila wadsworthia* is similar to species of the *B. fragilis* group and to certain *Fusobacterium* species that grow in the presence of 20% bile, several of the phenotypic properties of *Bilophila wadsworthia* are different from those of the *Bacteroides fragilis* group and *Fusobacterium* spp. *Bilophila wadsworthia* differs from the *B. fragilis* group by its failure to ferment carbohydrates, its production of urease, and its failure to produce a major amount of succinic acid. The production of strong catalase activity and lack of butyric acid production are key characteristics that separate *B. wadsworthia* from *Fusobacterium* species. In addition, *B. wadsworthia* is bile-resistant and on a BBE plate will produce large colonies like

B. fragilis group, but there will be a "black-eyed" appearance, rather than a completely blackening of the colony.[238]

***Fusobacterium* spp.** The genus *Fusobacterium* includes several species of anaerobic, gram-negative, NSF rods that can be differentiated from species of *Bacteroides, Prevotella, Porphyromonas,* and *Leptotrichia* by their production of major amounts of butyric acid but no isobutyric or isovaleric acid (Table 16-15) (Chart 16-10). The

species of *Bacteroides* and *Porphyromonas* that produce butyric acid also produce isobutyric and isovaleric acids. Although the name *Fusobacterium* would seem to suggest that all of the fusobacteria are "fusiform" or "spindle-shaped," only a few of them have this morphology. Although *F. nucleatum* is fusiform, the majority of other species are rod-shaped with parallel sides and rounded (not pointed) ends (Image Plate 16-1E and 16-1G). Additionally, some have a very bizarre and pleomorphic appearance on Gram's stain. While they grow well on anaerobic blood agar under anaerobic conditions, they are readily killed by exposure to ambient air. Most species are either nonfermentative or only weakly fermentative.[238,263] They are resistant to the vancomycin antibiotic potency disk, but susceptible to colistin and kanamycin. Most species are bile-susceptible; however, *F. mortiferum, F. ulcerans,* and *F. varium* can grow on BBE agar.[238]

The most common and well-known species of *Fusobacterium* is *F. nucleatum*. As mentioned, in the Gram's stain, it is gram-negative and the cells are spindle-shaped, long, and slender with tapered ends (Image Plate 16-1).[238,263,313] There are also spherical swellings found in the cells. Cells are usually 5 to 10 μm long, but shorter forms are also often seen. Colonies on anaerobe blood agar are 1 to 2 mm in diameter, slightly convex with slightly irregular margins, and have a characteristic internal flecking referred to as crystalline internal structures; some describe *F. nucleatum* colonies as "breadcrumb-like" (Image Plate 16-1F). Biochemically, *F. nucleatum* is relatively inactive (Table 16-15).

The cells of *F. necrophorum* measure about 0.6 × 5 μm, and are pleomorphic, often with curved forms and spherical areas within the cells (Image Plate 16-1G). Like *F. mortiferum* (discussed below), they also produce free coccoid bodies which, on direct examination, sometimes resemble degenerated leukocytes (i.e., neutrophilic debris). *F. necrophorum* has been divided into at least two subspecies: *F. necrophorum* subsp. *fundiliforme*, the more pathogenic strain and *F. necrophorum* subsp. *necrophorum*. Colonies are yellow to cream colored, round and smooth with entire edges (Image Plate 16-1H). They may fluoresce chartreuse under UV light like *F. nucleatum*, and they may green the blood agar. Both are usually lipase-positive on EYA; however, the reaction is weaker with subspecies *fundiliforme*. *F. necrophorum* can be occasionally misidentified as *F. nucleatum*; evaluations of the commercial anaerobic kit systems have included very few isolates of *F. necrophorum* to judge their performance. Initial studies with molecular techniques are still pending, so accurate identification can be difficult. If GLC is performed, butyric acid will be predominant, but that is true of the genus *Fusobacterium* and does not help with species level differentiation.[95,238] The cells of *F. mortiferum* are 0.5 to 2 μm wide by 2 to 10 μm long, highly pleomorphic, coccoid to filamentous, with spherical swellings near the center or one end of unevenly stained rods. Colonies on blood agar are 1 to 2 mm in diameter and have a distinctive fried-egg appearance, with raised, opaque centers and a flat, translucent margin (Table

16-15).

Other *Fusobacterium* species may be encountered rarely in infections. *F. ulcerans* is a rare isolate responsible for tropical ulcers, and resembles *F. varium* on Gram's stain. *F. varium*, however, is usually indole-positive and can be lipase-positive, whereas *F. ulcerans* is negative for both of these reactions.[98,238,263] For additional characteristics of these and other species, the following references are recommended.[95,98,238,263,302]

The differential characteristics of many other anaerobic gram-negative bacilli are shown in Table 16-16. References are included for many of the organisms for your interest in Table 16-2.

Identification of the Anaerobic NSF Gram-Positive Bacilli

Included in this group of anaerobes are species of the genera *Actinomyces, Arcanobacterium, Bifidobacterium, Eggerthella, Eubacterium, Lactobacillus, Propionibacterium, Pseudoramibacter,* and many others (Chart 16-11). An extensive list of recent changes in the taxonomy of these bacteria is provided in Table 16-2. Some key differential characteristics of the genera of NSF, gram-positive bacilli are given in Table 16-10. The microscopic morphology and colonial characteristics of *Actinomyces israelii* and *Eggerthella lenta* (formerly *Eubacterium lentum*) are shown in Image Plate 16-2 (Image Plate 16-2E and 16-2F).

Identification of the anaerobic, gram-positive NSF bacilli requires the use of GLC for metabolic product analysis and/or newer identification methods including sequencing and MALDI-TOF. Cellular morphology of many of these organisms tends to vary with the type of culture medium and growth conditions. On morphologic grounds alone, these bacteria can sometimes be confused with several other genera, including *Clostridium, Corynebacterium, Lactobacillus, Leptotrichia, Listeria, Nocardia, Peptostreptococcus,* and *Streptococcus*. Thus, GLC results and morphologic characteristics, considered together, aid in practical differentiation. At times, some strains of anaerobic bacilli resemble cocci, particularly in Gram-stained preparations of young colonies on blood agar. In addition, some streptococci, such *as S. mutans, S. intermedius, S. constellatus* and *Gemella morbillorum* and certain anaerobic gram-positive cocci, may appear rod-shaped when cells from colonies on blood agar are examined microscopically. Conversely, these latter bacteria usually form long chains of cells in enriched thioglycolate broth and other liquid media. It should be remembered that many gram-positive bacteria tend to stain in a gram-negative manner as they age. Also, some clostridia (e.g., *C. perfringens, C. ramosum* and *C. clostridioforme*) fail to produce spores in media routinely used in the clinical laboratory, whereas other clostridia do so as they age. Thus, Gram-stained preparations of very young cultures may aid in demonstration of gram-variability, and observation of smears from older cultures may aid in demonstrating spores of clostridia.

***Actinomyces* spp.** As discussed in the section on human infections with *Actinomyces* spp., *A. israelii* has long been associated with thoracic, abdominal, and pelvic actinomycosis. In Gram-stained smears prepared from

lesions, one may observe characteristic sulfur granules, which are granular microcolonies of the organism surrounded by purulent exudate. There are many

species of *Actinomyces,* as well as *Propionibacterium propionicum,* that are recognized as potential causes of actinomycosis and may produce sulfur granules (Chart 16-11). The cells of *A. israelii* are gram-positive rods, usually 1 μm in diameter, but they are extremely variable in length. The cells may be short diphtheroid rods, club-shaped, branched, or unbranched filaments (Image Plate 16-2). Rough colonies composed of branched rods or filaments usually develop slowly on blood agar. Young colonies (2 to 3 days old), when viewed with the dissecting microscope, appear as thin radiating filaments known as spider colonies. When the colonies are from 7 to 14 days old, they are often raised, heaped-up, white, opaque and glistening; the colonies have irregular or lobate margins, and are sometimes called "molar tooth" colonies (Image Plate 16-2F). However, smooth strains (about one-third of *A. israelii*) produce colonies more rapidly than rough strains. Smooth strains may produce 1- to 2-mm, circular, slightly raised white, opaque, smooth, glistening colonies after only 2 to 3 days of incubation. *A. naeslundii* may also produce smooth or rough colonies. Colonies of *A. viscosus* are most often 0.5 to 2 mm in diameter, entire, convex, grayish, and translucent. In *A. odontolyticus* colonies, a red color may develop on blood agar after 7 to 14 days of anaerobic incubation or after the plates have been left out in room air at ambient temperature for several days. As mentioned previously, cellular and colony characteristics of *Propionibacterium propionicum* are similar to those of the other *Actinomyces*. There is now a long list of other species of *Actinomyces*, as can be seen in Table 16-2, 16-17 and 16-18. Some of the species demonstrate the branching filaments on Gram's stain that are characteristically recognized as *Actinomyces* sp.; others appear more diphtheroidal and can be confused with *Corynebacterium* spp. Some species are quite aerotolerant, in some instances appearing more like aerobes than anaerobes. Most *Actinomyces* spp. are metronidazole-resistant, but highly penicillin susceptible, as well as being susceptible to many other anaerobic antibiotics.

Besides morphology, colony characteristics and metabolic products, characteristics useful for identification of the actinomycetes include relationship to oxygen, appearance and rapidity of growth in enriched thioglycolate medium, indole production, esculin and gelatin hydrolysis, fermentation of certain carbohydrates, results of testing with several rapid enzyme tests, 16S rRNA sequencing and other molecular methods. For further information relative to characterization and identification of isolates beyond that given in Tables 16-17 and 16-18, the following references are recommended.[99,238,414,499]

TABLE 16-17 Morphology of *Actinomyces, Propionibacterium,* and Related Species

Species	Gram's Stain Morphology	Colonial Morphology	Normal Flora/Clinical Relevance
***Actinomyces* spp.**			
A. cardiffensis	Pleomorphic, slender, straight to curved rods; beaded, branching filaments occurs	Pinpoint, convex, smooth colonies; opaque; cream to pinkish; nonhemolytic	Many clinical specimens; bacteremia; pulmonary infections
A. dentalis	Filamentous, beaded rod-shaped cells	Tiny, white, bread-crumb-like; can pit the agar	Oral cavity; dental abscesses
A. europaeus	Short rods; no filaments or branching	Smooth, translucent gray colonies; slightly β-hemolytic	Breast abscesses; UTI
A. funkei	Slender, curved rods with some branching	Small gray, nonhemolytic colonies; appear like "fried-egg" with age	Endocarditis
A. georgiae	Short rods that may have swellings; branching is seldom seen	1 mm colonies; circular, convex, entire; translucent; white, shiny and smooth; some strains may be tan	Gingival crevices; Endocarditis
A. gerencseriae	Short rod; filamentous; branches or beads	Difference strains produce pleomorphic colony types: Circular, white; smaller colonies are translucent or transparent; irregular opaque; "lumpy."	Cervicofacial, thoracic, & abdominal-pelvic actinomycosis
A. gravenitzii	Short rods; some swollen ends; some branching	Opaque colonies; may adhere to agar; may form molar tooth colonies like *A. israelii;* may produce pinkish pigment on laked rabbit blood agar	Cervicofacial, thoracic, & abdominal-pelvic actinomycosis
A. hongkongensis	Straight rods	Nonhemolytic, pinpoint colonies	Isolated from case of pelvic actinomycosis
A. israelii	Short rods; characteristic branching; pleomorphic	White, opaque; "molar tooth" colonies	GU tract; pulmonary; all forms of actinomycosis; pericarditis; IUD-association infections
A. johnsonii	Short rods; branching or beading	Gray-white; translucent	Gingival crevices
A. massiliensis	Straight rods	Circular, white, shiny; pinpoint at 48 hours	Bacteremia

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A. meyeri	Short rod; branching or beading with some swollen cells; clubbing	White colonies; strict anaerobe	Pulmonary; osteomyelitis; abscesses; pericarditis
A. naeslundii	Short rods; branching or beading	Gray-white; translucent	Oral flora; bacteremia; IUD-associated infections
A. nasicola	Branching rods; pleomorphic; some coccoid forms	Pinpoint, white, or gray; opaque, shiny, convex, and entire	Isolated from pus in the nose of patient during polypectomy
A. neuii	Diphtheroids and coccoid forms; in clusters or "X" and "Y" forms	Circular, smooth, convex; opaque, white, entire edge; subsp. *neuii* is α-hemolytic, but subsp. *anitratus* is nonhemolytic.	IUD-associated infections; abscesses; soft tissue infections; endophthalmitis; endocarditis
A. odontolyticus	Short rods; branching filaments or beading	Smooth and granular colonies; may have orange-reddish pigment	Oral flora; bacteremia; IUD-associated infections; soft tissue infections in head and neck
A. oricola	Rods with branching filaments	Pinpoint, bread-crumb-like; white; nonhemolytic	Dental abscess
A. oris	Short rods with clubbed ends	Smooth to granular surface with an irregular edge	Oral
A. radicidentis	Coccoid	Smooth and brown pigmented	Oral; isolated from infected root canals
A. radingae	Coccoid; pseudobranching	Small gray convex colony; butyrous; α-hemolytic	Abscesses; IUD-associated infections
A. turicensis	Straight to curved rods; pseudobranching; gram-variable	Glistening; opaque; butyrous; originally described as aerotolerant	Abscesses; IUD-associated infections
A. urogenitalis	Slightly curved rods	Smooth, nonadherent possibly with reddish pigment	Isolated from GU tract; pelvic actinomycosis; bacteremia
A.viscosus	Short diphtheroidal rods with branching and beading	Circular, convex, smooth; entire; creamy white; soft to mucoid; opaque	Oral organism; involved in cases of actinomycosis
***Actinobaculum* spp.**			
A. massilae	Straight or slightly curved rods; may branch singly or in clusters	1 mm nonhemolytic colonies	UTI and bacteremia
A. schaali	Straight to slightly curved rods; some branching	Circular with irregular margins	UTI; endocarditis
A. urinale	Straight to slightly curved rods; no branching seen	Small colonies (<1 mm), convex, smooth, entire edge; gray or white; weakly β-hemolytic	Isolated from urine
Varibaculum cambriense	Short, straight or slightly curved rods; diphtheroidal	Nonhemolytic, translucent colonies that are gray-to-white, pinpoint, convex and entire	Skin & soft tissue infections; IUD-associated
***Propionibacterium* spp.**			
P. acnes	Pleomorphic short rods; diphtheroidal; some may appear club-shaped; some may appear to branch; the term *anaerobic diphtheroids* is often used to describe them.	1–2 mm in diameter early on, becoming larger with age; circular, convex, white to gray-white, opaque, with an entire edge and a butyrous consistency	Skin flora; bacteremia; endocarditis; post–cataract-surgery endophthalmitis; shunt infections; implant infections, esp. shoulder implants
P. avidum	Not well described	Similar to *P. acnes*; β-hemolytic	Cutaneous abscess
P. granulosum	Not well described	White to gray colonies that are smooth and entire	Rarely, endocarditis
P. propionicum	Pleomorphic rods; may branch and look like *Actinomyces* spp.	Colonies similar to *A. israelii*	Cervicofacial, thoracic, & abdominal-pelvic actinomycosis; lacrimal canaliculitis
Propionimicrobium lymphophilum	Pleomorphic; diphtheroid-like; singly, pairs or short chains or clumps; may form "X" and "Y" forms.	Small white colonies; punctate, circular, convex; glistening with age	Initial clinical isolate was from a lymph node

Adapted from a variety of references including 178, 238, 477, and 499.

TABLE 16-18 Characteristics of *Actinomyces* spp. and Related Genera

Species	AERO	CAT	URE	ESC	NIT	Fermentation of MANN	RAFF	NAG	PIG	Branching
***Actinomyces* spp.**										
A. cardiffensis	(+)	–	–	–	V	–	V	–	–	+
A. dentalis	–	–	–	+	–	–	+	–	–	–
A. europaeus	+	–	–	V	V	–	–	–	–	–
A. funkei	+	–	–	–	+	–	–	V	–	+ (some)
A. georgiae	+	–	–	+	V	–	–	–	–	NA
A. gerencseriae	+	–	–	+	V	V	V	–	–	+
A. gravenitzii	+	–	–	–	V	–	V	+	+[a]	+ (some)
A. hongkongensis	–	–	–	–	–	–	–	–	–	NA
A. israelii	(+)	–	–	+	+	–	+	–	–	+
A. johnsonii	+	V	V	–	+	NA	NA	NA	–	
A. massiliensis	+	–	–	NA	+	–	–	–	–	–
A. meyeri	–	–	–	–	V	–	–	+	–	+
A. naeslundii	+	–	+	V	+	–	+	–	–	+
A. nasicola	(+)	–	–	–	–	–	–	+	–	NA
A. neuii	+	+	–	–	V	+	V	–	–	–
A. odontolyticus	+	–	–	V	+	–	–	–	+	+
A. oricola	+	–	–	+	–	–	+	–	–	NA
A. oris	+	V	–	V	+	NA	NA	NA	–	NA
A. radicidentis	+	+	V	+	V	+	+	–	+	NA
A. radingae	+	–	–	+	V	–	V	+	–	Pseudobranching
A. turicensis	+	–	–	–	–	–	V	–	–	Pseudobranching
A.urogenitalis	+	–	–	+	+	–	–	+	+	
A. viscosus	+	+	V	V	+	V	+	–	–	+
***Actinobaculum* spp.**										
A. massiliense	+	–	–	–	–	–	+	–	–	May branch
A. schaali	+	–	–	–	–	–	–	–	–	May branch
A. urinale	(+)	–	+	–	–	–	–	–	–	May branch
Varibaculum cambriense	(+)	–	–	–	+	V	–	–	–	Short rods

[a] *A. graevenitzii* produces brown colonies on RLB, but no pigment on Brucella blood agar; other *Actinomyces* that pigment on Brucella agar produce pinkish/brownish colonies and on rabbit laked blood agar (RLB), darker colonies.
(+), the organism is aerotolerant, but grows better anaerobically; +, positive reaction; −, negative reaction; V, some strains positive and some negative; w, weak reaction; NA, data not available; AERO, aerotolerant, grows in CO_2 incubator; CAT, catalase; URE, urease; ESC, esculin hydrolysis; NIT, nitrate reduction; MANN, fermentation of mannitol; RAFF, fermentation of raffinose; NAG, β-*N*-acetyl-glucosaminidase; PIG, pigment production; usually seen as pink-brown colonies on Brucella blood agar.
Adapted from references 178, 238, and 499.

***Propionibacterium* spp. and Related Genera.** *Propionibacterium acnes* is by far the most common gram-positive, NSF anaerobic rod encountered in clinical specimens. *P. avidum* and *P. granulosum* are seldom encountered in the clinical laboratory, and usually are not clinically significant. The cells of *P. acnes* usually measure 0.3 to 1.3 µm in diameter × 1 to 10 µm in length.[238] Their morphology has often been described as diphtheroidal or in appearance. The cells are markedly pleomorphic and occur in varying shapes and sizes, ranging from coccoid to definite rods. Cells are often unevenly stained by Gram's procedure. Like the corynebacteria, the cells reveal "Chinese letters," "birds-in-flight," and "picket-fence" arrangements, presumably because of "snapping" after they divide. *P. acnes* typically grows as an obligate anaerobe on primary isolation. Although some strains show growth when incubated in a CO_2 incubator, they usually grow better anaerobically, and hence have been described as aerotolerant or microaerophilic. Colonies of *P. acnes* on anaerobe blood agar are 1 to 2 mm in diameter, circular, entire, convex, glistening and opaque. Some strains produce a narrow zone of hemolysis. Analysis of short-chain acid metabolic products by GLC reveals the production of a major amount of propionic acid, a smaller amount of acetic acid, and only a minor amount of isovaleric acid [abbreviated aP (iv)]. *P. acnes* can be recognized without the use of GLC when it produces both indole and catalase. *Actinomyces viscosus*, which has a similar morphology on some media, produces catalase (Chart 16-11).

***Propionibacterium propionicum*.** In 1988, *Arachnia propionica* was reclassified as *Propionibacterium propionicum*.[89] Previously, the name *Arachnia propionica* had been created to accommodate the bacteria once called *Actinomyces propionicus*. This species, while its pathogenicity and morphologic characteristics resemble those of certain species of *Actinomyces*, differs from *Actinomyces* spp. by producing propionic acid as a major metabolic product, by its cell wall murein content and menaquinone composition that resemble those of *Propionibacterium* spp. *P. propionicum* is morphologically indistinguishable, in tissue and in culture, from *Actinomyces israelii*. Both *P. propionicum* and *A. israelii* form pleomorphic "diphtheroidal" rods and long, branched, filaments (described in more detail in the section on *Actinomyces* spp.). Both species are microaerophilic to anaerobic and grow optimally under anaerobic conditions. Tables 16-17 and 16-19 provide specific information on the characteristics of *Propionibacterium* spp. and the clinical relevance of selected species.

***Eubacterium* spp., *Eggerthella* spp., and Related Genera.** Most *Eubacterium* spp. and related genera produce small gray colonies on anaerobic blood agar, often requiring ≥48 hours for visible growth to be detected (Image Plate 16-2H). Strains of *Eggerthella lenta* are biochemically quite inert small rods without any branching; they are arginine dihydrolase positive and alkaline phosphatase-negative; catalase-positive and nitrate-positive, but usually urease- and indole-negative; they are sensitive to

kanamycin and vancomycin, but resistant to colistin on antibiotic disk tests (Chart 16-11). *Eubacterium* spp. are similar, but can be spot indole-positive and are catalase- and nitrate reduction-negative (Image Plate 16-2G). *Paraeggerthella hongkongensis* (i.e., the new nomenclature of what previously was *Eggerthella hongkongensis*) and *E. sinensis* are similar to *E. lenta* but nitrate negative. There are some data about using API 20A or Rapid ID 32 tests for identification of these organisms.[280] Definitive identification requires nucleic acid sequencing or MALDI-TOF assessment. Table 16-20 provides information on the phenotypic differentiation of these organisms.

***Bifidobacterium* spp. and Related Genera.** Currently, more than 30 species of the genus *Bifidobacterium* are recognized taxonomically (Chart 16-11). Recent changes in this group include the transfer of *B. inopinatum* to *Scardovia* as *Scardovia inopinata*, and the transfer of *B. denticolens* to *Parascardovia* as *Parascardovia denticolens.*[233] The morphology of *B. dentium* is somewhat similar to that of *Actinomyces*, but it differs in not producing branched filaments in thioglycolate medium. Gram-stained smears prepared from solid media or broth cultures show gram-positive diphtheroidal forms that are much more variable in size and shape than *P. acnes*. Cells vary from coccoid forms to long, often curved forms, with characteristic swollen ends and/or bifid forms that are regularly produced by *B. dentium*. Using the antibiotic potency disks, most of the *Bifidobacterium* and related genera are susceptible to kanamycin and vancomycin, but resistant to colistin. *Alloscardovia omnicolens* are catalase-negative, arginine dihydrolase-negative, nitrate-negative, but do ferment glucose and a number of other sugars with exception of rhamnose, sorbitol and mannose.[227] On GLC analysis of short-chain acid metabolic products, the bifidobacteria produce major amounts of acetic and lactic acids. For more information on the species of this group and their phenotypic characteristics, refer to Table 16-2 and 16-20.

TABLE 16-19 Characteristics of *Propionibacterium* spp. and *Propionimicrobium lymphophilum*

Species	Aerotolerance	CAT	IND	NIT	ESC	Comments
P. acidifaciens	–	–	–	–	–	Pleomorphic rods; colonies are circular, convex to domes, opaque, white to cream colored; nontranslucent internal appearance
P. acnes	+	+	+	+	–	Short rods; diphtheroidal
P. avidum	+	+	–	–	+	Pleomorphic and irregular rods; may have slight branching
P. granulosum	+	+	–	–	–	Pleomorphic rods, with slight branching; colonies gray and creamy
P. propionicum	–	–	–	+	–	Can see branching on Gram's stain; resembles *A. israelii* in colonial and microscopic morphology
Propionimicrobium lymphophilum[a]	–	V	–	+	–	Small colonies; circular, convex, white, and glistening; pleomorphic rods, can be diphtheroidal or club-shaped and some coccoid; arranged singly, in pairs and chains; may form X and Y forms

[a] *P. lymphophilum* may grow weakly on 20% bile agar; it is a fermenter.
+, positive reaction; −, negative reaction; V, variable reaction; CAT, catalase; IND, indole production; NIT, nitrate reduction; ESC, esculin hydrolysis.
Modified from references 178, 238, and, 499, and others as indicated in Table 16-2 for each organism.

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TABLE 16-20 Characteristics of *Bifidobacterium* spp. and Related Species, *Eubacterium* spp., and *Eggerthella* spp. and *Eubacterium*-Like Species

Species	AERO	CAT	IND	NIT	ESC	ADH	GLC Products	Prior Designation	Morphology
Bifidobacterium spp.	−/+	−	−	−	+	−	A,L		Rods with some bifurcation or "fork-like" branching; usually larger in diameter than cells of *Actinomyces* spp. or *Propionibacterium* spp.
B. scardovii	+	−	−	−	+	−	A,L	None	Rod with some curving; colonies are convex, shiny, with dense white centers
Alloscardovia omnicolens	−[a]	−	−	−	+	−	A,L	New genus and species	Short, irregular rods singly or pairs; with rudimentary branching; colonies are small
Parascardovia denticolens	−	−	−	−	NA	NA	A,L	*Bifidobacterium denticolens*	Slender rods; some may branch; some "V" forms colonies are smooth, Convex, irregular edges; cream-white
Scardovia inopinata	−	−	−	−	NA	−	A,L	*Bifidobacterium inopinatum*	Small coccoid cells, singly and in pairs; colonies are smooth, convex, irregular edges; cream-white
Eubacterium spp.	−	−	V	−+	NA	V	A or B,L or AB or A,B,L		Variable: straight rods to coccobacillus; some may branch. Many different colonial morphologies
Eggerthella. lenta[b]		+	−	+	−	+	a(l,s)	*Eubacterium lentum*	Pleomorphic bacilli; speckled colonies that fluoresce red
Eggerthella. sinensis		+	−	−	NA	+	NA	New species	
Paraeggeggerthella hongkongensis		+	−	−	NA	+	NA	*Eggerthella hongkongensis*	

[a] CO_2 enhances growth anaerobically; no growth aerobically.
[b] H_2S +; can grow in presence of 20% bile.
Most of the *Bifidobacterium* spp. are glucose fermenters; most of the *Eubacterium* spp. are non–glucose-fermenters.
+, positive reactions; −, negative reactions; −+, most species are negative, although an occasion species might be positive; NA, data is not available; AERO, growth under aerobic conditions; CAT, catalase; IND, Indole production; NIT, nitrate reduction; ESC, esculin hydrolysis; ADH, arginine hydrolysis.
Adapted from references 178, 238, and 499.

***Lactobacillus* spp.** Although the majority of *Lactobacillus* isolates are not strictly anaerobic, many grow better anaerobically. Clues that aid in the recognition of lactobacilli include their growth on Rogosa's selective tomato juice agar at relatively low pH (Becton Dickinson Microbiology Systems), their negative catalase reaction, their tendency to form chains of relatively uniform gram-positive rods, and their production of lactic acid as a major product with only a small amount of acetate or succinate (detected using GLC). The biochemical characteristics of some of the *Lactobacillus*-like organisms can be found in Table 16-21. However, the identification of most of the *Lactobacillus* spp. and related organisms are beyond a presumptive level is difficult and beyond the scope of this textbook. It is noteworthy that many of the lactobacilli are resistant to vancomycin. For additional information on identification and the susceptibility of *Lactobacillus* species to antimicrobial agents, see Chapter 17.

***Mobiluncus* spp.** *Mobiluncus* spp. grow on several kinds of nonselective plating media, including anaerobe blood agar (e.g., brain–heart infusion, Brucella agar, and

CDC anaerobe blood agar–based media) and chocolate agar. After 3 to 5 days of incubation, colonies are 2 to 4 mm in diameter, colorless, translucent, smooth, and flat, sometimes with a spreading appearance. The cells are less than 0.5 µm in width by about 1.5 to 3 µm in length. Succinate and acetate are major products (with or without minor amounts of lactate) after growth in peptone yeast extract broth supplemented with glycogen and 2% rabbit serum. Differentiation between the two currently recognized species, *M. curtisii* and *M. mulieris*, based on differences in morphology, negative reactions for indole and catalase, growth in the presence of arginine, hippurate hydrolysis, variable nitrate reduction and other characteristics is difficult.[238,456]

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TABLE 16-21 Characteristics of *Lactobacillus* spp. and Related Genera

Species	AERO	CAT	IND	NIT	ESC	ADH	Acid Phospatase	β-GAL	FERM	GLC	MORPH	Prior Designation
Lactobacillus spp.	V	–	–	$-^{+}$	$+^{-}$	V	V	V	+	L,a,s	Variable, but usually rods with straight sides; chains of rods common; no branching	
Olsenella uli	–	–	–	–	+	+	NA	NA	+	L	Small elliptical rods, singly, pairs or short chains; colonies are 1–2 mm, translucent to transparent, convex and entire.	*Lactobacillus uli*
O. profusa	–	–	–	–	+	–	NA	NA	+	L	Small elliptical rods, singly, pair and short chains; circular, entire, cream colored and opaque.	*Lactobacillus profusa*
Atopobium minutum[a]	–	–	–	–	ND	+	–	–	+	a,L,s	Small, elliptical rods, singly or in short chains; pinpoint colonies, convex, entire, gray, white, or yellow.	*Lactobacillus minutus*
A. parvulum	–	–	–	–	V	–	+	+	+	a,L,s	Small cocci singly, in pairs or short chains; some may have swellings; pinpoint colonies, raised, convex, entire edges' may be white or gray	*Streptococcus parvulus*
A. rimae	–	–	–	–	V	V	+	–	+	a,L,s	Small, elliptical rods which may have central swellings singly or in pairs or short chains; small colonies, convex, entire, translucent or transparent.	*Lactobacillus rimae*
A. vaginae[b]	–	–	–	–	–	+	+	–	+	a,L,s	Very small, elliptical cells singly, pairs or in short chains; colonies are very small.	New species

[a] *A. minutum* has been shown to carry a vancomycin resistance gene[166e]
[b] *A. vaginae* was initially described as a facultative anaerobe[198d]
+, positive reaction; −, negative reaction; $+^{-}$, most strains are positive, but may have occasional negative reactions; $-^{+}$, most strains are negative, but an occasional strain may be positive; a, minor acetic acid peak; L, major lactic acid peak; s, minor succinic acid peak; V, variable reactions among the species; AERO, ability to grow aerobically; CAT, catalase; IND, indole production; NIT, nitrate reduction; ESC, esculin hydrolysis; ADH, arginine dihydrolase; FERM, fermentative; β-GAL, β-galactosidase.
Adapted from references 178, 238, and 499.

Other Anaerobic NSF Gram-Positive Bacilli. Table 16-2 lists many additional changes in taxonomy and the new genera of NSF anaerobic gram-positive rods that have been described. Table 16-22 provides some phenotypic and morphologic information on these organisms, but definitive identification of the gram-positive NSF bacilli can be quite difficult and requires either nucleic acid sequencing or a combination of morphology, phenotypic characterization and GLC. The only way to assess their clinical significance, however, will be to correctly identify them, correlate the culture results with the clinical and pathologic findings and publish the findings. At this point, it is important to recognize these at least at the genus level, if possible, in routine clinical laboratories and to refer isolates that are thought to be clinically relevant to specialized labs for definitive identification.

Identification of Clostridium *Species*

Overview. The anaerobic gram-positive, spore-forming bacilli encountered in human clinical materials are members of the genus *Clostridium*. In 1994, Collins and associates,[104] on the basis of 16S rRNA gene sequences, found that the genus *Clostridium* is extremely heterogeneous. Thus, Collins et al. named five new genera of spore-forming rods.[104] Included were the following: *Caloramator, Filifactor*, *Moorella, Oxobacter,* and *Oxalophagus* species. In addition, 11 new species combinations were proposed. None of these new genera and species designations were relevant to infectious diseases of humans. Newly named clinically relevant species at the time of this writing include *Clostridium bartlettii*,[452] *C. bolteae*,[451] *C. hathewayi,*[461] and *C. neonatale*.[7] The original manuscripts should be consulted for information on their clinical significance.

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TABLE 16-22 Characteristics of Other Anaerobic Gram-Positive, Non–Spore-Forming Bacilli

Species	Site in Body or Clinical Relevance	CAT	IND	NIT	ESC	ADH	FERM	Clues to ID	GS and Colonial Morphology	Prior Designation
Anaerofustis stercorihominis	GI microbiota	–	NA	NA	NA	NA	+	Resistant to 20% bile; GLC = A, B	Thin rods in short chains	New genus and species
Anaerotruncus colihominis	GI microbiota; a case of bacteremia		+	–	–	+	+		Thin rods	New genus and species
Bulleidia extructa	Oral cavity; prosthetic hip infections	–	–	–	–	+	+	Stimulated by Tween	Short slightly curved rods singly and in pairs	New genus and species
Catabacter hongkongensis	GI microbiota; bacteremia cases	+	–	–	v	–	+	Motile; sensitive to kanamycin	Short rods or coccobacillus	New genus and species
Catenibacterium mitsuokai	GI microbiota	–	NA	–	–	NA	+	+	Short rods; long tangled chains	New genus and species
Colinsella aerofaciens	GI microbiota	–	–	–	V	V	+	Stimulated by Tween; H_2S +	Coccoid to short rods in chains	*Eubacterium aerofaciens*
Colinsella intestinalis	GI microbiota	NA	NA	NA	NA	NA	+	NAG + *	Rods in chains; white or gray colonies in center with clear margins	New species
Colinsella stercoris	GI microbiota	NA	NA	NA	NA	NA	+	NAG +	Rods in chains; white or gray colonies in center with clear margins	New species
Cryptobacterium curtum	Oral microbiota; chronic periodontitis	–	–	–	–	+	–	May decolorize with age	Short rods; diphtheroidal	*Eubacterium curtum*
Dorea formicigenerans	GI microbiota	–	–	–	–	NA	+	Produces H_2S; growth inhibited by 6.5% salt; GLC = a, F, I	Short or long rods in pairs and chains; colonies are circular, irregular, convex, opaque; white to tan and shiny, smooth	*Eubacterium formicigenerans*
D. longicatana	GI microbiota	–	–	–	+	NA	+	May decolorize with age	Short or long rods in pairs and long chains; opaques colonies that are circular, convex, entire, smooth and sticky	
Flavonifractor plautii	GI microbiota; isolates from blood, abdominal wounds; soft tissue infections	ND	V	–	+	–	+[w]	May become gram-negative with age; variably motile; variably H_2S +; reduced susceptibility to vancomycin; GLC = A,B	Straight or slightly curved rods singly or in pairs; small colonies, circular, convex, gray or white, smooth	*Eubacterium plautii* and some strains of *Clostridium orbiscindens.*
Holdemania filiformis	GI microbiota	–	–	–	+	–	+	Stimulated by Tween; may be resistant to 20% bile; GLC = A,L,S	Short to longer cells; may have swellings in cells; arranged in pairs and chains; colonies are circular, convex, granular	New genus and species; *Eubacterium*-like
Mobiluncus curtisii	Vagina; Associated with Bacterial Vaginosis	–	–	–	–	+	+	In fresh preparations, these have a corkscrew motility. GLC = S, L, A	Curved, gram-variable bacilli	

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Mogibacterium spp.		–	–	–	–	–	–	May decolorize with age	Rod-shaped	
Mogibacterium timidum	Oral; periodontal infections	–	–	–	–	–	–	Growth is very slow with tiny colonies even after 5 days	Short rods; diphteroidal, in clumps; colonies are round, white or cream; entire; rough appearance	*Eubacterium timidum*
Moryella indoligenes	GI microbiota probably; isolated from abdominal abscess	–	+	–	–	NA	+[w]	Indole +	"Warped" cells, singly and pairs and in short chains; nonpigmented and non-hemolytic colonies	New genus and species
Pseudoramibacter alactolyticus	Oral	–	–	–	–	–	+	F,a,B	Rods in pairs resembling flying birds, clumps, or Chinese characters; colonies are punctuate, circular, smooth or shiny; translucent and opaque colony types	*Eubacterium alactolyticum*
Roseburia spp.	GI microbiota	–	NA	NA	+	NA	+	Produces H_2S; gram-variable; motile	Thin pleomorphic rods; translucent colonies with entire edges	New genus and species
Shuttleworthia satelles	Oral cavity; endocarditis case	–	+	–	+	+	+	GLC = A,B,L	Short or slightly curved; diphtheroidal in pairs and short chains; very variable colonial morphologies; many with satellite colonies	New genus and species
Slackia exigua	Oral; periodontitis	–	–	–	–	+	–	Growth stimulated by 0.5% arginine; may stain gram-negative with age	Coccobacilli or short rods, singly or in clumps; colonies are circular, convex, translucent	*Eubacterium exiguum*
Solobacterium moorei	GI microbiota; bacteremia cases; wound infections	–	–	–	+	+	+		Straight or slightly curved rods, singly or in pairs	New genus and species
Turicibacter sanguinis	GI microbiota; blood culture isolate	–	–	–			+		Irregular long rods in long chains; colonies are gray-white, convex, undulating margins	New genus and species

*PCR primers sets have been described that effectively differentiate *C. intestinalis* from *C. stercoris*.[244]
+, positive reaction; –, negative reaction; +⁻, most strains are positive, but may have occasional negative reactions; –⁺, most strains are negative, but an occasional strain may be positive; V, variable reactions among the species; w, weak reactions; CAT, catalase; IND, indole production; NIT, nitrate reduction; ESC, esculin hydrolysis; ADH, arginine dihydrolase; FERM, fermentative; GS, Gram's stain.
Adapted from references 178, 238, 499.

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Currently, species of *Clostridium* encountered clinically vary in their relationships to oxygen and in their anabolic and catabolic physiologic activities (Chart 16-12). Certain clostridia, for example *C. haemolyticum* and *C. novyi* type B, are among the strictest of obligate anaerobes. At the other end of the spectrum, *C. histolyticum, C. tertium* and *C. carnis* are aerotolerant, and form colonies on anaerobe blood agar plates incubated in a candle jar or in a 5% to 10% CO_2 incubator. In the clinical laboratory the problem sometimes arises of determining whether an isolate is an aerotolerant *Clostridium* or a facultatively anaerobic *Bacillus*. Aerotolerant clostridia rarely form spores when grown aerobically and are catalase-negative, whereas species of the genus *Bacillus* rarely form spores when grown under anaerobic conditions, and they produce catalase.[238,464] Although the clostridia are considered gram-positive, many stain gram-negative by the time smears of growing cultures are prepared. For example, *Clostridium ramosum, C. symbiosum,* and *C. clostridioforme* group are usually stained gram-negative.

The demonstration of spores is frequently difficult with some species, such as *C. perfringens, C. ramosum,* and *C. clostridioforme* group. In those species in which spores are usually present, Gram-stained preparations are usually sufficient; special spore stains generally offer no particular advantage. In order to enhance spore

formation, a heat shock or alcohol spore selection technique may be used.[238,264] Identifying characteristics for most of the clostridia encountered in human infections are given in Tables 16-23 and 16-24.

C. perfringens. Some of the key reactions for identifying *C. perfringens* are illustrated in Image Plate 16-3C and 16-3E. The double zone of hemolysis on blood agar, production of lecithinase on EYA and stormy fermentation of litmus milk (or proteolysis of milk agar) are characteristic of this species. The cells of *C. perfringens* are usually 0.8 to 1.5 µm in diameter × 2 to 4 µm long and have blunt ends (Image Plate 16-3A, 16-3B and 16-3D). They are often described as boxcar-shaped. However, cells examined during early growth in broth culture tend to be short and coccoid, while older cultures contain longer cells that may be almost filamentous. After overnight incubation on blood agar, colonies are usually 1 to 3 mm in diameter, but may reach a diameter of 4 to 15 mm after prolonged incubation. Colonies are usually flat, somewhat rhizoid, and raised centrally. Some colonies tend to spread, but they do not swarm. *C. perfringens* is nonmotile. Traditionally, the Nagler has been used for the identification of *C. perfringens*; similarly, the "Reverse CAMP" test has also been used. These are not commonly performed in clinical practice, but the principles are reviewed in Chart 16-8.

Identification of Other Selected *Clostridium* spp. *C. perfringens* is by far the most commonly isolated species of *Clostridium* from human sources. However, there are many other clostridia that are isolated from clinical specimens. Cells of *C. ramosum* are usually less than 0.6 µm in diameter × 2 to 5 µm long, but are extremely pleomorphic, sometimes producing short chains or long filaments; many strains will be gram-negative. On blood agar, colonies are often 1 to 2 mm in diameter, usually nonhemolytic, slightly irregular or circular, entire, low convex and translucent. Isolates of *C. ramosum* are characteristically resistant to rifampin by the 15-mg disk method discussed above, but inhibited by the 2-U penicillin disk and the 1-mg kanamycin disk, similar to *F. mortiferum* and *F. varium*. *C. ramosum* is indole-negative, whereas *F. mortiferum* and *F. varium* are indole-positive. *C. ramosum* also shows enhanced growth on bile agar, hydrolyzes esculin, but is negative for catalase, lipase and lecithinase. It is among the few clostridia that ferment mannitol. Acetic, lactic and succinic acids are the major metabolic products. The cells of *C. septicum* are usually about 0.6 µm wide and 3 to 6 µm long (Image Plate 16-3H). It tends to be pleomorphic, sometimes producing long, thin filaments. Chain formation is common, as are intensely staining citron (lemon-shaped) forms. Spores are oval, subterminal and distend the organism. After 48 hours of incubation on blood agar, colonies are 2 to 5 mm in diameter, surrounded by a 1- to 4-mm zone of complete hemolysis; they are flat, slightly raised, gray, glistening, semitranslucent and have markedly irregular to rhizoid margins, often surrounded by a zone of swarming (Image Plate 16-3G).

Extremely motile strains may swarm across a wide area of the plate. Stiff blood agar, which contains 4% to 6% agar, instead of the usual 1.5%, is sometimes used in plating media to minimize swarming. Some key characteristics of *C. septicum* are that it hydrolyzes gelatin, does not produce indole, lipase, or lecithinase and ferments lactose but not mannitol, rhamnose, or sucrose. Acetic and butyric acids are the major metabolic products.

C. clostridioforme complex is made up of at least six species, *C. aldense, C. bolteae, C. citroniae, C. clostridioforme, C. hathewayi,* and *C. lavalense*. They all stain predominantly gram-negative, and they have been referred to as containing cigar-shaped cells. All ferment glucose and are negative for gelatin hydrolysis. Three are indole-positive, *C. aldense, C. citroniae,* and *C. lavalense*; two can be esculin-positive, *C. bolteae* and *C. hathewayi*. *C. hathewayi* is β-NAG positive and the others negative or have an unknown reaction. *C. symbiosum* also stains gram-negative and may have cigar-shaped cells like the *C. clostridioforme* complex, but it is usually gelatin hydrolysis–positive, although weakly, and urea-positive. *C. symbiosum* grows very slowly and usually forms a film of growth on the agar surface, so it is distinguishable from the complex.

C. innocuum, a commonly isolated *Clostridium*, is a gram-positive bacillus with oval, terminal spores; colonies will fluoresce chartreuse under UV light similar to *C. difficile* but *C. innocuum* is negative for motility and gelatin hydrolysis, whereas *C. difficile* should be positive for these biochemicals. *C. tertium* is the most commonly isolated of the aerotolerant *Clostridium* spp.; it has oval, terminal spores and is motile and esculin hydrolysis–positive.

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TABLE 16-23 Phenotypic and Colonial Characteristics of *Clostridium* spp.

Species	Gel	Glu	Lec	LIP	IND	URE	ESC	NIT	MOT	Spores	Colony[a]
C. aldenense	–	+	NA	NA	+	–	–	–	NA	ST, but rarely seen; α-galactosidase +; β-galactosidase +; Stains gram-negative; β-NAG –	Part of *C. clostridioforme* complex; spores are rarely seen. Colonies are 1–2 mm, flat, opaque to white, nonhemolytic; found in GI tract
C. argitenense	+	–	–	–	–	NA	–	–	NA	ST	
C. barati	–	+	+	–	–	–	+	–	–	ST; rarely seen on Gram's stain	Small, nonhemolytic colonies
C. bifermentans	+	+	+	–	+	–	+	–	+	O; ST	Gray; irregular edge; narrow zone of hemolysis; **chalk white on EYA**; may swarm or spread; translucent to opaque; flat or raised; mottled internal structures
C. bolteae	–	+	–	–	–	NA	–[+]	–	NA	ST, but rarely seen; cigar-shaped cells stain gram-negative; can contain the vanB gene;β-NAG –	A member of *C. clostridioforme* complex
C. botulinum	+	+	–	+	–	NA	V	–	+	ST or T	Variable hemolysis seen
C. butyricum	–	+	–	–	–	–	+	–	+	O; ST	Large, irregular colonies with mottled to mosaic internal structures
C. cadaveris	+	+	–	–	+	–	–	–	+	O;T	White to gray; entire or slightly irregular edges; raised to slightly convex
C. carnis	–	+	–	–	–		+	–	NA	ST	**Aerotolerant**
C. citroniae	–	+	NA	NA	+	–	–	–		ST; spores rarely seen; stain gram-negative; β-NAG –	Flat, opaque to white, nonhemolytic; member of *C. clostridioforme* complex
C. clostridioforme	–	+	–	–	–	–	+	–	+	O; ST; cigar or football-shaped cells that may taper but spores rarely seen on Gram's stain; stains gram-negative; can be β-lactamase positive	Small; convex and translucent colonies; mottled or mosaic internal surface; when exposed to air, colonies may cause greening of blood agar
C. difficile	+	+	–	–	–	–	+	–	+	O;ST or T	Slightly raised; umbonate with filamentous edge; translucent with crystalline internal speckling; **chartreuse fluorescence**; **horse barn odor**
C. hastiforme	+	–	–	–	–	–	–	–[+]	+	T	
C. hathewayi[d]	–	NA	–	–	–	NA	+[w]	–	NA	ST; spores rarely seen; cigar-shaped cells stains gram-negative; rods with non-tapered ends; β-NAG +; **can contain *vanB* gene**	Nonhemolytic; gray-white, convex, shiny colonies with irregular margins; member of *C. clostridioforme* complex
C. histolyticum	+	–	–	–	–	–	–	–	+	O; ST	Smooth or rough colonies; if rough, have rhizoid edges; **aerotolerant**

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C. innocuum	–	+	–	–	–	–	+	–	–	O;T	White, glossy, raised colonies; **chartreuse fluorescence**
C. lavalense	–	+	–	–	+	–	–	NA	NA	ST large rods with tapered ends; **stains gram-negative**; spores are rarely seen; α-galactosidase – and β-galactosidase +; may be vancomycin resistant	Large colonies that are gray-white; nonhemolytic; strongly adhere to agar; member of *C. clostridioforme* complex
C. limosum	+	–	+	–	–	–	–	–	+	ST	
C. novyi A	+	+	+	+	–	–	–	–	+	O; ST	Gray, translucent; irregular surface; **may have double zone of hemolysis**
C. perfringens[b]	+	+	+	–	–	–	–	+⁻	–	ST; rarely seen in Gram's stain	Gray; opaque; flat; edges somewhat rhizoid, but tend to spread and not swarm; **double zone of hemolysis**
C. putrificum	+	+	–	–	–	–	–⁺	–	+	T or ST	Not distinctive
C. ramosum	–	+	–	–	–	–	+	–	–	O or R; T; cells are thin variable rods	Translucent; circular or slightly irregular edges; convex; brownish discoloration on blood agar with air exposure; **red fluorescence**
C. septicum	+	+	–	–	–	–	+	V	+	O or ST	Medusa-head like growth; becomes heavy film that cover agar surface; **(swarms)**; glistening flat gray colonies
C. sordellii	+	+	+	–	+	+⁻	–⁺	–	+	O; ST; free spores often seen	Translucent to opaque; flat or raised; can have mottled internal structure; colonies may spread over agar surface (Image Plate 16-4G and 16-4H)
C. sporogenes	+	+	–	+	–	–	+	–	+	O; ST; many free spores	Raised gray-yellow center; rhizoid edges; colonies adhere firmly to agar; may show some swarming (Image Plate 16-4C)
C. subterminale	+	–	–⁺	–	–	–	–⁺	–	+	O;SR; rarely seen in Gram's stain	
C. symbiosum	W	+ʷ	–	–	–	+	–	–	+	ST; stains gram-negative; may have cigar-shaped cells that form chains; spores may or may not be seen	Very slow growing; sometimes produces only a film of growth, described as "dusty growth"
C. tertium[c]	–	+	–	–	–	–	+	–	+	O;T	Small; translucent; glossy colonies; **aerotolerant**
C. tetani	+	–	–	–ʷ	V	–	–	–	+	R;T; drumstick shape	Translucent; gray; irregular edges; may form thin film of growth on entire plate; may have narrow zone of hemolysis

[a] If there is a unique characteristic of the species, it has been bolded.
[b] Some strains of *C. perfringens* may be catalase-positive; some may be slightly aerotolerant.
[c] *C. tertium*, many strains of *C. histolyticum*, and *C. carnis* (not in Table) are aerotolerant *Clostridium* spp.
[d] There is a proposal to rename *C. hathewayi* to *Hungatella hathewayi*.[249]
+, positive; –, negative; –⁺, most strains negative with an occasional strain positive; +⁻, most strains positive with an occasional strain negative; w, weak reaction; ND, not determined; V, variable reactions, Gel, gelatin hydrolysis; Glu, glucose fermentation; Lec, lecithinase; LIP, lipase; IND, indole production; URE, urease; ESC, esculin hydrolysis; NIT, nitrate reduction; MOT, motility; β-NAG, *N*-acetyl-β-glucosaminidase, O, oval shape; R, round spores; T, terminal spore location; ST, subterminal spore location; EYA, Egg yolk agar.
Adapted from references 178, 238, 464.

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TABLE 16-24 Useful Characteristics for Identifying *Clostridium* Species

Characteristic	Species
Aerotolerant	*C. carnis, C. histolyticum, C. tertium*
Nonmotile[a]	*C. innocuum, C. perfringens, C. ramosum*
Usually stains gram-negative	*C. clostridioforme complex (C. clostridioforme, C. aldenense, C. bolteae, C. citroniae, C. hathewayi, C. lavalense), C. symbiosum*
Terminal spores	*C. baratii, C. cadaveris, C. innocuum, C. ramosum, C. tertium, C. tetani* (Image Plate 16-4D)
	C. perfringens, C. clostridioforme complex (see above

Spores not seen in stains	*for species); C. symbiosum, C. baratii, C. ramosum, C. citroniae, C. subterminale*
Lecithinase produced on EYA	*C. bifermentans, C. limosum, C. novyi, C. perfringens, C. sordellii, C. subterminale, C. baratii*
Lipase produced on EYA	*C. botulinum, C. novyi A, C. sporogenes* (Image Plate 16-3F)
Both lecithinase and lipase produced	*C. novyii A, C. sporogenes*
Asaccharolytic and proteolytic (i.e., gelatin-positive)	*C. histolyticum, C. limosum, C. subterminale, C. tetani, C. hastiforme*
Urease-positive	*C. sordellii*[b], *C. symbiosum (original description)*
Do not hydrolyze gelatin	*C. butyricum, C. clostridioforme complex, C. paraputrificum, C. baratii, C. ramosum*
Mannitol fermented[c]	*C. difficile, C. innocuum, C. ramosum, C. tertium*
Rhamnose fermented	*C. clostridioforme, C. ramosum, C. sporogenes*
Saccharolytic and proteolytic	*C. bifermentans, C. botulinum, C. cadaveris, C. difficile, C. perfringens, C. putrificum, C. septicum, C. sordellii, C. novyi A, C. sporogenes, C. symbiosum*
Saccharolytic but nonproteolytic	*C. baratii, C. butyricum, C. clostridioforme, C. innocuum, C. ramosum, C. tertium, C. carnis, C. aldenense, C. bolteae, C. carnis, C. lavalense*

[a] There may be other nonmotile species of *Clostridium*, that have been more recently described.
[b] *C. sordellii* resembles *C. bifermentans*, but *C. bifermentans* is urease-negative; *C. symbios*um stains gram-negative and may have tapered ends so it is easy to distinguish from *C. sordellii.*
[c] There are a few other species that are mostly negative, but an occasional strain may be positive: *C. botulinum*, Type B, E, F; *C. butyricum* and possibly other species not in Table 16-26.
EYA, egg yolk agar.

C. difficile-*Associated Intestinal Diseases: Epidemiology and Laboratory Identification*

Description of Disease and Epidemiology. *C. difficile*, first isolated in 1935, was believed to be nonpathogenic for humans until the late 1970s, when it was implicated

as a causative agent in AAD and PMC.[33,34,464] Benign, self-limited diarrhea frequently develops in hospitalized patients who are being treated with antibiotics. *C. difficile* has been found responsible for 25% to 30% of the cases of AAD.[74] The diarrhea often subsides once the inciting antibiotic is discontinued. In other patients, the intestinal symptoms may be more severe, and the diarrhea persists. These patients may have antibiotic-associated colitis (AAC), a condition caused by *C. difficile* 60% to 75% of the time. The more severe and life-threatening PMC is caused primarily by *C. difficile*, although may be infrequently caused by *S. aureus* or other pathogens.[33,34,139,464] In the United States, there are >300,000 cases per year of AAD, AAC, or PMC caused by toxigenic *C. difficile*. One review reported that their hospital had medical expenditures associated with *C. difficile*-associated disease were almost $1,000,000 per year and another review article suggested that healthcare costs in the United States were approaching $1 billion annually.[453] The severity of illness, as well as pathologic findings, are highly variable, depending on whether the patient has PMC, AAC, AAD or is simply colonized with *C. difficile* or is an asymptomatic carrier. The pathologic findings in PMC and AAC have been adequately reviewed elsewhere.[34,35,139] In a report of gastroenteritis-associated deaths in the United States, 1999 to 2007, the CDC reported that *C. difficile* mortality increased fivefold from 10/1,000,000 person years in 1999 to 2000 to 48/1,000,000 person years in 2006 to 2007. This was statistically significant. Norovirus was found to be the second leading infectious cause of death in this report.[201] *C. difficile* can also be a rare cause of abscesses, wound infections, osteomyelitis, pleuritis, peritonitis, bacteremia, and urogenital tract infections.[323,464]

Two toxins are involved in *C. difficile*-associated GI disease: toxin A, which is an enterotoxin capable of producing fluid accumulation in ligated rabbit ileal loop assays, and toxin B, which is a potent cytotoxin capable of producing cytopathogenic effects in several tissue culture cell lines.[464] Toxin A is also cytotoxic in certain cell lines, but is not as cytotoxic as toxin B in the cell lines that have long been used for toxin B testing.[306] The majority of isolates of toxigenic *C. difficile* carry the genes for both toxin A (*tcd*A) and toxin B (*tcd*B). In addition, there are strains in which a third gene,

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*tcd*C, is present, which causes the overproduction of *C. difficile* toxins, resulting in more severe disease. These hypervirulent strains have been called NAP1/027 or PCR ribotype 027, and the toxin is called a binary toxin. These latter hypervirulent strains have caused outbreaks of disease in Canada, Europe, and the United States, and there is some evidence that they may be triggered by extensive use of fluoroquinolones.[326,464] Decreased cure and increased recurrence rates are associated

with this hypervirulent strain as well.[375] For additional information on *C. difficile* and its toxins, the following references are recommended.[33,34,49,64,306,464,496]

C. difficile is ubiquitous in nature, and has been isolated from soil, water, intestinal contents of various animals, the vagina and urethra of humans, and feces of many healthy infants, but from the stools of only about 3% of healthy adult volunteers. However, the organism is more prevalent in the feces of some hospitalized adults who do not have diarrhea or colitis. It has been found in the feces of about 13% to 30% of hospitalized adults who were colonized but had no evidence of disease caused by *C. difficile* nor any antecedent antibiotic treatment. Toxigenic *C. difficile* can commonly be found in the feces of many healthy infants, and may remain as colonizers for long periods of time. It is often difficult to evaluate what role *C. difficile* has in a infantile diarrhea in hospitalized children; in many laboratories, *C. difficile* tests are not routinely performed on the stool of infants less than 2 years old.[2] Some infants, however, with severe and protracted diarrhea, associated with antecedent antibiotic therapy, have had PMC and at the same time, *C. difficile* cytotoxin present in their feces without any other putative etiologic agent. These patients have often responded clinically to oral vancomycin. There are some issues as well with association of *C. difficile* and diarrhea in children greater than 2 years, but recent review articles address this and can be referred to if the question arises in the laboratory.[64,344,411] The American Academy of Pediatrics recently issued a policy with information and recommendations in regard to *C. difficile* in children.[418]

The great majority of patients with *C. difficile* infection have had prior exposure to antibiotics whether they have been diagnosed while in the hospital or long-term care facility or are seen in an ambulatory care setting when the diagnosis is made. In a study of patients recently discharged from an acute-care hospital, Chang et al. found that 92% of patients diagnosed in the ambulatory care setting had received antibiotics during a prior hospitalization and 65% of these patients received antibiotics both as an in and outpatient.[86] Antimicrobial agents implicated in *C. difficile*-associated GI illness have included most antibiotics; however, the disease is most commonly associated with clindamycin, cephalosporins, and the fluoroquinolones.[33–35,180,464] For more information on the clinical findings and clinical diagnosis of AAD, AAC, and PMC, the reader is referred to other sources.[33,34,35,101,464]

Collection and Transport of Specimens Containing *C. difficile*. Ordinarily, watery or semisolid, unformed fecal specimens (about 5 g or 1,020 mL of liquid stool) are the preferred specimens for testing. Swab specimens, because of the small volume obtained, are inadequate. Formed stool specimens are inappropriate for diagnostic testing unless an epidemiologic study of stool carriage is being conducted. Other suitable specimens include biopsy material or lumen contents obtained by colonoscopy

and involved bowel (surgical removal; autopsy). However, lumen contents or biopsy material from colonoscopic procedures may excessively dilute the sample, or the quantity obtained by biopsy may be insufficient for the laboratory work required. The testing of biopsy specimens, however, is less sensitive than the testing of stool, which is the preferred specimen. Leak-proof plastic containers should be used for the transport of specimens. If specimens are to be processed by the laboratory on the same day they are collected, then transportation at room temperature will suffice. If a specimen arrives late in the day and cannot be processed by assays that detect toxin until the following day, it may be held in the refrigerator without demonstrable loss of cytotoxic activity, but the directions for any commercial tests should always be followed. Optimally, specimens should not remain at room temperature longer than 2 hours before being cultured; if processing for culture cannot occur within 2 hours of collection, then refrigerate in an anaerobic environment (transport vial or bag), although this may reduce the number of vegetative cells. If specimens are to be shipped a reference laboratory for a toxin assay, they should be shipped on dry ice and stored in lab at −70°C before shipment. Storage at −20°C will not guarantee the stability of the toxins. Most laboratories have migrated to PCR for the detection of *C. difficile.* If this is the case, then the manufacturer's guidelines for specimen transport should be followed.

Laboratory Diagnosis of Toxigenic *C. difficile*. To diagnose C. *difficile* infections, stool samples are submitted to the laboratory to detect the presence of toxigenic *C. difficile*. Culture for the recovery of toxigenic *C. difficile* was the first method used in clinical laboratories in the late 1970s when the association of *C. difficile* and diarrhea was initially recognized, and it continues to be considered the "gold standard" for detection and the reference method when evaluating newer tests. Plating of samples of stool, with or without prior heat or alcohol shocking, onto selective CCFA agar (Cycloserine, cefoxitin, fructose agar) and nonselective media (anaerobic blood agar) is used for the recovery of *C. difficile* (Image Plate 16-4E). Then, any *C. difficile* isolate recovered is tested for the production of toxins. (Culture media and methods are discussed below). The cytotoxin B tissue culture assay is the test most commonly performed to determine if the *C. difficile* isolate recovered is toxigenic. Alternatively, direct cytotoxin B tissue culture assays for detection of toxigenic *C. difficile* could also be performed directly on stool samples instead of culture. Many refer to this test as the cell culture neutralization assay (CCNA). The procedure for the tissue culture assays consist of inoculation of specific tissue culture panels, usually containing McCoy epithelial cells or human foreskin cells with the stool sample and examining it in 24 to 28 hours for presence of cytopathic effect (CPE), a rounding up of the fibroblast cells as a result of the presence of Toxin B (Chart 16-9). *C. difficile* antitoxin added to a companion well would neutralize the toxin, confirming that the CPE was indeed due specifically to *C. difficile*. For many years, the cytotoxin B

assay was felt to be equivalent to culture for detection of toxigenic *C. difficile*, but more recently with the advent of molecular methods of detection, the sensitivity of the Toxin B assay when used directly, as

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compared to toxigenic culture, was found to be <70% with a specificity of 99%, thus limiting its use as a primary test in most situations.[458] Information on the cytotoxin procedure is in Chart 16-9. Extended turnaround times for culture and the need to keep confirmatory tests available for detection of the toxin in isolated colonies have limited the usefulness of these tests in most clinical laboratories. However, culture is the method used for epidemiology, when susceptibility testing is requested, and as the preferred comparator method when new tests are being evaluated. Tissue culture assays are still used in some laboratories for confirmation of newer screening assays and for the purpose of confirming culture results, but less commonly as the primary diagnostic test.

Culture Methods and Cytotoxin Identification. *C. difficile* can be isolated from stool specimens by the use of a spore selection technique (i.e., heat shock or alcohol spore selection procedures) in conjunction with use of selective plating media such as PEA blood agar (Remel, Lenexa, KS; or BD Microbiology Systems) or CCFA plus nonselective CDC anaerobe blood agar.[464] Detailed descriptions of the alcohol and heat-treatment spore selection procedures are given elsewhere.[238] If either of these procedures is used, the alcohol or heat-treated sample is inoculated onto anaerobe blood agar and/or EYA. Selective media like CCFA could also be inoculated since typical morphology may be easier to identify, but should not be used alone since it might be too inhibitory after the stool has been treated to reduce other microbiota organisms. There is a third method of stool treatment that has been described more recently which optimizes recovery of *C. difficile* with the use of an enrichment broth that has been supplemented with sodium taurcholate to enhance spore germination. In two separate studies, results were superior to pretreatment with alcohol or heat before plating.[16,220] Following 48 hours of incubation anaerobically, colonies of *C. difficile* on anaerobe blood agar are nonhemolytic, about 2 to 4 mm in diameter, grayish translucent, slightly raised, flat, and spreading, with rhizoid margins. As seen through a dissecting microscope, the colonies show an iridescent, "speckled-opalescent" appearance. *C. difficile* is negative for lipase and lecithinase on EYA and produces the characteristic flat, irregular to circular colonies with a rhizoid edge. The colonies have ground glass appearance on the surface and a yellowish to white pigmentation on CCFA agar. If a UV lamp is shone on the colonies, they will fluoresce a chartreuse color. In addition, there is a characteristic horse manure (horse barn) odor due to production of *para*-cresol (Table 16-24). Often when an anaerobic jar is opened, the

odor will be immediately detected.[238]

In addition to the spore selection procedure, a plate of PEA medium and a CCFA plate should be inoculated with untreated stool, or a stool suspension prepared in buffered gelatin diluent. After 48 hours of incubation, colonies of *C. difficile* on PEA medium will be virtually identical to those on anaerobe blood agar (described above). The appearance of *C. difficile* colonies on CCFA medium is described above and in Table 16-24. The gram-positive to gram-variable rods have subterminal spores and are motile. Metabolic product analysis reveals acetic, propionic, isobutyric, butyric, isovaleric, valeric, and isocaproic acids. Esculin and gelatin are hydrolyzed. Indole, nitrate, and urease are negative. Most strains ferment glucose, mannitol and mannose. Salicin and xylose are variable. Once a typical colony of *C. difficile* is isolated and phenotypic identification is complete, evidence that it is toxigenic needs to be accomplished using the cytotoxin B assay or a molecular test for detection of a toxin gene(s).

Two new culture media have been developed to select for strains of toxigenic *C. difficile* to avoid the necessity of performing a confirmatory toxin assay. One is called the Cdifftox plate assay, reported on by Darkoh et al. in which CCFA, along with sodium taurocholate, added to enhance the germination of spores, blood and a chromogenic substrate (X-gal) provide a single plate upon which toxigenic *C. difficile* strains will produce a blue color and nontoxigenic strains a white colony. The toxins, if present, will cleave the chromogenic substrate in place of their natural substrate, UDP glucose. Culture results are usually available in 24 to 48 hours. The product was described to have 99.8% accuracy when testing 528 isolates that came from 50 tissue culture cytotoxicity assay positive specimens.[112] There is a commercially produced chromogenic media, chromID *C. difficile*, that can detect toxigenic *C. difficile* in <3days. It has been evaluated in a few laboratories and the results were very good and available more rapidly than what can be obtained on CCFA agar plus toxin detection.[77,140,304,432]

Enzyme Immunoassays for Toxin Detection. Because of the labor-intensive and time-consuming nature of culture and lack of sensitivity of the cytotoxin tissue culture assay, enzyme immunoassays (EIAs) have been developed for the detection of *C. difficile* toxin(s). Assays to detect toxin A were introduced into clinical laboratories over 20 years ago. These were more rapid and less time-consuming than culture or CCNA and provided good information in formats that allowed for batch or individual specimen testing. A number of these tests in a variety of formats were developed and adapted by the majority of routine laboratories. When a few toxin B-positive/toxin A-negative strains were discovered, a number of EIA products that detected both toxin A and toxin B were developed and implemented in most clinical laboratories to replace the Toxin A EIA assays.[243] With the widespread use of PCR and the use of culture as gold standard in more recent comparator studies, the EIA for toxin A + B assays were

determined to be performing at sensitivities as low as 52% and not much higher than 81%.[440,510] Authors who have compared EIA or lateral flow assays for detection of toxins A and B have in general concluded that the assays should not be the only test that is done routinely for the diagnosis of *C. difficile*. Even when these tests were examined to detect severe disease versus mild disease, EIA assays were still much less sensitive even with severe disease than would be acceptable for primary stool testing.[226] Although insensitive, the specificity of the EIA toxin assays is good, in the range of 91% to 94% or greater. Therefore, they can be used for confirmation of a positive result by an antigen assay, for example, or another highly sensitive, but less specific methods.[49,138,226] Today, laboratories should no longer rely on these EIAs alone for the diagnosis of toxigenic *C. difficile*.

Nucleic Acid Amplification Test for *C. difficile.* Amplification of the *tcd*B or *tcd*A genes has consistently demonstrated good sensitivity and specificity when compared to toxigenic culture as the gold standard.[49,138,362,458] Although laboratories have developed their own real-time PCR assays,

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there are many commercial products available. There is ample evidence indicating that these molecular methods can significantly increase the sensitivity over EIA assays and consequently the incidence of *C. difficile* detected in the stool of hospitalized patients, in some cases by as much >50% with good specificity as compared to culture.[74,138,297,458,335,366,373] Table 16-25 lists many of the commercially available assays in the US presently and a few bullet points of where they may differ from each other. Table 16-26 provides results of published studies comparing the most commonly used nucleic acid amplification tests (NAATs) alone, against other NAATs or versus toxigenic culture as the "gold standard." A review article in 2011 provided excellent information on the then commercially available PCR assays; the author's conclusion were that the NAAT assays all compared well with culture and were superior to EIAs and tissue culture. The turnaround time was adequate, ranging from 45 to 180 minutes and would allow for same shift resulting.[374] A meta-analysis of 25 publications that evaluated NAAT for the detection of *C. difficile,* which included >11,000 stool specimens that met inclusion criteria, found that most labs that presented data on a *C. difficile* molecular test were using BD GeneOhm, followed by Cepheid GeneXpert. In addition, laboratories also used a Light Cycler PCR (Roche Molecular, IND), or Hologic's ProGastro CD. A few laboratories reported use of Illumigene's LAMP assay, but were not included in the analysis because the studies were too heterogeneous for comparison at that time. The authors concluded that molecular tests were highly accurate for detection of *C. difficile* toxins and that the

LAMP assay looked promising, which has subsequently been proven.[362]

There are often questions about repeat testing for the presence of *C. difficile* with molecular assays. The utility of repeat testing for *C. difficile* has been evaluated and in most studies, it has not proved useful for the diagnosis of *C. difficile*, but if done, it should always be done using a different method than what was initially done. Once a cohort of patients has initially been determined to be negative for *C. difficile* by a highly sensitive assay, such as a NAAT, then repeat testing is more likely to generated false-positive results than true-positive results. Furthermore, laboratory testing should not be performed as a "test of cure." The resolution of *C. difficile* disease should be assessed on a clinical basis, regardless of whether or not *C. difficile* remains as part of the microbiota of the patient. The Society for Healthcare Epidemiology of America (SHEA) also discourages repeat testing for *C. difficile*.[74,101,305]

***C. difficile* GDH Antigen Testing.** As an alternative to primary PCR testing, there are at least three commercially available assays that detect glutamate dehydrogenase (GDH), an antigen common to *C. difficile* and other *Clostridium* spp.[73,110,138,390,476]These assays are very highly sensitive, but are not specific enough for *C. difficile* to be used alone. However, they can be used as a negative screen initially for *C. difficile* stool requests; when positive, confirmatory tests that detect *C. difficile* toxins specifically, can be employed before reporting results. *C. DIFF CHEK*-60 (TechLab, Blacksburg, VA) is a 96-well ELISA that can be performed in 60 minutes. The sensitivity of this assay is very high (>99%) as reported in most studies; in one study, only 22 of 37 GDH positive/cytotoxin assay positive, were positive with a well-known EIA for toxin A and B (59.5% sensitivity). The positive predictive value of the GDH was only 50%, so it must be used in an algorithmic fashion with an assay that confirms presence of cytotoxin.[183]A combination GDH antigen + toxin EIA assay is now available from the same manufacturer to run both tests simultaneously; C. DIFF Quik Chek Complete (TechLab, Blacksburg, VA) is a rapid membrane enzyme immunoassay to which the diluted stool sample is added and incubated for 15 minutes. The formation of a blue line on the left of the reaction window would indicate the presence of GDH and a blue line on right would indicate the presence of *C. difficile* toxin. Use of the antigen as a negative screen, followed by a cytotoxin B assay on the samples that are positive, has been shown to produce very sensitive results with high negative predictive values in a cost-effective approach.[390,476] When the antigen assay is negative, and this can usually be done in less than 10 minutes, then the results of a negative *C. difficile* can be reported immediately. However, nontoxigenic *C. difficile* can also produce a positive GDH result; hence the need to confirm the antigen positive stool samples.[75] This can be done as mentioned above by means of an EIA for toxin A/B, a cytotoxin assay in tissue culture or PCR.[110,362] The latter approach would provide the best overall sensitivity and specificity as a one-step confirmation.

However, many labs use a three-step approach as follows: positive antigen results are followed up with toxin A/B EIA; if the two are positive, results are signed out as positive for toxigenic *C. difficile*, but if negative, further confirmation testing is performed with PCR or culture. One study examined the use of two different molecular assays for this confirmation; they performed equally well, although the authors felt one was slightly more efficient than the other.[3]

TABLE 16-25 Characteristics of Commercial NAAT for the Detection of Toxigenic *C. difficile*

NAAT	Manufacturer	Target[a]	Format	Turnaround Time[b] (minutes)
Prodesse ProGastro Cd Assay[248,421,458,488]	Hologics, Inc, CA	*tcd*B	Smart Cycler	75–90
Gen-Ohm Cdiff Assay[3,248,421,473,488]	BD-GeneOhm, CA	*tcd*B	Smart Cycler	180
BD Max[226,288, c]	BD Gene Ohm, CA	*tcd*B	BD-Max	160
Xpert *C. difficile* Assay[3,199,223,433,510]	Cepheid, CA	*tcd*B	GeneXpert	45
Illumigene[d,23,49,199,271,358]	Meridian Biosciences, Inc.,	*tcd*A	Illumigene Instrument	75
Portrait Toxigenic *C. difficile*[63]	Great Basin, WI	*tcd*B	Slide array; Visual: chip readout	60
Simplexa Universal Direct PCR[113,273]	Focus Diagnostic, CA	*tcd*B≈	3M Integrated Cycler	60
Verigene *C difficile* Test[76]	Nanosphere, III	*tcd*A, *tcd*B, PCR Ribotype 027	Verigene Reader and Processor SP	<120
Amplivue *C. difficile* Assay[113]	Quidel Corp., CA	*tcd*A	Manual read; lateral flow in cassette	80

[a] Target: gene for production of Toxin A,B, or C that is being targeted by the assay.
[b] Turnaround time as listed in package insert and/or on website of manufacturer.
[c] BD Max is an automated format for the Gene Ohm assay.
[d] Illumigene assay is often referred to as LAMP, or loop amplification.
NAAT, nucleic acid amplification test.

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TABLE 16-26 Evaluations of Commercially Available *C. difficile* NAAT Assays[a]

Comparators	# Specimens/ % Positive	Sensitivity (%)	Specificity (%)	Predictive Value Positive (%)	Predictive Value Negative (%)	REF
GeneOhm						
EIA, GDH/EIA, & ProGastro Cd		100				449
PCR[b] and ProGastro Cd[c]	346/8.4	84	99			230
Quik Chek & ProGastro Cd	200	90	97	90	97	387
Illumigene[c]	139/15	95	100	100	99	44
Xpert, Illumigene & Portrait	549/21	97	99			58
No comparators	360/12	95	99.7	98	99.4	265
No comparators	404/10	84	98	90	97	423
XPert						
No comparators	253/19	100	95	83	100	398
EIA and PCR[b,e]	138	100	98			468
GeneOhm, Illumigene & Portrait	549/21	100	92			58
No comparator[c]	566/12	99	81	90	97	101
Illumigene	568/19	100	97			183
Illumigene	200/22	100	100			337
CCNA[c]	220	97	93	72	99	206
ProGastro Cd						
EIA, GDH/EIA, & GeneOhm		94				440
PCR[b] and GeneOhm	346/8.4	92	99			230
EIA and CCNA	285/15.7		77	99	94	423
Quik Chek and GeneOhm[d]	200	100	93	83	100	387
Portrait						
GeneOhm, XPert, Illumigene	549/21	98	93			58
Illumigene						
Vidas EIA	302/29	95	99	98	98	330
EIA and GDH	810	92	98	84	99	20
GeneOhm[c]	139/15	95	97	83	99	44
GeneOhm, XPert & Portrait	549/21	93	95			58
XPert	568/19	84	100			183
No comparator[e]	141/19	89	98			334
XPert	200/22	83	100			337
CCNA	472	92	99	92	99	249
Verigene						
Direct culture[f]	1,875/15	99	88	42	99.9	70
Enriched culture	1,875/15	91	92.5	68	98	70
Light Cycler						
EIA	200/12	86	97	90	96	404
Simplexa						
QuikChek[e] and CCNA	342/26	98				251
Amplivue and Illumigene	200	98	100			104
Amplivue						
Simplexa and Illumigene		96	100			104

[a] Toxgenic cultures were used as the gold standard in study unless otherwise indicated.
GeneOhm (BD, MD); XPert (Cepheid); ProGastroCd (GenProbe, Inc., CA); Portrait (Great Basin, WI); Illumigene (Meridian Bioscience, Inc.); Light Cycler (Roche, Ind); Verigene; Simplexa (Focus Diag., CA); Amplivue (Quidel Corp).
[b] PCR was a laboratory-developed assay.
[c] Culture only done on discordants.
[d] Frozen stools; pediatric patients.
[e] Culture not performed.
[f] For comparison, two cultures were performed, one with CCFA and one with CCFA containing taurcholate to enhance spore germination. Assay detects *tcd*A and *tcd*B and *tcd* C; 24% of positives were hypervirulent (mostly ribotype 027) strains.
EIA, enzyme immunoassay for toxin A and/or B; QuikChek, combination test with GDH and EIA for toxin B; CCNA, cytotoxin neutralization assay.

There is also a latex agglutination assay for GDH combined with a toxin A immunoassay from Biosite Labs. One study examined 557 fresh stool samples comparing its performance to a toxin A/B immunoassay and cytotoxin B tissue culture assay. The sensitivity of the GDH was 97%, with a specificity of 87%. Seventeen percent of the positive GDH samples were not confirmed with the toxin A assay and another confirmatory test was needed.[322] No toxigenic culture or PCR was used as a comparator, however, so it is difficult to know if the sensitivity was as high as reported since tissue culture sensitivity, the comparator arm of the study, does not have sensitivity as high as toxigenic culture and/or PCR. Laboratories that have the capability for performing PCR routinely on all stool specimens may instead perform amplification on all samples routinely in order to reduce the amount of steps and potentially the time-to-results. This approach may provide the best overall sensitivity and specificity, PPV and NPV as compared to toxigenic culture. SHEA has suggested that NAAT appears to be sensitive and specific for detection but more data is needed before they would endorse PCR as primary test for all laboratories.[101] The two- or three-step algorithm does have the advantage of more rapid reporting, especially with specimens that are antigen and toxin A/B positive and those that are negative. More time, however, is needed for further confirmation of those specimens that are positive for the GDH antigen, but EIA negative. In a study examining results of the GDH/toxin A/B assay two-step algorithm, the authors reported that they were able to report 36% of the *C. difficile* positive results and 94% of the negative results within 30 minutes. Only 13% of their workload was utilized to confirm GDH antigen positive/toxin A/B negative specimens before reporting results.[110] In another study using Premier EIA for GDH, positive results were followed by a Illumigene's LAMP assay for confirmation; the sensitivity was 92% compared to other algorithms used and the authors suggested that the LAMP assay was much less expensive than other PCR assays and was very user friendly; they found no advantage to a three-step assay.[23]

Prevention of *C. difficile*. Prevention of *C. difficile* disease is the most important way to reduce the disease. Stewardship programs in healthcare systems that work toward reducing the inappropriate use of antibiotics, and implementation of hand washing, use of chlorine-containing cleaning agents in the hospital, and other infection control precautions to limit spread of toxigenic *C. difficile* are the ways in which this can be done.[101,152,272] Using appropriate tests that are sensitive and specific and enforcing appropriate specimen collection and transport guidelines are procedures that laboratories can do to participate in prevention and control. Culture of *C. difficile*, although not a timely nor efficient routine method for laboratory diagnosis, remains a useful tool in epidemiologic studies when strain typing and/or antimicrobial testing may be useful in defining an outbreak and preventing further disease. For routine testing,

use of an algorithm that includes glutamate dehydrogenase antigen testing or direct amplification tests would be good choices for laboratories to consider.[52,110,390,464,488] A cost-effectiveness study was performed on a two-step algorithm using the C. DIFF Quik Check (GDH plus EIA assay) confirmed with a PCR assay when the antigen and EIA were discordant. Eighty-six percent of the stool sample results could be resulted quickly; the remaining 14% required a PCR for confirmation of the result before it was resulted. The cost per test was $13.50 for this approach versus performing a PCR on all samples, which they estimated to cost $26 per test.[488] Possible algorithms for processing *C. difficile* specimens is given in Box 16-12. Box 16-13 presents some bullet points to remember when handling requests for toxigenic *C. difficile.*

Identification of the Anaerobic Cocci

Anaerobic Gram-Positive Cocci. *P. anaerobius* is a large, elongated coccus occurring in pairs and chains; colonies are larger than other anaerobic cocci and are gray-white and opaque in appearance. A sweet odor due to the production of isocaproic acid and susceptibility to the SPS disk can be distinguishing characteristics. *P. anaerobius* is catalase-, indole-, and urease-negative, but is positive for production of α-glucosidase. *Finegoldia magna* and *Parvimonas micra* can commonly appear in similar clinical situations, but can be distinguished in the laboratory because of their size initially. *P. micra* cells are <0.6 µm and colonies have a milky halo which is quite distinctive; the cells of *F. magna*, in contrast, are >0.6 µm. Both of these organisms are catalase-, indole-, α-glucosidase-, and urease-negative. Colonies of *Peptoniphilus asaccharolyticus* have a musty odor and are positive for indole, which helps distinguish them for the above-mentioned common cocci. *Staphylococcus saccharolyticus* is one of the few anaerobic gram-positive cocci that can reduce nitrate; it is also somewhat aerotolerant on transfers. It appears in clusters on Gram's stain. It is coagulase-negative, although that test would not usually be performed on an anaerobe. It is also a fermenter of glucose, lactose and maltose. Identifying characteristics of the most commonly encountered species are given in Table 16-27. For additional information, the following references are recommended.[238,351,499] A series of 16S rRNA-based fluorescent probes have been used to successfully identify many of the common anaerobic gram-positive cocci; these may prove useful to clinical laboratories in the future in conjunction with other newer methods.[513]

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BOX 16-12

Algorithms for *C. difficile* Testing

A. **Perform PCR on all stool samples and result when completed**
   a. Advantage:
      i. High sensitivity and specificity
      ii. Relatively rapid turnaround time
      iii. Need to have only one procedure and QC for *C. difficile* testing
   b. Disadvantage: instrumentation and reagents may be expensive
      i. Contamination issues need to be monitored
      ii. Both infected patients and asymptomatic carriers will be detected.

B. Perform toxigenic culture on all stool samples
   a. Advantage:
      i. High sensitivity and specificity
      ii. Isolate is available for epidemiologic testing, AST, or other studies
   b. Disadvantage:
      i. Long turnaround time
      ii. Need to have assay for confirming that isolate is toxigenic
      iii. Processing of samples for culture can be laborious

C. Two-step algorithm
   a. Step 1. Perform assay, such as Quik Chek, which combines detection of GDH antigen and EIA for toxin A and B or B alone
      i. If positive for both: sign out as positive
      ii. If negative for both, sign out as negative
      iii. If positive for antigen and negative for toxin, perform another confirmatory assay
   b. Step 2. Perform toxigenic culture or PCR for confirmation of toxigenic C. *difficile* and sign out according to results
      OR a two-step could be performance of a GDH assay and reflex a positive result to PCR or culture

D. Three-step algorithm
   a. Step 1 Perform GDH assay
      i. If negative, sign out as negative
      ii. If positive, move to next step
   b. Step 2. Perform EIA for toxin B or toxin A and B or CCNA
      i. If positive, sign out as positive
      ii. If negative, go to next step
   c. Step 3. Perform PCR or toxigenic culture and sign out accordingly

Anaerobic Gram-Negative Cocci. The most commonly encountered anaerobic gram-negative coccus is *Veillonella parvula*. Cells are <0.5 μm and colonies are small,

transparent to opaque, and yellowish gray. Some are catalase-positive; they are susceptible to kanamycin and colistin when the special antibiotic potency disks are examined. It is a nonfermenter, and positive for nitrate reduction. One new species, *V. montipelliernsis* is different from other *Veillonella* species in being resistant to colistin on the antibiotic disk test. *Acidaminococcus* spp. are nitrate- and catalase-negative, but do weakly ferment carbohydrates; the cells of this organism are larger than those of *Veillonella*. Cells of *Megasphaera* spp. are usually >1.7 μm, a very distinguishing characteristic; the organisms are fermentative, catalase and nitrate negative and this genus is susceptible to vancomycin, whereas most gram-negative cocci are resistant. A newly named species, *Negativicoccus* has very small cells, <0.4 μm and they can be microaerophilic. They are also colistin resistant on the antibiotic disk test. Other characteristics can be seen in Table 16-28.

Box 16-14 provides approaches to the identification of an anaerobic isolate in the routine clinical laboratory. There are also additional references for alternative approaches, especially for presumptive or less than full identification for instances where that is appropriate.[28,202,238]

Management of Diseases Involving Anaerobic Bacteria

Comments on Usual Antibiotic Susceptibility/Treatment Protocols for Anaerobes

Successful management of diseases involving anaerobic bacteria requires selection and treatment with appropriate antimicrobial agents, often in conjunction with removal of bacteria by drainage of abscesses, elimination of foreign bodies, debridement of necrotic tissue, and other surgical measures. It was once believed that most anaerobes had predictable antimicrobial susceptibility patterns and that accurate identification of isolates was all that was necessary to predict the susceptibility of individual isolates to various antibiotics. This is an oversimplification. While some antimicrobial agents are active against nearly all anaerobic bacteria (e.g., carbapenems, piperacillin-tazobactam, ampicillin-sulbactam, and metronidazole), there are several other antimicrobial agents that might be used in treatment, the susceptibility of which are not nearly as predictable. Most members of the *B. fragilis* group are resistant to penicillins and cephalosporins and there is increasing resistance to clindamycin. Resistance genes for tetracycline, clindamycin, cefoxitin, carbapenems, chloramphenicol, and metronidazole have been identified in many anaerobes and are usually found to be transferable in in vitro mating experiments.[214,215] Metronidazole remains highly active against most gram-negative anaerobic bacteria, including the *B. fragilis* group tested in the United

States, but resistance to metronidazole has emerged in *B. fragilis* strains in other countries.[190,214,215] A recent CDC report supported the fact that isolates of *B. fragilis* group that demonstrate metronidazole resistance in the United States should be investigated for acquisition outside of the United States. They report of a case of a multidrug-resistant (MDR)-*B. fragilis* group isolate in a patient whose intra-abdominal abscess and bloodstream infection were a result of health care given in India, prior to the patient traveling to the United States for diagnosis and treatment.[83] Metronidazole resistance does, however, occur innately in *Actinomyces* species, *Propionibacterium* species, *Lactobacillus* species, some of the anaerobic cocci, and other gram-positive anaerobes.[190] Although some of the fluoroquinolones, especially moxifloxacin, showed good activity against anaerobes initially, resistance to these agents has developed rapidly.[190,214] In a 2010 survey by Snydman et al., 27% of *B. fragilis* and 55% of *B. vulgatus* were resistant to moxifloxacin, as compared to an earlier surveillance in which >86% of the anaerobes tested were susceptible to moxifloxacin, again indicating variability of susceptibility in isolates tested.[190] The penicillins and ureidopenicillins, the second- and third-generation cephalosporins, and clindamycin may or may not be active against certain commonly encountered species of anaerobic gram-negative bacilli and clostridia; resistance to these agents can be quite variable.[5,214] Resistance to the carbapenems is very rare among anaerobes; however, in Japan, where imipenem is highly prescribed, resistance to it is as high as 3.2%.[215]

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BOX 16-13

Important Consideration Points to Consider About Processing Specimens for Toxigenic *C. difficile* Requests:

1. Do not perform any diagnostic tests on formed stools.
2. Only one specimen per patient per day. If PCR is used on all samples, no repeat testing should be done.
   a. Repeat PCR testing on patients with previous PCR positive results should not be performed for "test of cure."
   b. Repeat PCR testing on patients with previous PCR negative results should not be performed, unless there is a change in the clinical findings suggesting a new onset of disease.
3. Appropriate patients only should be tested: patient with diarrhea (>3 watery stools in a 24-hour period of time).
4. Rectal swabs should not be performed for diagnosis of toxigenic *C. difficile*,

although they might be used for epidemiologic studies.

5. Do not perform an enzyme immunoassay for toxin A/B as the only *C. difficile* test.
6. If a toxigenic cell culture neutralization assay (CCNA) is performed alone, there will be a reduced sensitivity as compared to toxigenic culture or PCR, but CCNA is very specific and can be used to confirm that culture isolates are toxigenic.
7. If a PCR alone is performed on all stool samples, carriers as well as infected individuals will be detected.
8. Toxigenic culture is still considered the gold standard (i.e., has the highest sensitivity and specificity) for recovery of *C. difficile*, but does have the longest turnaround time (TAT).
   a. Confirmation that isolates are toxigenic needs to be performed before reporting a positive result.
   b. Chromogenic media may be used to decrease TAT for initial recovery of *C. difficile*; if the media is selective for toxigenic *C. difficile* culture, this may further reduce the TAT.
9. In patients who have been hospitalized for >3 days, the detection of toxigenic *C. difficile* should be the primary request for new onset diarrhea that meets the criteria given above; the yield for common bacterial and parasite GI pathogens is low in this setting and often such requests will be rejected.

Because of the time it takes for recovery and identification of anaerobes, it is usually necessary for the attending physician to start antimicrobial therapy empirically before results of susceptibility testing are available. Therefore, tabulated susceptibility results and treatment protocols reported in the literature or in the local hospital, and clinical experience of the physician may be the basis for the initial choice of antibiotics. Excellent sources of additional information relative to selection of antimicrobial agents for treatment purposes have been published recently elsewhere.[182,214,215,232,302] Publication of antibiograms for anaerobes specifically should be developed by clinical laboratories, even if they include data of more than 1 year in order to accumulate enough data to validate the results. CLSI provides a document to help us with development of such an antibiogram and have published national statistics of anaerobe susceptibilities.[100] However, there is enough variability in the susceptibility patterns of clinically significant anaerobes that in vitro susceptibility testing of individual isolates is indicated at times to aid clinicians with the management of serious infections, particularly those that require prolonged therapy such as brain abscess, endocarditis, lung abscess, infections involving joints, osteomyelitis, infections involving prosthetic devices, vascular grafts, recurrent or refractory bacteremia or when infection does not respond to empirical therapy. A survey of isolates from intra-abdominal and diabetic

foot infections recently even demonstrated that there were not only differences in what organisms were found but also differences in susceptibility patterns of similar anaerobes from the different infections, providing another reason for performing susceptibilities on selected isolates.[97]

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TABLE 16-27 Phenotypic Characteristics of Anaerobic Gram-Positive Cocci

Species	IND	NIT	URE	PYR	Glucose FERM	GLC	Gram's Morph	Colonial Morph	Previous Name
Peptostreptococcus anaerobius[a]	–	–	–	–	–	A,IC	Large cocci in pairs and chains; usually much larger than other cocci	Gray with raised center; opaque; nonhemolytic; sweet odor	None
Parvimonas micra	–	–	–	+	–	A	Clusters, short chains or pairs; <6 μm	Small; domed; dull-white; yellow-brown "halo" around colonies	*Micromonas micros, Peptostreptococcus micros*
Finegoldia magna	–	–	–	+	–[w]	A	Pairs, chains, tetrads and clusters; ≥6 μm	Small white colonies; some may be translucent	*Peptostreptococcus magna*
Peptoniphilus spp.									
P. asaccharolyticus	d	–	–	–	–	A,b	Appear in clumps; may almost appear gram-negative due to poor staining	Small, white to yellow; convex; musty odor	*Peptostreptococcus asaccharolyticus*
P. gorbachii	D		–	–	–	A,b	Large cells, >7 μm; round, entire, opaque	Gray, flat, convex. circular, opaque; entire edge	New species
P .harei	d		–	–	–	A,b	Variable in size and shape	Flat, translucent colonies	*Peptostreptococcus harei*
P. indolicus	+	+	–	–	–	A,b	Clumps, like *P. asaccharolyticus*	Small convex white to yellow colonies	*Peptostreptococcus indolicus*
P. ivorii	–		–	–	–	IV	Variable in size; in clumps	Convex, slightly yellow	*Peptostreptococcus ivorii*
P. lacrimalis	–		–	–	–	A,b	Short chains or clumps	White to pink colonies	*Peptostreptococcus lacrimalis*
P. olsenii	D		–	–	–	A,b	Larger cocci; ≥7 μm	Gray, flat, convex; circular; opaque with a whiter central peak; entire edge	New species
***Anaerococcus* spp.**									
A. hydrogenalis	+	–	d	–	+	B,a	Variable size; tetrads, clumps and short chains	Gray-white; convex; unpleasant odor	*Peptostreptococcus hydrogenalis*
A. lactolyticus	–	NA	+	–	+	B,a	Short chains or clumps	Pink or white colonies	*Peptostreptococcus lactolyticus*
A. murdochii	–	–	–	+	+	B,A	Larger cocci, ≥7 μm	Gray with a whiter center; flat, convex, circular, opaque	New species

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A. octavius	d	NA	–	+[w]	+	B,a,c	Clumps	Yellow-white; glistening; raised; entire edge	*Peptostreptococcus octavius*
A. prevotii	–	–[+]	+	+	–	B,a	Variable size; clumps or tetrads	Nonglistening; gray, convex	*Peptostreptococcus prevotii*
A. tetradius	–	–	+	+[w]	+	B,a	Variable in size; clumps and tetrads	Nonglistening; gray; convex	*Peptostreptococcus tetradius*
A. vaginalis	d		–	–	+	B,a	Variable in size; clumps and tetrads	Nonglistening; gray; convex	*Peptostreptococcus vaginalis*
Atopobium parvulum[b]	–	ND	ND	ND	+	ND	Elongated cocci; short chains	Resemble lactobacilli on agar	*Streptococcus parvulum*
***Staphylococcus* spp.**									
S. saccharolyticus	NA	+	+	+	+	A	Clusters and tetrads	Small and smooth; nonhemolytic; glistening; opaque; coagulase-negative	*Peptostreptococcus saccharolyticus*
S. aureus subsp. *anaerobius*	NA	+	NA	+	+	NA	Clusters	Small white colonies	None
***Blautia* spp.**[c]									
B. producta	–	–	–		+	A	Ovoid; in pairs or chains	Gray and glistening	*Peptostreptococcus productus*
B. wexlerae	–	NA	+		+	A,S	Coccobacillus	Gray; opaque; umbonate; entire edge; esculin-positive	New species
Gallicola barnesae	w	NA	–	NA	–	A,B	Single and in pairs	NA	*Peptostreptococcus barnesae*
Murdochiella asaccharolyticus	+	–	–	–	–	L,a,b,s	Pairs and chains; <0.6 μm	Gray-white; convex; flat	New species

[a] *P. anaerobius* is inhibited by sodium polyanetholsulfonate (SPS), which is present in most blood culture bottle. It is the only species inhibited so that can be used as a distinguishing characteristic.
[b] *Atopobium parvulum* is a coccus; however, other members of this genus are considered non–spore-forming rods and will be described in those tables.
[c] *Blautia* is a genus of non–spore-forming rods, which have a lineage with Clostridium more than cocci. *B. wexlerae* and *B. producta* appear as cocci and are included here, but other species may be considered as rods and not cocci.
+, positive; –, negative; w, weak reaction; IND, indole production; NIT, nitrate reduction; URE, urease; PYR, pyrrolidonyl arylamidase test; Glucose FERM, glucose fermentation; GLC, from PYG broth.
Adapted from references 178 and 446.

TABLE 16-28 Characteristics of Anaerobic Gram-Negative Cocci

Species	CAT	NIT	Gluc FERM	Microaero Growth	Gas Prod	Main GLC Products	Antibiotic Disk Test	Size of Cells on Gram's Stain (μm)	Comments	Clinical Comment
Acidaminococcus spp.[a]	–	–	–	–	+	A,B (P,L)	R,S,S	<1.0 (ovoid and in pairs)	Entire, slightly raised colonies, round; transparent. GI flora	Found very rarely in clinical specimens with unknown significance
Anaeroglobus spp.	–	–	+[w]	–	–	A,P,IB,B,IV	R,S,S	<1.0 (ovoid—ellipsoidal cells in pairs and short chains)	Circular, convex, translucent colonies. Oral and GI flora	None
Megasphaera spp.[b]	–	–	+	–	+	A,P,B,V,C	V,S,S	>1.5 (chains; may stain gram-positive)	Circular, entire, translucent colonies. GI flora	Reported as a cause of endocarditis[50]
Negativicoccus spp.[c]	–	–	–	+	–	A,P	R,S,R	<0.5 (ovoid cells)	Small colonies; circular and convex; translucent. Recently described.	Isolated from blood culture of patient with underlying illness[94]
Veillonella spp.[b,c]	V	+	–	–	+	A,P, (L)	R,S,V[d]	<0.5 (spherical; pairs and short chains)	Smooth, entire, opaque colonies; butyrous, gray-white. Oral and GI flora; most commonly isolated	Bacteremia and endocarditis, as well as recovered in other clinical specimens, wherein the significance was unknown[94,312]

[a] *Acidaminococcus* can use amino acids as its main source of energy.
[b] These species can ferment lactate.
[c] These species can decarboxylate succinate.
[d] *V. montipellierensis* is resistant to colistin disk.
+, positive; –, negative; w, weak; V, some strains are positive and strains are negative; CAT, catalase; NIT, nitrate reduction; Gluc FERM, fermentation of glucose; Gas Prod, production of gas.
Antibiotic disks: Vanco, 5 μg disk; Kanamycin, 1,000 μg disk, Colistin, 10 μg disk; S, Susceptible; R, Resistant.
Adapted from reference 446.

BOX 16-14

Strategies for Identification of Anaerobic Isolates in Routine Laboratories

1. Aerotolerance testing confirms presence of an anaerobe and it is in pure culture or felt to be clinically relevant, warranting further identification (ID).
   Consider Gram's stain and colonial morphology to group the anaerobe.
   Observe selective and/or differential media where appropriate, such as BBE and EYA, and perform antibiotic potency disk test.
   Perform commercial ID kit and if identification agrees with above results, consider that as the identification, especially if the ID is a well-known species
   If GLC is available, could consider using this in conjunction with above.
   If commercial ID is performed and does not match Gram's stain and colonial morphology and/or the organism is not a common species, consider reporting to the genus (i.e., if certain of that level) or refer for sequencing or MALDI-TOF. This decision can also depend on request of clinician.
2. Aerotolerance testing confirms presence of an anaerobe and it is in pure culture or felt to be clinically relevant, warranting further ID.
   Consider Gram's stain and colonial morphology to group the anaerobe. Observe selective and/or differential media where appropriate, such as BBE and EYA, and perform antibiotic potency disk test.
   OR
   Go directly to nucleic acid sequencing or MALDI-TOF, if available, or refer to a reference or commercial lab for this testing.
   Compare ID to morphology and report ID; if new name for old genus and species, sign out with new name and old name in parenthesis; if new genus/species without prior designation, include on report any information known about the organism, such as description about Gram's morphology and/or potential clinical relevance.

For either scenario above, one could stratify workup based on whether the site of specimen collection was sterile or nonsterile; whether monomicrobial or polymicrobial culture; whether organism is considered clinically relevant by clinician. Full identification should be considered for blood culture isolates, CSF isolates (although rarely found); endocarditis and osteomyelitis.

Another indication for testing is the setting in which there has not been a clear-cut clinical precedent on which to base treatment decisions because the identification of the organism is new or an uncommon isolate is found in a disease usually attributed to another anaerobe or aerobe. In addition, clinical microbiologists experienced with anaerobes are encouraged to investigate the activities of newly marketed and/or investigational antimicrobial agents against anaerobes isolated in their hospital

laboratory. Lastly, there is a need to monitor susceptibility patterns of anaerobes at the local, regional, national, and worldwide levels.

Organisms to be considered for antimicrobial susceptibility testing, because of their virulence, or because they are commonly resistant to certain antimicrobial agents include species of the *Bacteroides fragilis* group, species of the pigmented *Prevotella-Porphyromonas* group, other *Prevotella*, *Fusobacterium mortiferum, F. varium* and *F. necrophorum*, *Bilophila, Sutterrella*, and some *Clostridium* species. Information on what antibiotics to test versus a particular anaerobe or groups of anaerobes can be found in the CLSI document and in handbooks that provide information on therapeutic options for various anaerobic infections.[32,100,182]

Methods for Antimicrobial Susceptibility Testing of Anaerobes

At present, CLSI recommends the agar dilution method for antimicrobial susceptibility testing (AST) for most anaerobes in the routine clinical laboratory. However, for *B. fragilis* group isolates, they do provide a broth microdilution method as an alternative. Also included in the newest

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document (CLSI M11-A8, 2012) is a method for β-lactamase testing of anaerobic isolates.[100] AST is suggested in specific situations in the document; minimally, laboratories should consider testing isolates (or sending isolates out to reference laboratories) from sterile sites, especially BSI and in infections with uncommon anaerobes for which the susceptibility is unknown. On a routine basis, some surveillance studies of susceptibilities should be performed to monitor for the development of new resistance patterns.

The medium recommended for agar dilution is Brucella agar supplemented with 5 μg of hemin and 1 μg of vitamin K_1 per mL, and 5% (v/v) laked sheep blood. Dilutions of appropriate antibiotics and sheep blood are added after the agar has been prepared. After preparation, agar plates can be stored for up to 72 hours at 2°C to 8°C. Inoculation of the plates is done using a replicating device that will deliver 1 to 2 μL of inocula (e.g., Steers replicator). This inoculum "spot" is delivered to obtain approximately 10^5 CFU for *B. fragilis* and *B. thetaiotaomicron*; some variation in colony counts may occur with other organisms. After about 10 minutes to allow for absorption of the inocula, plates are inverted and incubated in an anaerobic jar or chamber at 35°C to 37°C for 42 to 48 hours. The CLSI document includes images of end points and their interpretations to assist in reading the plates to best determine the MIC of each isolate. These are very helpful for the laboratory to use especially when first employing anaerobic AST in your laboratory or training new personnel. With agar

dilution, at least 30 isolates can be tested at one time, making this method excellent for performing those surveillance studies. For broth microdilution of the *B. fragilis* group, the recommended medium for this Brucella broth supplemented with 5 μg/mL hemin, 1 μg/mL vitamin K_1, and lysed horse blood (5%). Methods for performing both agar and broth microdilution AST are provided in appendices of the CLSI document.[100] Another, perhaps more user-friendly method for performing anaerobic AST is the Etest (bioMérieux Vitek) gradient test strip method. Procedures for this are not provided in the CLSI document, but rather, from the manufacturer, or from the literature. Both the Etest procedure and the broth microdilution procedure are practical ways for testing individual isolates against a limited number of antimicrobial agents; these and other methods, including the spiral gradient methods are reviewed in detail elsewhere.[61,263,266] Broth disk elution and disk agar diffusion techniques have been used in the past but should not be used for routine anaerobe testing despite their convenience. Most anaerobes other than *Bacteroides* spp. grow too slowly for the disk diffusion procedure to work; the Bauer–Kirby interpretive charts were not designed for anaerobes, and interpretive charts based on standardized media and methods for disk diffusion testing of anaerobes are lacking; also, there has been poor correlation between zone size measurements and the results from minimal inhibitory concentration dilution tests.[232]

In 2008, a survey of whether routine laboratories performed anti-anaerobe susceptibility testing demonstrated that susceptibility testing was performed in-house by 21% of hospital laboratories and sent out to reference laboratories an additional 20% of the time. Susceptibility testing was attempted for all blood culture isolates by both hospital (21% of total laboratories) and reference laboratories, but only performed by 17% for sterile body site and 14% of the time for wound isolates. Etest was used most often followed by broth microdilution.[191] For details of the recommended procedures, the reader is referred to the most current Clinical Laboratory Standards Institute document.[100]

Antimicrobial Susceptibility Results for Groups of Anaerobes

Susceptibility of the Gram-Negative Bacilli. *B. fragilis* group isolates produce β-lactamases and consequently are resistant to penicillin (ampicillin, amoxicillin) and most cephalosporins, but are usually susceptible to ampicillin-sulbactam and piperacillin-tazobactam. They are uniformly susceptible to metronidazole and the carbapenems (at least in the United States), but increasingly resistant to clindamycin. In the most recent National Survey for the Susceptibility of *Bacteroides fragilis* group (2005 to 2007) that tracks emergence of resistance among this group of anaerobes, the carbapenems and piperacillin-tazobactam were again found to be the most active anti-anaerobic agents, but there was some resistance seen in certain members of the group

to other agents: *P. distasonis* had a 21% resistance to ampicillin/sulbactam and proved to be the most resistant species overall among the >5,000 isolates included in their study; *B. uniformis* and *B. egegerthii* were approximately 7% resistant to tigecycline; and *B. vulgatus* was >50% resistant to moxifloxacin. Overall, the resistance to clindamycin by the *Bacteroides* spp. in general was high, at >40%. There was some metronidazole-resistant *Bacteroides* spp. found in their survey.[444] The authors suggest that the increased resistance seen in non-*B. fragilis* specifically makes a case for species-level identification of isolates in the *B. fragilis* group which is not always timely or possible without the use of newer identification tools in the routine laboratory. Attempts at such differentiation should at least be considered for isolates from blood and other sterile sites. In a survey of the in vitro susceptibility of anaerobes in eight Belgian hospitals, there were not many changes noted in 2011 to 2012 as compared to a survey in 2004. However, *Prevotella* and other anaerobic gram-negative bacilli were found to have declining susceptibility to clindamycin, with 61% susceptible in 2011 to 2012 as compared to 82% in 2004.[528] In addition, *Fusobacterium* spp. had decreased susceptibility to moxifloxacin in 2011 to 2012, with 71% susceptible compared to 90% susceptibility in 2004.[528] Data from a survey in Taiwan reported that the proportion of susceptible isolates of *Bacteroides* spp, *Prevotella* spp. and/or *Fusobacterium* spp. to many antibiotics, including clindamycin and ampicillin-sulbactam, decreased from 2000 to 2007. In addition, there were some carbapenem resistant isolates found among all three groups.[263] Species of *Prevotella* have been found that are resistant to metronidazole and this certainly needs to be monitored in the future along with all trends toward resistance.[345] Data on metronidazole susceptibilities from ~1,100 anaerobes in 2001 in the United States showed that *Bacteroides fragilis, B. distasonis, B. ovatus, B. thetaiotaomicron, B. vulgatus, Fusobacterium,* and *Clostridium* spp. were among the groups that met the FDA criteria of >90% susceptibility (MIC ≤8 μg/mL). *Eubacterium* and the gram-negative cocci were the only isolates that did not meet the criteria for susceptibility.[147]

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B. ureolyticus are uniformly susceptible to the various penicillins, cephalosporins, and other antimicrobial agents tested (i.e., clindamycin, chloramphenicol, metronidazole, and even aminoglycosides), whereas the related *C. gracilis* isolates were commonly resistant to the penicillins, cephalosporins, and clindamycin.[234]

Susceptibility of Anaerobic Gram-Positive NSF Bacilli. Gram-positive NSF are so diverse that a single susceptibility pattern does not exist; in general, isolates are susceptible to penicillins (with and without β-lactamase inhibitors combinations),

metronidazole and carbapenems, but resistant to aminoglycosides and colistin. As mentioned above, however, *Actinomyces, Propionibacterium*, *Atopobium,* and other more aerotolerant strains of NSF are resistant to metronidazole. These species and many others are susceptible to vancomycin and other glycopeptides, but there are notable exceptions to this as well; for example, *Lactobacillus* spp. are often resistant to vancomycin.[499]

Susceptibility of *Clostridium* spp. Including C. *difficile*. *Clostridium* spp. are usually susceptible to penicillins and cephalosporins, metronidazole, carbapenems, and other agents. However, this varies among the species. In a survey of *Clostridium* spp. susceptibilities in 2011 to 2012 in eight Belgian hospitals, not many changes were noted except that moxifloxacin susceptibility had decreased from 88% to 66%.[528] The susceptibility of *C. perfringens* to clindamycin is still high, but many other species of *Clostridium*, including *C. difficile*, can be resistant. *C. difficile* is most often treated with vancomycin or metronidazole; there is still controversy about whether one is better than the other, although there are studies which have shown equivalent efficacy. There is evidence, however, that vancomycin is superior to metronidazole in patients with severe disease.[533] In vitro susceptibility is not often performed because all isolates are predictably susceptible to these agents as well as many penicillins, cephalosporins and carbapenems. In the anaerobic bacteria antibiogram in the Appendix of the associated CLSI document, *C. difficile* isolates were 100% susceptible to metronidazole, ampicillin/sulbactam, piperacillin/tazobactam, cefoxitin. Only 5% were susceptible to clindamycin, whereas 78% were susceptible to moxifloxacin. All were 100% resistant to penicillin. MICs to ertapenem were very low, but isolates were resistant to imipenem and meropenem.[100]

A study in Ireland found an outbreak of toxin A-negative and toxin B-positive strains of *C. difficile* that were resistant to fluoroquinolones, clindamycin, and clarithromycin. Toxin A- and B-positive isolates, not involved in the outbreak, had much lower MICs to the fluoroquinolones and were susceptible to the macrolides and clindamycin.[136] It is difficult to know how widespread this pattern of susceptibility is since most laboratories do not isolate *C. difficile* nor perform susceptibility tests.

About 22% to 26% of patients who experience *C. difficile* disease will go on to have at least one recurrence due to either relapse with same strain or reinfection with a new strain.[35,75] For recurrent disease, many nonantibiotic methods have been tried along with or in place of vancomycin and metronidazole. These include the use of probiotics, fecal implantation, anion exchange resins to absorb the toxins, immune therapy with intravenous immunoglobulins, the yeast *Saccharomyces boulardii* and pulsed doses or tapering doses of vancomycin, all with variable success.[35,38,194] Fidaxomicin, a drug more recently approved for treatment of *C. difficile*, was found

noninferior and as safe as vancomycin in a double-blind controlled study.[107,239] In patients who are being treated for other infections along with their *C. difficile*, fidaxomicin was found to be more efficacious than vancomycin in achieving clinical cure, suggesting that the action of vancomycin on toxigenic *C. difficile* may be diminished with concomitant antibiotic use.[347] There is no in vitro activity of fidaxomicin versus gram-negative aerobes or anaerobes nor against yeast.[189] There is some evidence that neither fidaxomicin is nor vancomycin are as effective in patients who are infected with the hypervirulent, NAP1 strain of *C. difficile*, as compared to infections caused by non-NAP1 strains.[375]

Susceptibility of Anaerobic Cocci. The in vitro susceptibility of the anaerobic cocci to antimicrobial agents has been described in the past in review articles.[348,351] According to recent guidelines, the antimicrobial agents of choice for the empirical treatment of patients with infections involving anaerobic cocci are penicillin (e.g., penicillin G, ampicillin, amoxicillin) or clindamycin.[32,182] Although not commonly isolated as a pathogen, several *P. anaerobius* isolates have demonstrated resistance to penicillin G.[5] Metronidazole is active against >90% of the obligately anaerobic cocci, but is not active against most strains of the microaerophilic streptococci (e.g., *Streptococcus intermedius*).[182] Other compounds that are active in vitro against most of the anaerobic cocci include chloramphenicol, imipenem, ampicillin/sulbactam, piperacillin/tazobactam and cefoxitin, but not cefoperazone, cefotaxime or cefotetan.[5,348] Compounds that show promise against the gram-positive anaerobic cocci include moxifloxacin, quinupristin/dalfopristin and linezolid.[182,320,446] A study in the Netherlands of susceptibilities of 115 anaerobic gram-positive cocci demonstrated some variability in MICs among the various genera tested. All were resistant to doxycycline. Of the three most often tested, *F. magna* had the highest MIC_{90} values for penicillin G, amoxicillin-clavulanic acid, clindamycin, tigecycline, levofloxacin and moxifloxacin. *P. micra* has the lowest MIC_{90} for levofloxacin, metronidazole, doxycycline and amoxicillin-clavulanic acid. *P. harei* has the highest MIC_{50} for levofloxacin and doxycycline and the lowest MIC_{90} for cefoxitin, ertapenem, meropenem and chloramphenicol. This isolate can be easily mistaken for *P. asaccharolyticus*, and the authors point this out in the paper as well. There was one strain of *P. micra* found resistant to metronidazole.[489]A European study with data from 10 countries of the susceptibilities of 299 anaerobic gram-positive cocci (predominantly *F. magna* and *P. micra*) showed susceptibility overall to imipenem, vancomycin, metronidazole, and linezolid. There was a little resistance (7%) to penicillin and clindamycin, mostly among *F. magna* isolates from blood, abscesses and soft tissue infections.[53] *Veillonella* species are resistant to vancomycin and some of

the quinolones. The US study of metronidazole mentioned above did show resistance to metronidazole among some of the anaerobic cocci.[147]

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Comments on Susceptibility Testing of Anaerobes

What we can anticipate in the future is much more information about the response to antibiotics by newly named organisms with the increased use of sequencing and MALDI-TOF for anaerobic identification, and subsequent publication of their susceptibilities to antibiotics. In addition, as more molecular methods are developed for detection of specific resistance markers, these may be used alone or in combination with more conventional susceptibility testing methods. Such methods were used in a study of carbapenem resistant *B. fragilis* isolates in Spain and demonstrated agreement with AST testing. The authors concluded that AST should be done at least periodically in order to detect emerging resistance.[481] An example of an antibiogram for anaerobes that can then be developed for use by clinicians and laboratories for empiric treatment and for monitoring purposes, is given in the 2012 CLSI document in Appendix D for the *B. fragilis* group and Appendix E for other anaerobes.[100] Table 16-29 provides a summary of overall susceptibilities for selected anaerobes based on those data and from the literature as noted in the table for specific isolates.

Summary

First, much credit for this chapter needs to be given where it is due, to Dr. Stephen Allen, who passed away in 2013 and left behind a great void in the area of anaerobic bacteriology. Much of what he wrote in prior editions of this text was work that he pioneered throughout his years at the University of Indiana. The Image Plates throughout and figures were mainly his and those of colleagues including Jean Siders and Linda Marler and we owe all of them for what they contributed to this great field. I only hope that Dr. Allen is not unhappy with the results of this 7th edition. Dr. Allen, you will be greatly missed.

The contribution of anaerobic bacteria to the maintenance of the health and well-being of the human host as well as their role in various infectious syndromes continues to be well recognized in clinical microbiology. Their taxonomy can be complex and confusing, only heightened by newer methods of genetic analysis and identification. Sometimes there is a disparity between the latter and the more conventional morphologic and phenotypic approaches to identification and classification. Keeping up with the changes in taxonomy can appear over-whelming at times. We are in a

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where the new and old are colliding or intermingling, depending on one's perspective, but it is important for all of us to try to understand the differences between the old and new methods and devise a rational approach to their incorporation in our routine laboratories. In this chapter, an attempt was made to provide information available in older editions for those who wish to continue to pursue presumptive identifications of anaerobes using spot tests, biochemicals and even GLC, which can be very appropriate in routine situations. We have, however, also tried to delineate places where full identification may be needed and to emphasize that the use of sequencing and/or MALDI-TOF methods may be needed. Understanding the principles and results of the newer methods is as essential in anaerobic bacteriology as it has become in the identification of aerobes, fungi, and mycobacteria.

TABLE 16-29 Percent Susceptibility of Selected Anaerobes

Organism	Amp/Sulb	Pip/Tazo	Cefox	Carb	Pen	Clinda	Moxi	Metron
B. fragilis group	86	95	65	>96	0	~50	~50	100
B. fragilis	89	98	85	>96	0	>60	~50	100
Fusobacterium[a]	100	100	100	100	40	100	95	100
Prevotella	98	99	99	100	40	66	59	100
Gram-positive cocci	98	100	100	100	96	78	82	98
Veillonella	100	61	100	100	57	89	79	86
P. acnes[b]	100	100	100	100	100	100	100	3
C. perfringens	100	100	100	100	100	96	99	100
C. difficile	100	100	100	V[c]	0	5	78	100
C. bifermentans MCM; stevens	100	100		100	100	100		100
C. clostridioforme MCM; stevens	75				67	90		100
C. ramosum	100	100		100	100	82		98
C. septicum						100		100
C. sordellii	100				100	94		95
C. tertium	100				100	100		100

[a] *F. nucleatum* and *F. necrophorum*.
[b] Isolates of *P. acnes* are susceptible to vancomycin.
[c] *C. difficile* is resistant to imipenem and meropenem, but MIC are very low versus ertapenem. Data for all agents is from intestinal sources, but does not imply efficacy; vancomycin MICs are all low and indicate susceptibility.
Modified from reference 100, 463, and 464. Data is given in % susceptible.
Amp/Sulb, ampicillin/sulbactam; Pip/Tazo, piperacillin/tazobactam; Cefox, cefoxitin; Carb, carbapenems; Pen, penicillin (or ampicillin); Clinda, clindamycin; Moxi, moxifloxacin; Metron, metronidazole.

The chapter has increased the amount of information on specific diseases such as

Lemierre's syndrome, *C. difficile*-associated diarrhea and bacterial vaginosis, along with some increased information on the association of newly named organisms and the associated infections. There is a combination of older and newer references given throughout the chapter because in some cases, there may not be anything new, but the older classical works need to be shared and remembered. Finally, we have attempted to update information on developments in anaerobic antibiotic resistance and its detection and surveillance. Not everything that is new in the area of anaerobic microbiology could be included, but for those of us who have been anaerobe addicts over the years, the authors hope this chapter will be helpful to keep that enthusiasm going.

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CHAPTER 17

Antimicrobial Susceptibility Testing

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Evolution and Spread of Antibiotic Resistance

Mechanisms of Action of Major Antibiotic Classes

Genetic Basis of Resistance to Major Antibiotic Classes

Mechanisms of Antibiotic Resistance in Bacteria

- Prevention of Access to the Target
  - Porins
  - Efflux Pumps
- Antibiotic Inactivation by Destruction or Modification
  - β-Lactamases
  - Aminoglycoside Modifying Enzymes
  - Other Enzymes
- Target Modification
  - Penicillin-Binding Proteins
  - DNA Gyrase
  - Other Targets
- Bypass as a Resistance Mechanism

Multiple Antibiotic Resistance Mechanisms in Bacteria

Laboratory Guidance of Antimicrobial Susceptibility Testing

Antibiotic Susceptibility Testing Methods

Standardization of Antibiotic Susceptibility Testing Methods

Growth Medium
Specialized Medium and Additives
pH
Serum
Cation Concentration
Environmental Conditions
Inoculum
Antimicrobial Agents
Selection of Antimicrobial Agents
Reference Strains
Quality Control
Quality Assurance and Proficiency Testing

Antimicrobial Susceptibility Test Procedures

Diffusion Susceptibility Methods

Disk Diffusion Susceptibility Test
Gradient Diffusion Susceptibility Test

Dilution Susceptibility Testing

Agar Dilution Susceptibility Test
Macrodilution Broth Susceptibility Test
Microbroth Dilution Susceptibility Test

Automated Systems

Vitek 2
MicroScan Walkaway SI
BD Phoenix

Choice of Test

Detection of Specific Types of Antibiotic Resistance

β-Lactamase Testing

Detection of Resistance in Gram-Positive Bacteria

Staphylococci

Methicillin (Oxacillin)-Resistant Staphylococci
Decreased Vancomycin Susceptibility in Staphylococci
Inducible Clindamycin Resistance

Enterococci

β-Lactam Resistance
High-Level Aminoglycoside Resistance
Decreased Susceptibility to Vancomycin
Reporting
Molecular Detection of Vancomycin Resistance Genes
Enterococcal Resistance to Newer Agents
Streptococci
Streptococcus pneumoniae
Decreased Susceptibility to Penicillin
Decreased Susceptibility to Macrolides and Lincosamides
Other Viridans Group Streptococci
β-Hemolytic Streptococci
Reporting
Fastidious Gram-Positive Bacteria
Detection of Resistance in Gram-Negative Bacteria
Haemophilus influenzae and *Haemophilus parainfluenzae*
Reporting
Fastidious Gram-Negative Bacteria
Moraxella
Neisseria
Enterobacteriaceae
β-Lactamases
Extended-Spectrum β-Lactamases
AmpC β-Lactamase Detection
Carbapenemase Detection (*Enterobacteriaceae*)
1074
1075
Detection of Fluoroquinolone Resistance in *Salmonella* spp.
Gram-Negative Nonfermenters
P. aeruginosa (Other Pseudomonads)
Detection of MBL-Producing *P. aeruginosa*
Acinetobacter baumannii
Susceptibility Testing of Cystic Fibrosis Isolates

Bacterial Agents of Bioterrorism

Antibiotic Susceptibility Reporting

Formatting Antibiotic Susceptibility Reports

Clinically Relevant Antibiotic Reporting

Selective Reporting of Antibiotics

Cumulative Antibiogram Reporting

Antibiotic Stewardship

Future Perspectives

Evolution and Spread of Antibiotic Resistance

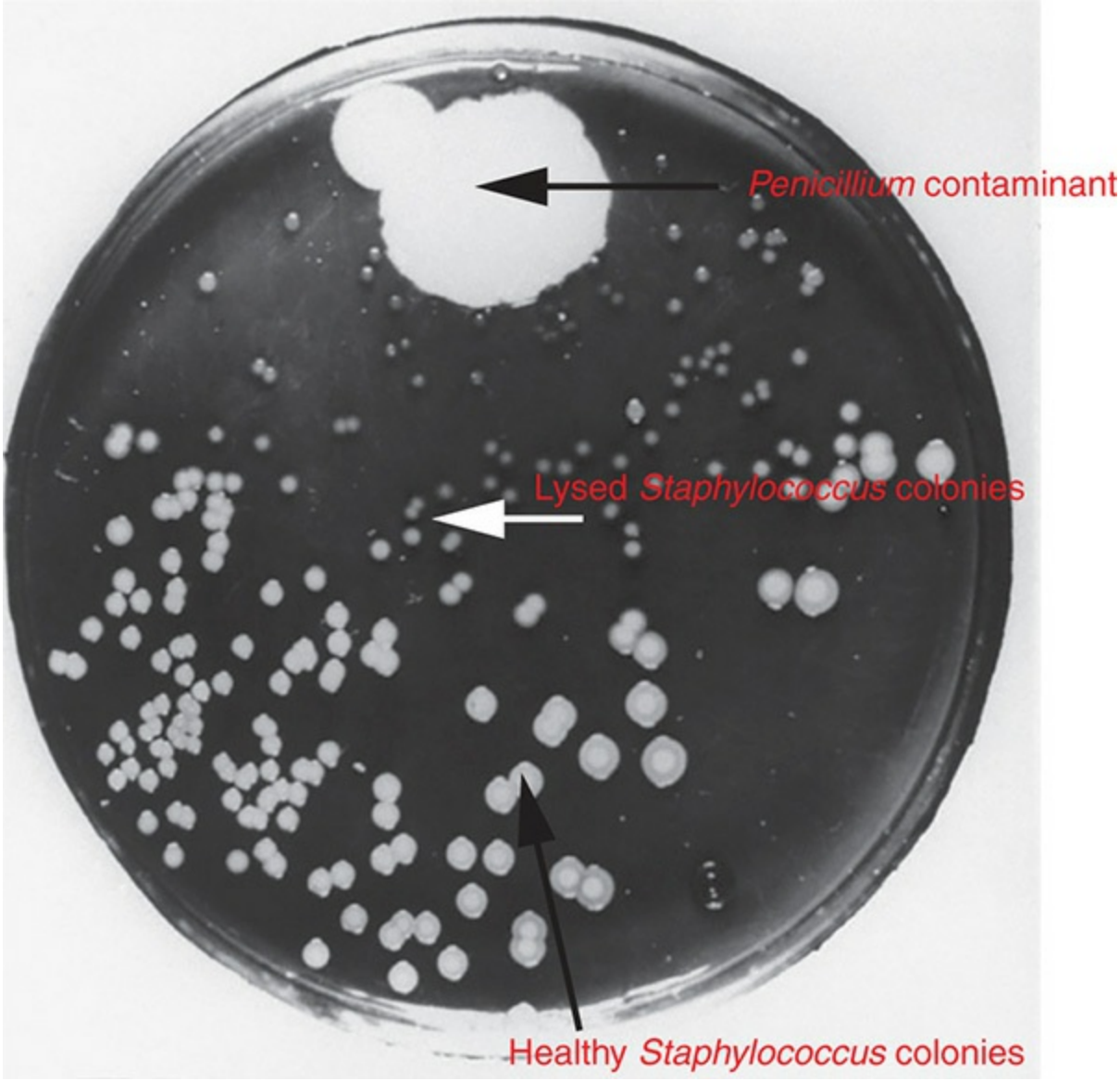
An antibiotic is a compound that is either derived from nature or chemically synthesized, which acts on bacteria through inhibiting normal biochemical functions (i.e., cell wall synthesis, protein synthesis, DNA replication/transcription, or cellular respiration). Bacteria in turn have developed several mechanisms to increase their resistance to the action of commonly prescribed antibiotics, and some types of resistance have globally spread to such an extent that the effectiveness of many drugs and even drug classes are being rapidly eroded. Rapid emergence of many types of antibiotic resistance is one of the major threats to modern medicine and public health of this age.[340] Without an acceleration in the development of new compounds that are effective against many different types of antibiotic-resistant organisms (AROs), modern medical progress will be slowed because of the risk of an adverse clinical outcome from an ARO infection.[66,101,257,562,614]

It is remarkable that we have gone from one of the great medical advances of the 20th century—the discovery of penicillin—to this current worldwide situation over the course of several decades. In 1928, Alexander Fleming observed that a strain of *Penicillium notatum* was producing a diffusible bacteriolytic substance capable of killing bacteria (Fig. 17-1).[196] The discovery of penicillin and its manufacture in sufficient purity to be used as a human drug heralded the advent of the modern antibiotic era. Penicillin production was limited and extremely expensive during World War II, but other antibiotics were discovered and the antibiogram profiles of various organisms were established. Streptomycin was discovered in 1943 because of Waksman's interest in soil organisms,[645] and gramicidin and tyrocidin soon followed. Chlortetracycline (Aureomycin) was also discovered by Duggar at Lederle Laboratories (Pearl River, NY) in 1944. Since that time, the pharmaceutical industry has developed a large number of antimicrobial agents (Table 17-1), but unfortunately

the ability and feasibility of producing novel compounds has not kept pace with the emergence of many types of drug resistance.[257,614]

Despite the initial optimism that antimicrobial agents would put an end to bacterial infection, it did not take long before resistant bacterial strains emerged after introduction and clinical prescription of new drugs. Rapid development of various types of antibiotic resistance has been driven by inaccurate prescribing and overuse of these "life-saving" drugs both in human and veterinary medicine as well as in animal husbandry. Several public health and clinical groups have now published guidelines for prudent prescription of antibiotics because of the recent recognition that chemotherapeutic resources must be managed wisely to control the development and spread of antibiotic resistance.[155,340,365,521,552,614]

Antibiotic susceptibility testing of clinical pathogens then became a clinical necessity in order for effective therapy to be prescribed. It is important to emphasize, however, that antimicrobial agent susceptibility tests are intended to be a guide for the clinician, not a guarantee that a particular agent or drug combination will be effective in therapy. Clinical microbiology laboratories can be of great assistance to clinicians. They can evaluate the *Klebsiella* in vitro interactions between an isolated microbe and antimicrobial agents that would be appropriate for treatment of an infection in vivo. The laboratory data also help the clinician decide whether the selected dose of an antimicrobial agent is adequate to provide effective levels at the site of infection. This chapter outlines procedures for the routine antimicrobial susceptibility testing of aerobic and facultative anaerobic bacteria. Testing of anaerobic bacteria, mycobacteria, and fungi is discussed in Chapters 16, 19, and 21, respectively.



■ **FIGURE 17-1** Fleming's discovery of the action of the antibiotic penicillin produced by the mold *Penicillium*. *Staphylococcus* growing around the mold, which was contaminating the culture, is being lysed by the antibiotic. *Staphylococcus* colonies growing further away from the mold are unaffected.

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TABLE 17-1 Major Classes of Antimicrobial Agents and Their Antibacterial Activity

Class	Major Subclasses	Agents Included	Mechanism of Action	Spectrum of Activity
Penicillins	Penicillin	Penicillin G, penicillin V, procaine penicillin, benzathine penicillin	Inhibits cell wall synthesis by binding to PBPs in the cell wall and inhibits cross-linking	Gram-positive aerobic bacteria including streptococci and enterococci. Most staphylococci are now resistant. Susceptible anaerobes (*Clostridium, Peptoniphilus, Finegoldia, Peptostreptococcus, Fusobacterium*), *Pasteurella, Treponema pallidum* (syphilis) and *Borrelia* (Lyme disease).
	Aminopenicillin	Amoxicillin, ampicillin	Same as penicillin	Same as penicillin but gram-negative spectrum also includes β-lactamase-negative *H. influenzae*, some *Enterobacteriaceae* genera (*Proteus mirabilis, E. coli, Salmonella, Shigella*) but susceptibility must be confirmed before use.
	Ureidopenicillin	Azlocillin, mezlocillin, piperacillin	Same as penicillin	Enhanced gram-negative activity including most *Enterobacteriaceae*. Piperacillin is active against *P. aeruginosa*.
	Carboxypenicillin	Carbenicillin, ticarcillin	Same as penicillin	Enhanced gram-negative activity but rarely used. Ticarcillin is active against enterococci.
	Penicillinase-stable penicillins	Cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin	Same as penicillin	Used for serious infection due to methicillin-sensitive *S. aureus*. No activity against enterococci, gram-negative or anaerobic bacteria.
β-Lactam/β-lactamase inhibitor combinations		Amoxicillin-clavulanic acid Ampicillin sulbactam Ceftaroline-avibactam Ceftazidime-avibactam Piperacillin-tazobactam Ticarcillin-clavulanic acid	Restores activity of the primary β-lactam antibiotic by inhibition of the β-lactamase activity via the inhibitor binding to enzyme's active site	Addition of a β-lactamase inhibitor extends the spectrum of the primary compound. Ticarcillin-clavulanic acid has activity against some *S. maltophilia*. Amoxicillin-clavulanic acid has broad activity against oral aerobes and anaerobes causing bite wound infection. Ampicillin sulbactam active against multidrug resistant *Acinetobacter* spp. Piperacillin-tazobactam has an extended gram-negative spectrum that includes *P. aeruginosa*, MSSA, and some anaerobes including *B. fragilis*.
Cephems	First generation	*Parenteral:* cephalothin, cefazolin, and cephapirin. *Oral:* cephalexin, cephradine, cefadroxil	Inhibits cell wall synthesis by binding to PBPs in the cell wall and inhibit cross-linking	Gram-positive bacteria including MSSA, β-hemolytic streptococci (A, B, C, and G), *Klebsiella* spp., and some *E. coli* and *Proteus mirabilis* but susceptibility must be checked before use.
	Second generation	*Parenteral:* cefamandole, cefonicid, cefuroxime. *Oral:* cefaclor, cefdinir, cefprozil, cefuroxime		Improved gram-negative activity with some loss of gram-positive activity compared to first-generation agents. Cefuroxime has good activity against community-acquired respiratory pathogens (MSSA, *H. influenzae*, and *S. pneumoniae*).
	Third generation	*Parenteral:* cefperazone, cefotaxime, ceftazidime, ceftizoxime, ceftriaxone. *Oral:* cefpodoxime, ceftibuten, cefditoren, cefetamet.		Enhanced gram-negative activity compared with second-generation agents. Ceftazidime has less gram-positive activity but is the only drug with *P. aeruginosa* activity. Cefotaxime/ceftriax; both have enhanced CNS penetration for treatment of bacterial meningitis pathogens. Good streptococcal activity and moderate activity against MSSA. Cefixime and ceftibuten have the best gram-negative activity of oral cephalosporins but neither is very active against gram-positive bacteria. Cefpodoxime and cefdinir have better gram-positive activity against staphylococci and streptococci.
	Fourth generation	*Parenteral:* cefepime. *Oral:* cefixime		Cefipime is highly active against gram-negative bacteria including *P. aeruginosa* and *Enterobacter* spp. Gram-positive activity is similar to ceftriaxone/cefotaxime.
	Cephamycin	Cefoxitin, cefotetan, cefmetazole		Expanded gram-negative and anaerobic activities useful for obstetric-gynecologic and colorectal surgery prophylaxis. Some resistance to *B. fragilis* make these agents less optimal for primary treatment of intra-abdominal and pelvic infections.

	Cephalosporins with activity against MRSA	*Parenteral:* ceftaroline, ceftobiprole		Only cephalosporins with activity against MRSA. Ceftaroline also has activity against CAP pathogens. Ceftobiprole has been approved for use in Canada and Europe but not in the United States.
Monobactams		*Parenteral:* aztreonam	Same as cephems	Active against most gram-negative bacteria including *P. aeruginosa*. Not active against gram-positive bacteria or anaerobes.
Penems	Carbapenem	*Parenteral:* doripenem, ertapenem, imipenem, meropenem, razupenem	Same as cephems	Very broad spectrum of activity against most gram-positive and gram-negative bacteria and anaerobes. Ertapenem is not active against enterococci, and *Acinetobacter* spp. or *P. aeruginosa*.
	Penems	*Parenteral:* sulopenem. *Oral:* faropenem	Same as cephems	Similar to carbapenems
Aminocyclitols		Spectinomycin	Same as aminoglycosides	Used for treatment of genital *N. gonorrhoeae* infections due to susceptible strains
Aminoglycosides		Amikacin, gentamicin, kanamycin, netilmicin, plazomicin, streptomycin, tobramycin	Inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome	Active against most aerobic gram-negative bacilli, *Nocardia* (amikacin), agents of bioterrorism (*Brucella* [streptomycin], *Francisella tularensis* [streptomycin], *Yersinia pestis* [streptomycin]), and some mycobacteria, but the activity is variable depending on the drug. Synergistic with some β-lactam drugs and vancomycin against enterococci (gentamicin and streptomycin), *P. aeruginosa* and some other gram-negatives and staphylococci, but these drugs are not used alone for gram-positive infections.
Ansamycins		Rifampin	Inhibits protein synthesis by binding to the β-subunit of DNA-dependent RNA polymerase	Active against mycobacteria and staphylococci
Folate pathway inhibitors		Sulfonamides, trimethoprim, trimethoprim-sulfamethoxazole	Interferes with folic acid synthesis at sequential steps in the pathway	Broad-spectrum gram-negative activity including many *Enterobacteriaceae*, *H. influenzae*, *Moraxella catarrhalis*, and *Stenotrophomonas* but not *P. aeruginosa*. Moderate activity against staphylococci including coagulase-negative strains and MRSA, but it has no activity against group A streptococci or enterococci. Also active against *Listeria monocytogenes*, *Nocardia*, and *Pneumocystis jiroveci* (PJP).
Glycopeptides	Glycopeptide	*Parenteral:* vancomycin	Inhibits cell wall synthesis at an earlier step than β-lactam compounds	Active against staphylococci including coagulase-negative strains and MRSA, susceptible enterococci, and *S. pneumoniae* including highly penicillin-resistant strains. Not active against *Lactobacillus*, *Leuconostoc*, *Actinomyces* and VRE. Also active against gram-positive anaerobes and oral formulation is used to treat *C. difficile* induced diarrhea.
	Lipoglycopeptides	Dalbavancin, oritavancin, teicoplanin, telvancin, ramoplanin	Same as vancomycin	Similar to vancomycin
Lincosamides		*Parenteral and oral:* clindamycin	Inhibits protein synthesis by binding to the 50S ribosomal subunit	Active against aerobic and anaerobic gram-positive bacteria including *Actinomyces* spp., *Clostridium* except for *C. difficile*, *Peptostreptococcus*, staphylococci, and group A streptococci, but resistance is increasing.

Lipopeptides		*Parenteral:* daptomycin	Inhibits nucleic acid and protein synthesis by causing rapid bacterial cell membrane depolarization and an influx of potassium ions	Active against aerobic gram-positives, including MRSA, group A *Streptococcus*, and VRE
	Polymyxins	Colistin, polymyxin B	Disrupts bacterial cell membrane permeability	Broad-spectrum activity against gram-negative aerobic bacteria
Macrocyclic		Fidoxomicin	Inhibits protein synthesis by blocking RNA polymerase	Active against gram-positive bacteria especially *Clostridium difficile*
Macrolides		*Parenteral and oral:* erythromycin (parent compound for other macrolides) *Oral:* azithromycin, clarithromycin, dirithromycin, erythromycin	Inhibits protein synthesis by binding to the 50S ribosomal subunit	Active against gram-positive bacteria including β-hemolytic streptococci, susceptible *S. pneumoniae* strains, and some MSSA strains. Active against atypical infections, including *Bordetella pertussis*, *Campylobacter jejuni*, *T. pallidum*, *Ureaplasma*, *M. pneumoniae*, *Legionella*, *Chlamydia* spp., and some mycobacteria. Azithromycin and clarithromycin have better activity against *H. influenzae* and mycobacteria than erythromycin.
	Ketolide	*Oral:* telithromycin	Same as macrolides	Similar to macrolides except it has enhanced activity against *S. pneumoniae* including penicillin-resistant strains
	Fluoroketolide	Solithromycin	Same as macrolides	Similar to telithromycin. First fluoroketolide in development.
Nitrofurans		*Oral:* nitrofurantoin	Highly active metabolites damage bacterial DNA	Active against enteric gram-negatives and enterococci causing urinary tract infection. Susceptibility should be tested before use.
Nitroimidazoles		*Parenteral or oral:* Metronidazole, tinidazole	Disrupts bacterial DNA by production of radical anions	Broad-spectrum activity against anaerobes
Oxazolidinones		*Parenteral or oral:* linezolid, tedizolid	Inhibits protein synthesis by inhibiting bacterial ribosome assemble	Linezolid is used for serious infections caused by MRSA. Tedizolid is a second-generation oxazolidinone undergoing clinical trials for use against MRSA infections.
Phenicols		*Parenteral:* chloramphenicol	Inhibits protein synthesis by inhibiting the peptidyl transferase activity of the bacterial ribosome	Broad-spectrum bacteriostatic activity against gram-positive and gram-negative bacteria including anaerobes
Pseudomonic acid		*Topical:* mupirocin	Inhibits protein and RNA synthesis in *S. aureus*	Active against gram-positive bacteria including MRSA
Quinolones	Quinolone	Cinoxacin, garenoxacin, nalidixic acid	Inhibits nucleic acid synthesis by binding to DNA topoisomerases II and IV	Parent compounds for development of fluoroquinolones
	Fluoroquinolone	*Parenteral or oral:* ciprofloxacin, finafloxacin, gatifloxacin, gemifloxacin, levofloxacin, grapafloxacin, lemefloxacin, moxifloxacin *Oral:* norfloxacin, ofloxacin	Same as quinolones	Active against most aerobic gram-negative bacilli in the *Enterobacteriaceae*, *H. influenzae,* and staphylococci. Ciprofloxacin has the best activity against *P. aeruginosa*. Ciprofloxacin has poor activity against *S. pneumoniae*. Levofloxacin, moxifloxacin, and gemifloxacin have good activity against gram-positive bacteria including *S. pneumonia*e, and atypical pathogens causing CAP (*Chlamydophila pneumoniae*, *M. pneumoniae,* and *Legionella*). Ciprofloxacin is also used for postexposure prophylaxis for inhalational anthrax. Levofloxacin is approved for treatment of inhalational anthrax. Moxifloxacin and gemifloxacin also have some anaerobic activity but are not the drugs

				of choice for serious infections. Norfloxacin is active against enteric gram-negatives causing urinary tract infection but it has no activity against enterococci or *P. aeruginosa*. Norfloxacin use has been restricted in many countries due to side effects.
Steroidal	Fusidanes	*Topical:* fusidic acid	Inhibits protein synthesis by preventing the turnover of elongation factor-G from the bacterial ribosome	Gram-positive activity includes staphylococci, β-hemolytic streptococci and corynebacteria. Many strains of MRSA remain susceptible but resistance may develop on therapy.
Streptogramins		*Parenteral:* quinupristin-dalfopristin	Two agents are synergistic in blocking protein synthesis by binding to the 50S ribosomal subunit	Active against gram-positive cocci including vancomycin-resistant *E. faecium* and MRSA. Drug is less active against *E. faecalis*.
Tetracyclines		*Parenteral or oral:* doxycycline, minocycline. *Oral:* tetracycline	Inhibit protein synthesis by binding to the 30S ribosomal subunit	Some broad-spectrum gram-negative but more limited gram-positive activity but prevalent resistance, so susceptibility should be tested before use. Active against some atypical pneumonia agents (*Chlamydophila, M. pneumoniae*), *Rickettsia, Borrelia burgdorferi* (early-stage disease), *Helicobacter pylori, Vibrio, Brucella, Treponema pallidum,* and some mycobacteria. Minocycline is active against MRSA, *Stenotrophomonas,* and *M. marinum*.
	Glycylcyclines	*Parenteral:* tigecycline	Same as tetracyclines	Expanded activity spectrum compared to the tetracyclines. Gram-positive activity includes MRSA and VRE. Gram-negative activity is broad (*Enterobacteriaceae, Acinetobacter* spp., *Stenotrophomonas, Haemophilus,* and *Moraxella*) but does not include *P. aeruginosa* or *Proteus*. Also good anaerobic and mycobacterial activity.
Fosfomycins		*Oral:* fosfomycin	Inhibits cell wall biogenesis as a mimic of phosphoenol-pyruvate (PEP) thereby blocking the action of MurA in catalyzing peptidoglycan	Active against *E. coli* and *Klebsiella* spp. that produce extended-spectrum β-lactamases

Adapted in part from Glossary I, supplementary tables, CLSI M100-S23[121] and M100-S24[122].

Mechanisms of Action of Major Antibiotic Classes

Antibiotics are designed to inhibit or block some essential cellular process, thereby controlling growth of the bacteria (i.e., bacteriostatic compound) or killing it outright (i.e., bactericidal). The effect of a bacteriostatic versus bactericidal antibiotic on a logarithmically growing bacterial culture is shown in Figure 17-2. Most of the major classes of antibiotics used in human medicine are derived from natural products, but three classes have been synthesized, including the sulfa drugs, the fluoroquinolones, and more recently an oxazolidinone. Development of available and new antibiotics is an expensive and arduous process. A stepwise procedure is followed in bringing forward new compounds of interest.[257,614] A set of pathogenic bacterial strains, including those that have developed clinically important resistance, are first tested using the new compound to determine its spectrum of activity and potency. The compound will move forward to be tested on animals with induced invasive infection

to determine the efficacy of the agent. Finally, the new compound may then be used in first phase clinical trials and compared with the efficacy of available antibiotics for infections caused by both susceptible and resistant bacteria. Potential toxicity of each compound is also established during animal testing before using the drug in human trials.

Antibiotics used in human medicine are grouped into major classes based on their mechanisms of action. Table 17-1 outlines the major classes of available antibiotics along with their structural targets and their spectrum of activity. Despite intensive research efforts into the mechanism of antibiotic action in bacterial pathogens, only four major targets exist: (1) inhibition of cell wall biosynthesis, (2) inhibition of protein synthesis, (3) inhibition of DNA replication and repair, and (4) inhibition of folate coenzyme biosynthesis. Antibiotic resistance therefore develops by three major mechanisms: (1) destruction of the compound by an enzyme produced by the bacterium, (2) cell impermeability and/or efflux, or (3) alteration of one of the drug target(s).Figure 17-3 provides a schematic outline of each of these mechanisms of antibiotic action and resistance development. A detailed description of the biochemical structure and mechanism of action of each antibiotic is beyond the scope of this chapter. Interested readers should consult *Mandell's Principles and Practice of Infectious Diseases*[370] as well as several other texts and reviews on this topic.[79,135,646]

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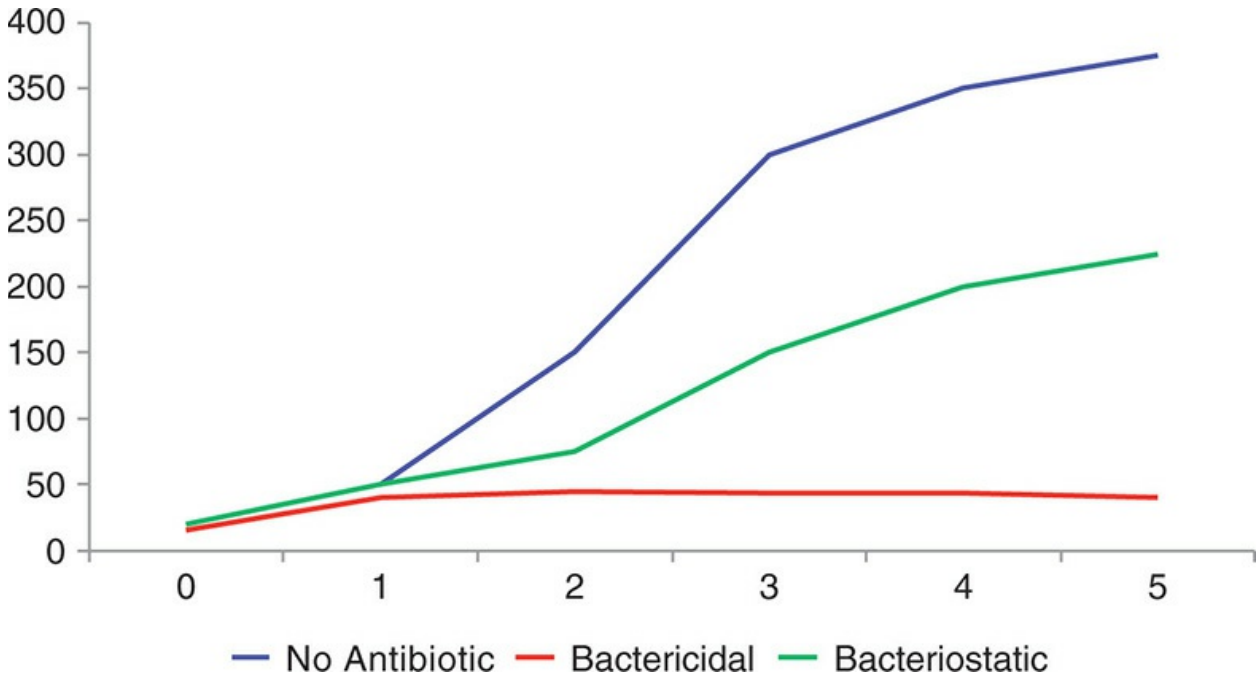
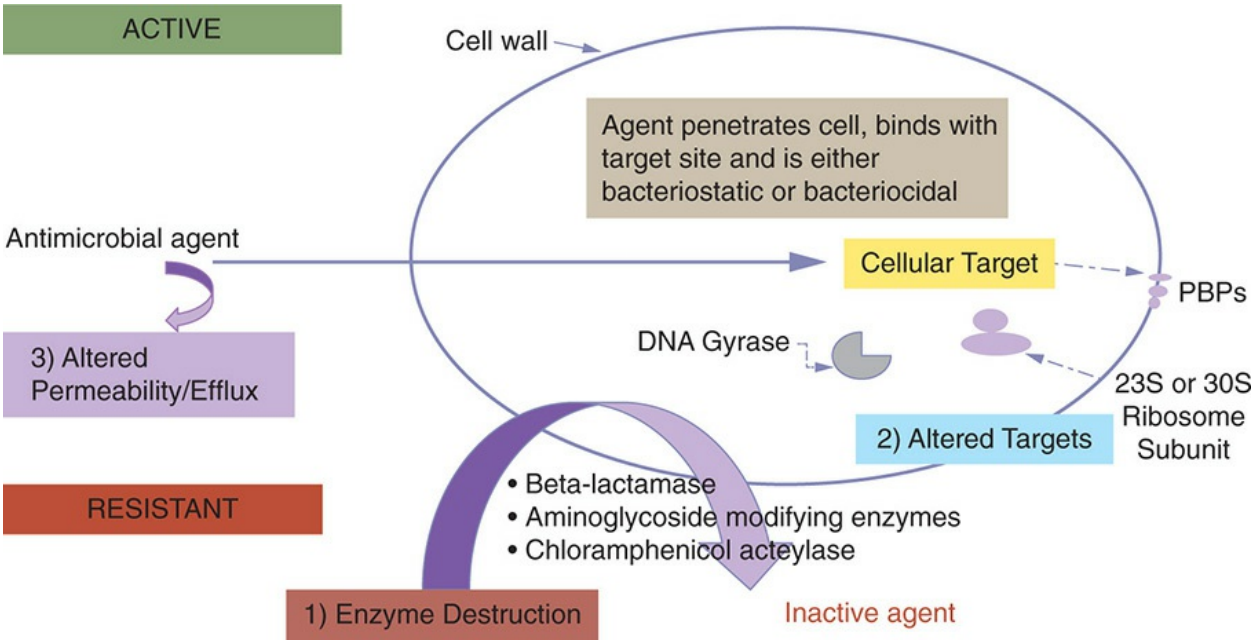


■ **FIGURE 17-2** Bacteriostatic versus bactericidal action of antibiotics on bacterial growth. Bacterial growth curves compare the following: (1) culture growing without antibiotics present (*Blue line*), (2) culture growing in the presence of a bacteriostatic antibiotic (*Green line*), and (3) culture growing in the presence of a bactericidal antibiotic (*Red line*).



■ **FIGURE 17-3** Main mechanisms of antimicrobial resistance in bacteria.

Genetic Basis of Resistance to Major Antibiotic Classes

To understand the mechanisms of bacterial resistance, it is necessary to understand bacterial physiology, the pharmacology of antimicrobial drugs, and the molecular biology of infectious agents. Bacteria are incredibly resilient and rapidly adapt to develop resistance under selection pressure from antibiotic use. A fascinating, but disturbing, finding is the documentation that some antibiotics elicit a transient increase in the mutation rate of bacteria.[48] Thus, antibiotics may not only serve as selectors of antibiotic-resistant clones, but also as primary promoters of de novo resistance.

The genes encoding for resistance may be located either on the chromosome or an extrachromosomal element called a plasmid. Plasmids are circularized pieces of DNA that act independently of the chromosome. The practical significance of the difference is that the chromosomal DNA

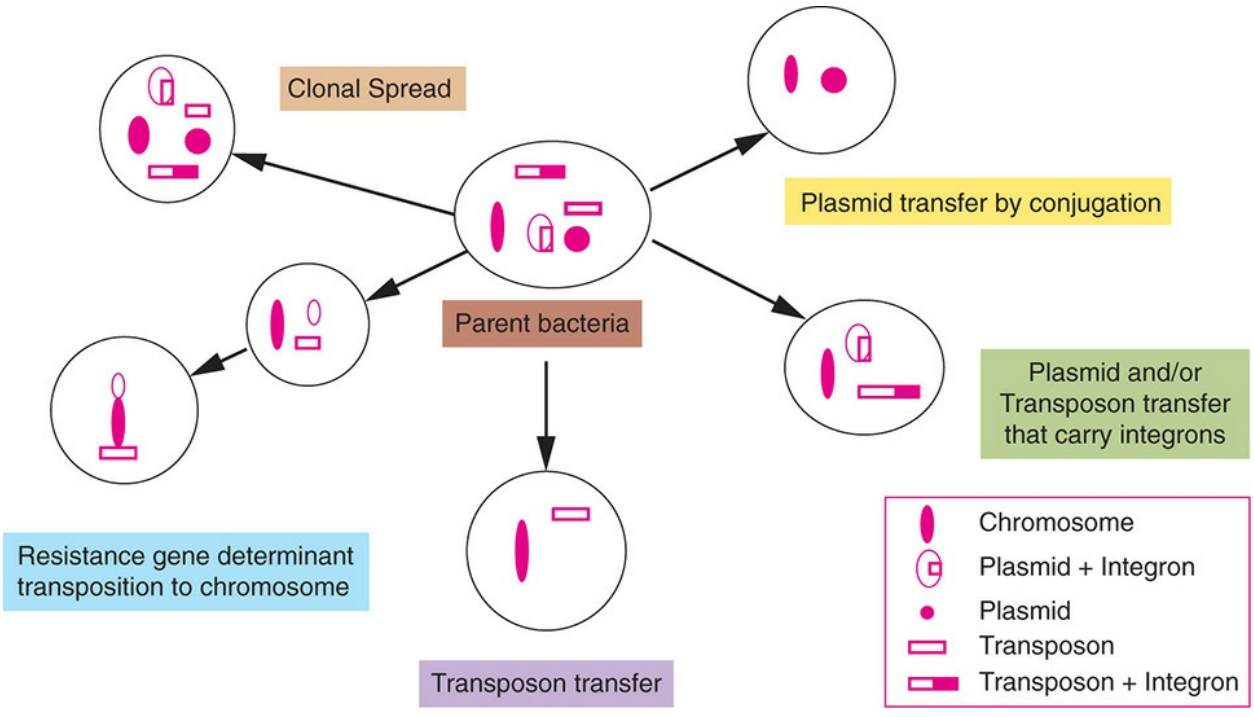
is relatively stable, whereas the plasmid DNA is easily mobilized from one strain to another, one species to another, or even one genus to another. The variables involved in bacterial horizontal gene transfer are described in Table 17-2, and various transfer mechanisms are illustrated in Figure 17-4. In addition, the linking of resistance genes for multiple antimicrobial agents on a plasmid allows the bulk transfer of resistance determinants that characterizes many newly resistant organisms.[37,533,585,684]

Bacteria can resist the effects of antibiotics by chromosomal mutation or inductive expression of a latent chromosomal gene. More commonly though, bacteria exchange genetic information and transfer one or more antibiotic resistance genes by several mechanisms. The most common mechanism by which resistance genes are transferred is conjugation. Most commonly, antibiotic resistance genes are carried on plasmids as part of a transposable genetic element called a transposon or "jumping gene."[134,510,546,574,675] Transposons have the ability to enter transmissible plasmids or chromosomes and subsequently undergo block conjugal horizontal transfer to other related or even unrelated bacterial species. The result may be a mosaic of genetic material from the donor and recipient bacteria.[366] Conjugation with plasmid transfer is particularly common among the *Enterobacteriaceae*, *Pseudomonas,* and anaerobic species.[78,99, 162,211,283,366] Transfer of antibiotic resistance determinants across a major barrier between gram-positive and gram-negative bacteria has also been documented,[133] but the reverse is uncommon. For example, spread of resistance can occur between staphylococci and enterococci and between *Enterobacteriaceae* and *Pseudomonas* or anaerobes such as *Bacteroides*.[19,69,596,621,651]

Bacterial transposons, plasmids, or chromosomes may contain an integron (Fig. 17-4), which is a two-component gene capture and dissemination system in bacteria that was initially discovered in relation to antibiotic resistance.[128,215,228,229,381,530–532] An integron consists of a first component gene encoding a site-specific recombinase along with a specific site for recombination, while fragments of DNA called gene cassettes that can be incorporated or shuffled make up the second component (Fig. 17-5). Integrons also contain an integrase (*int1*) similar to that found in bacteriophage, followed by an *attl* site for recognition of the integrase and integration of cassettes, and a promoter to drive expression. A repeat sequence that flanks the cassettes encoded by an *attC* sequence (also called 59-be) enables integration of the integron at the *attl* site, as well as excision and subsequent horizontal gene transfer. Because an integron cassette may encode several genes for antibiotic resistance to different classes of antibiotics, horizontal gene transfer by this mechanism is a very effective way for the bacterium to rapidly spread multidrug resistance determinants.

TABLE 17-2 Variables in the Expression and Transfer of Bacterial Resistance

Characteristic	Variable	Comments
Location	Chromosomal	Genetic stability; expression often constitutive
	Extrachromosomal	Plasmids easily mobilized for transfer from bacterial cell to cell
	Transposon	Moves genetic material between chromosome and plasmid or between bacteria
Transfer	Conjugation	Either by plasmids (R-factor) or a transposon
	Transduction	Transfer by bacteriophage
	Transformation	Direct transfer of DNA between compatible species
Expression	Constitutive	Produced with or without exposure to a stimulus
	Inducible	Produced only after exposure to a stimulus
	Constitutive-inducible	Produced at low level without stimulus; production greatly increased after stimulation



■ **FIGURE 17-4** Horizontal transfer of resistance determinants in bacteria.

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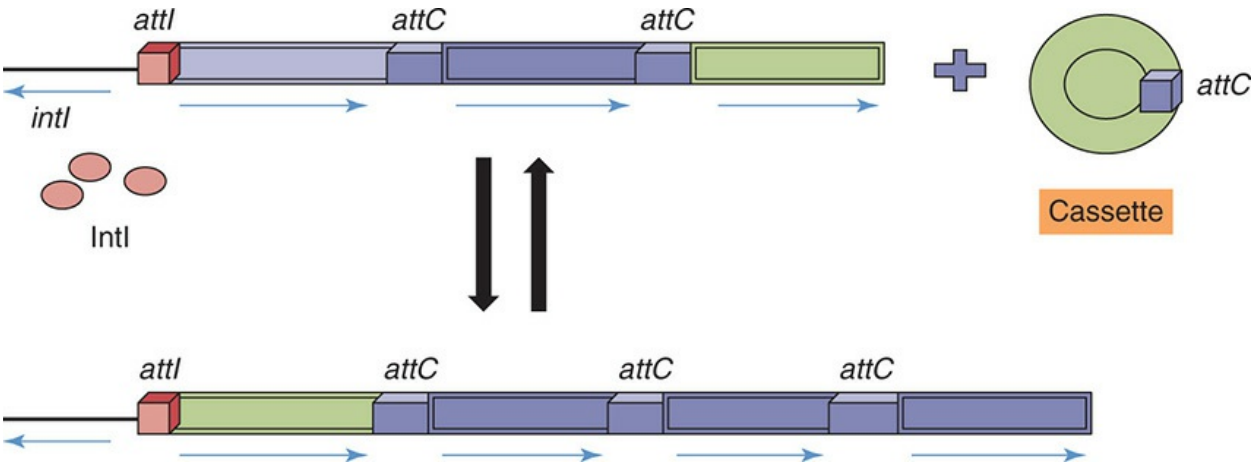

■ **FIGURE 17-5** Integrons carry multiple drug resistance determinants. Naturally occurring integrons use site-specific recombination for storing, expressing, and transferring genes. Integrons consist of a recombinase gene and a recombination site *attI* (integron attachment site) into which a circular gene cassette containing an *attC* (cassette attachment site) can be integrated. There is also a strong promoter upstream from *attI* that allows gene cassettes to be transcribed into mRNA. Cassettes can be deleted by recombination between pairs of *attC* sites, or by recombination between an *attC* and *attI* site. The order of genes in an integron can therefore be changed by excision and reintegration of the excised cassette into *attI*. (Adapted from references 215 and 381.)

Antibiotic resistance genes may be expressed continuously regardless of exposure to an antibiotic, or be inducible whereby expression only occurs in the presence of the drug (i.e., during antibiotic therapy). Staphylococcal β-lactamase (penicillinase) is an example of an inducible enzyme.[511] It is present on a plasmid and is not produced unless the bacteria are exposed to a β-lactam antibiotic, such as penicillin, after which production of the enzyme is turned on. Many β-lactamases of gram-negative bacteria are present on the chromosome and are produced constitutively, but may be induced to produce even greater levels of the enzyme when exposed to a β-lactam agent.

Finally, some resistance mechanisms are expressed homogeneously, while others are expressed heterogeneously. Homogeneous or uniform expression of a resistance factor facilitates detection of antibiotic resistance in the laboratory. If only a small fraction of the bacteria expresses the resistance mechanism (heterogeneous expression or heteroresistance), however, sampling error may compromise detection of the

resistance in the laboratory.

Mechanisms of Antibiotic Resistance in Bacteria

The mechanisms by which resistance is expressed in bacteria are summarized in Table 17-3 and Figure 17-3. The activity

of antimicrobial agents is diminished by three major mechanisms: (1) prevention of access to the target, (2) inactivation of the compound by destruction or modification, and (3) alteration of the target site of the antibiotic.[76,419,646] Inactivation of antimicrobials by bacterial β-lactamases and aminoglycoside-inactivating enzymes is the most common way that resistance develops to these two major drug classes. Cell wall impermeability or efflux, which prevents transport of the antibiotic to its intracellular site of action, also occurs for β-lactams, aminoglycosides, and the tetracyclines.[419] Antibiotic target modification is the primary mechanism of resistance to the fluoroquinolones, macrolides, and folate synthesis antagonist agents, but it may also occur for β-lactams.[419] It should be emphasized at the outset, however, that virtually any resistance mechanism may be found in most bacteria and that multiple mechanisms are often found in a single organism (Table 17-4).

TABLE 17-3 Mechanisms of Bacterial Resistance to Antimicrobial Agents

Mechanism	Antimicrobial Agent Group	Examples
Enzymatic inactivation	β-Lactams	β-Lactamases; penicillinases; cephalosporinases; carbapenemases
	Aminoglycosides	Aminoglycoside modifying enzymes
Altered receptors	β-Lactams	Altered penicillin-binding proteins (PBPs)
	Ribosomal alterations	Tetracycline; erythromycin; aminoglycosides
	DNA gyrase alterations	Fluoroquinolones
	Altered bacterial enzymes	Sulfamethoxazole; trimethoprim
Altered antibiotic		

transport		
	Reduced protein motive force	Aminoglycosides and gram-negative bacteria; decreased influx
	Active transport from bacterial cells	Tetracycline; erythromycin; active efflux

Prevention of Access to the Target

Accumulation of antimicrobial agents at their site of action in the bacterial cell is the sum of transport into the cell, inactivation during the transport process, and clearance of the antimicrobial agent from the cell. To understand transport of molecules to the active site, it is necessary to consider the structural differences between gram-positive and gram-negative bacteria. Bacteria are prokaryotes, and in both gram-positive and gram-negative species, the cell membrane is surrounded by the cell wall, and the cytoplasm contains nucleic acids, ribosomes, and other cellular components (Fig. 17-6). The cell wall is considerably different in gram-positive bacteria compared to that found in gram-negative species. Gram-positive bacteria have a single cell membrane with a thick external cell wall that is composed of peptidoglycan that is interwoven with lipoteichoic acid (Figs. 17-6 and 17-7). For β-lactam and glycopeptide antibiotics, transport to the target site is not an issue in gram-positive species because their penicillin-binding protein (PBP) targets in the cell wall are freely accessible and the plasma membrane does not have to be crossed.

The cell wall of gram-negative bacteria is far more complex and is composed of an internal plasma membrane and an outer cell membrane, between which is the periplasmic space that contains an attenuated peptidoglycan layer (Figs. 17-6 and 17-7). The outer membrane (OM) of gram-negative bacteria not only performs an important protective role for the bacterium, but also the permeability of this layer has a major impact on the susceptibility of the microorganisms to antibiotics that have intracellular targets. Several excellent reviews of OM permeability and antibiotic resistance have been published.[152,233,441,485] The OM is made up of an asymmetric bilayer of phospholipid and lipopolysaccharides (LPS), with LPS being located in the outer leaflet. The OM phospholipids composition is similar to the cytoplasmic membrane, being made up of mostly phosphatidylethanolamine with lesser amounts of phosphatidylglycerol and cardiolipin.[152] This lipid bilayer is impermeable to large, charged molecules. LPS is composed of three parts: (1) hydrophobic, fatty acid chains bearing lipid A (a glucosamine-based phospholipid), (2) a short core oligosaccharide, and (3) a distal polysaccharide, the O-antigen.[501] *E. coli* strains can have varying lengths of these structures (e.g., R or "rough" mutants or Ra to Re chemotypes have varying lengths of core oligosaccharide while "deep rough" mutants have a truncated

varying lengths of core oligosaccharide while "deep rough" mutants have a truncated core and "smooth" strains have an intact O-antigen) because only the inner part of LPS, consisting of lipid A and two 3-deoxy-D-manno-oct-2-ulsonic acid molecules linked to the core oligosaccharide, is required for growth.[501] A large number of other different types of proteins are also found in the OM, including murein lipoprotein (Lpp), OmpA, and several general diffusion porins. Lpp and OmpA together play a structural role in maintaining the shape of the cell.

Porins. Influx into the bacterial cell is largely controlled by porins, which are water-filled open "pores" or channels that span the OM and allow the passive transport of hydrophilic molecules (Figs. 17-6 and 17-8).[425,427,428,441,681] Porins in gram-negative bacteria have been characterized and classified according to their functional structure (monomeric to trimeric), their activity (nonspecific or specific channel/selective pore), and their regulation and expression.[152,425,427,428,681] The classical porins produced by *E. coli* have been the best studied, and this has formed the basis for studies of other porins in gram-negative species. *E. coli* produces three major trimeric porins: (1) a large porin channel called OM protein F (OmpF), (2) a small porin channel called OM protein C (OmpC), and (3) PhoE.[681] The third channel called PhoE is produced in mutants that lack both OmpF and OmpC, but this channel does not appear to be important for movement of antimicrobial agents.[427] PhoE selects for inorganic phosphate and anions while the other two trimeric porins regulate cations.[428,441,681] Most porin proteins involved in antibiotic transport in other enteric gram-negative bacteria also belong to the classical OmpC and OmpF families and behave in a similar fashion to those of ***E. coli***. However, ***P. aeruginosa*** and ***A. baumannii*** have an intrinsic low susceptibility to β-lactams because of their reduced **OM** permeability. ***P. aeruginosa*** has a low number of distinct porins called OprD, which have different properties compared to the ***Enterobacteriaceae***.[356]Imipenem is the only antimicrobial agent for which resistance in ***P. aeruginosa*** occurs by altered membrane transport. However, in some situations, imipenem gains entry, not through the main porin protein, but through a specific transport protein, designated OprD.[499,500] It appears, however, that the presence of a chromosomal class C β-lactamase is necessary for resistance in addition to the altered porin protein.[354] ***A. baumannii***, *Neisseria gonorrhoeae,* and other gram-negative species also utilize nonclassical porins.[189,439]

For many antimicrobial agents with intracellular targets, efficient transport across the OM of enteric bacteria is critical for their effective action. Small hydrophilic drugs such as the β-lactams use the porins to gain access to the interior of the cell, while hydrophobic drugs such as the macrolides are able to directly diffuse across the lipid bilayer. Factors such as the charge on the molecule and the hydrophobicity of the compound play an important role in the movement of antibiotics through the OM (Fig.

positively charged molecules or zwitterions (i.e., compounds with balanced positive and negative charges). Presumably, the negative charges cause the antibiotic to "hang up" as it crosses the negatively charged porin channel. The exclusion of hydrophobic compounds from the aqueous environment of the porin may explain the lack of efficacy of the hydrophobic compound methicillin against gram-negative bacteria. Likewise, β-lactam antibiotics with large bulky side chains, such as mezlocillin, piperacillin, and cefoperazone, also cross the membrane poorly. In one study, the "best performer" among the β-lactam antibiotics was imipenem, which is a zwitterionic, hydrophilic compound with a very compact structure.[109] It has been proposed that the explanation for susceptibility of *Enterobacter cloacae* to imipenem in the presence of resistance to third-generation cephalosporin drugs is the greater accessibility of imipenem to targets, mediated by rapid transit probably through several porin channels.[454]

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TABLE 17-4 Mechanism(s) of Antimicrobial Resistance in Clinically Important Bacteria

Bacterial Type	Antibiotic Group	Common Mechanism(s)	Other Mechanism(s)
Staphylococcus spp.	Penicillins	β-Lactamase (penicillinase)	Altered penicillin-binding proteins (PBPs)
	Penicillinase-resistant penicillins	Altered PBPs (*mecA* and *mecC* genes)	Borderline: altered PBPs; methicillinase; hyperproduction of β-lactamase
	Fluoroquinolones	Active reflux; altered DNA gyrase	Poor transport across membrane
	Erythromycin and/or clindamycin	Altered ribosomal targets (Constitutive or inducible)	Active efflux of erythromycin
	Glycopeptides	Altered cell wall binding proteins	Acquisition of a *vanA/B* gene (rare to date)
S. pneumoniae	β-Lactams	Altered cell wall; low-affinity PBPs	
	Erythromycin and/or clindamycin	Altered ribosomal targets (*erm* gene) (Constitutive or inducible)	Active efflux of erythromycin
Enterococcus spp.	β-Lactams	Altered cell wall; low-affinity PBPs	β-Lactamase
	Aminoglycosides	Intrinsic; poor transport across membrane	Altered ribosomal binding sites
		High level: aminoglycoside modifying enzymes (AME)	
	Glycopeptides	Altered cell wall binding proteins	
H. influenzae	Penicillins	β-Lactamase (penicillinase)	Altered PBPs
	Trimethoprim-sulfamethoxazole	Altered enzyme targets	
	Chloramphenicol	Chloramphenicol acetyltransferase	Altered membrane transport
N. gonorrhoeae	Penicillins	β-Lactamase (penicillinase)	Altered PBPs
	Cephalosporins	Altered PBPs	Altered membrane transport
	Fluoroquinolones	Altered DNA gyrase	Altered transport through outer membrane
	Tetracycline	Active efflux	
	Spectinomycin	Altered ribosomal target	AME
Enterobacteriaceae	β-Lactams	β-lactamases; poor diffusion or altered porins	Altered PBPs; low protein motive force; extended-spectrum β-lactamases (ESBLs); carbapenamases (e.g., KPCs, NDMs, etc.)
	Aminoglycosides	AME; poor diffusion or altered porins	
	Fluoroquinolones	Altered DNA gyrase	Altered transport through outer membrane
	Tetracycline	Active efflux	
	Trimethoprim-sulfamethoxazole	Altered enzyme targets	
Pseudomonads	β-Lactams	β-lactamases; poor diffusion or altered porins	Altered PBPs; low protein motive force; metallo-β-lactamases (MBLs) (e.g., VIM, IMP etc.)
	Aminoglycosides	AME; poor diffusion or altered porins	
	Fluoroquinolones	Altered DNA gyrase	Altered transport through outer membrane
Acinetobacter spp.	β-Lactams	β-Lactamases; poor diffusion or altered porins	Altered PBPs; low protein motive force; carbapenemases (e.g., OXA enzymes)
	Aminoglycosides	AME; poor diffusion or altered porins	
	Fluoroquinolones	Altered DNA gyrase	Altered transport through outer membrane

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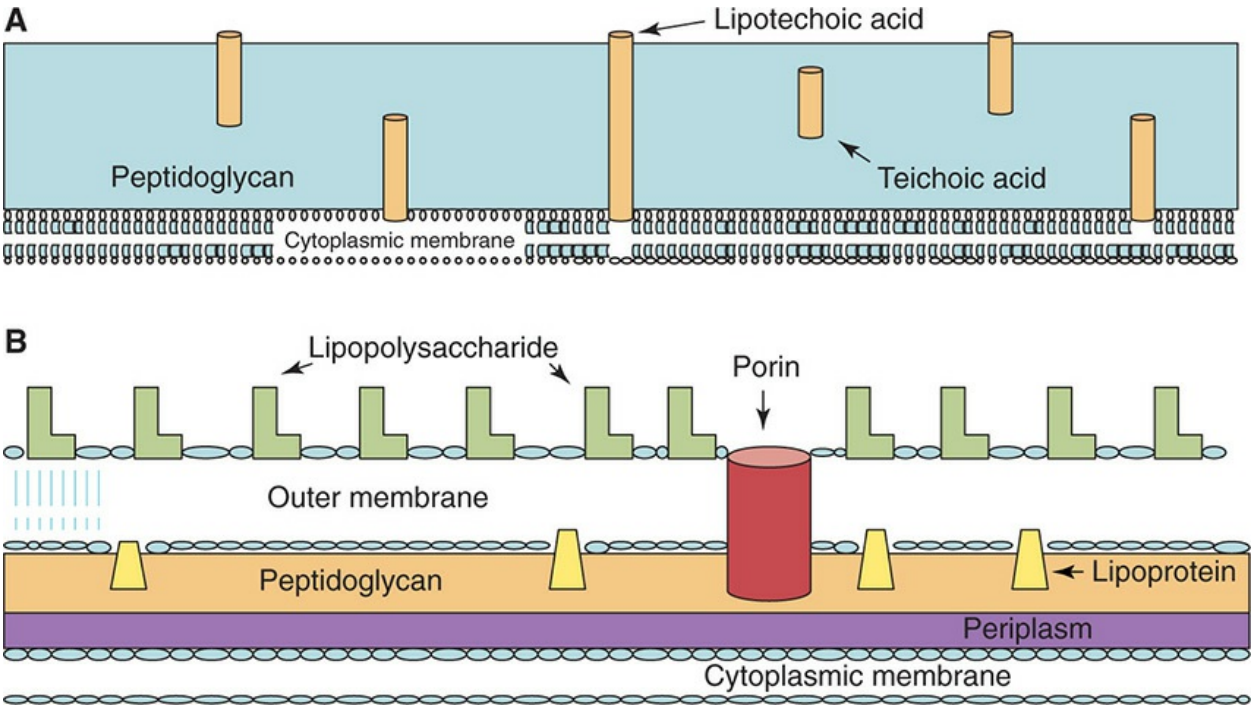
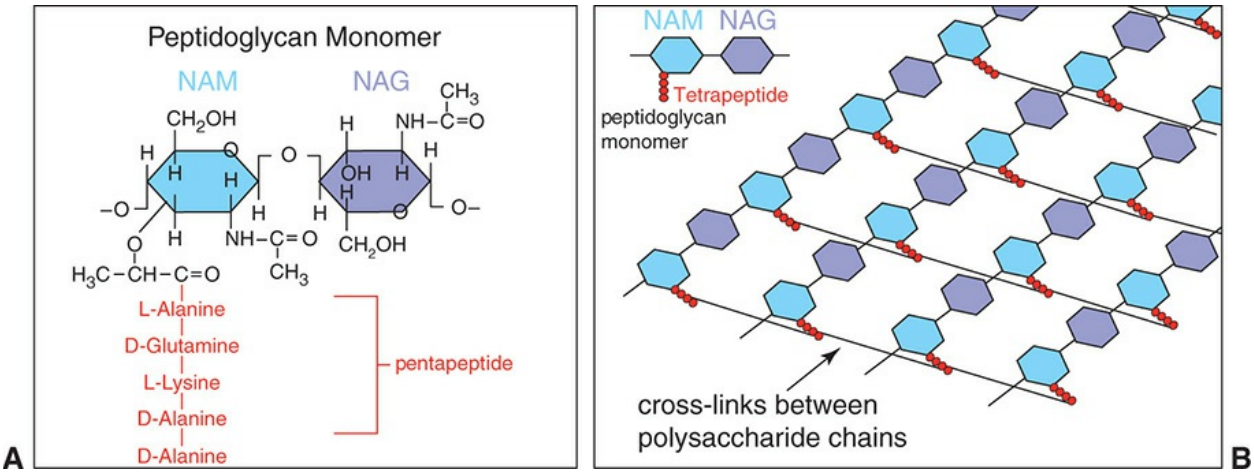


■ **FIGURE 17-6** Gram-positive and gram-negative cell walls. **A.** The gram-positive cell wall is composed of a thick, multilayered peptidoglycan (PG) sheath outside of the cytoplasmic membrane. Techoic acids are linked to and embedded in the PG, and lipoteichoic acids extend into the cytoplasmic membrane. **B.** The gram-negative cell wall is composed of an outer membrane linked by lipoproteins to thin, mainly single-layered PG. The PG is located within the periplasmic space that is created between the outer and inner membranes. The outer membrane includes porins, which allow the passage of small hydrophilic molecules across the membrane, and lipopolysaccharide molecules that extend into extracellular space. (Adapted from Cabeen, MT, Jacobs-Wagner C. Bacterial cell shape. Nat Rev Microbiol 2005;3:601–610.)



■ **FIGURE 17-7** Structure of peptidoglycan in the bacterial cell wall. **A.** Peptidoglycan

(PG) is composed of chains of PG monomers (NAG–NAM–tetrapeptide). **B.** PG monomers join together to form chains, and the chains are then joined by cross-links between the tetrapeptides to provide strength. (Courtesy of Gary E Kaiser.)

Gram-negative species have, however, developed mutations in the porin expression or composition of their OM as a means to confer drug resistance, and a large number of strains have been characterized that have this type of resistance.[441] The influence of the balance of porin expression and β-lactam susceptibility is well illustrated by an interesting case reported by Medeiros and colleagues.[389] Two strains of *Salmonella enterica* serovar Typhimurium developed complete resistance to all cephalosporin drugs while a patient was on therapy. Although the original isolates were susceptible to the cephalosporins, shortly after starting therapy with cephalexin, a cephalosporin-resistant strain was isolated from a wound sample. Both strains failed to show an increase in β-lactamase activity, but further studies demonstrated disruption in the osmoregulation of porin synthesis in the clonal descendants of the original pretherapy strain. Osmoregulation of porin synthesis in the susceptible parent strain was normal with both OmpC and OmpF porins being present in low osmolality conditions, but in high osmolality conditions only OmpC porins were expressed. The expression of OmpC porin was thought to be favored in vivo because of the high osmolarity conditions in the tissues of the patient. The resistant isolate, however, expressed only OmpF porins in low osmolarity conditions, while the synthesis of both types of porins was repressed at high osmolarity conditions. Porin expression modification may occur stepwise during exposure in vivo to subinhibitory levels of a β-lactam, or in this case, be totally repressed, resulting in loss of the OmpC channel and high-level resistance because of complete cellular impermeability.

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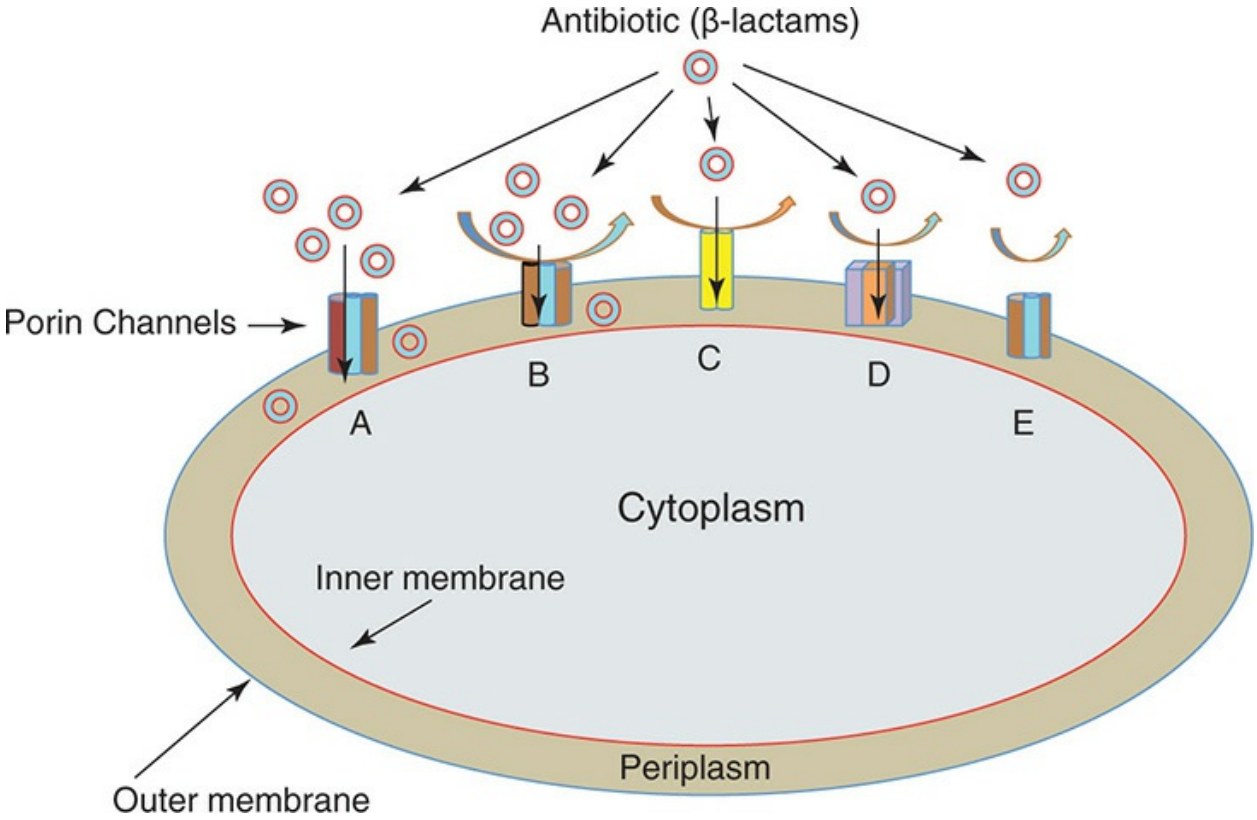


■ **FIGURE 17-8** Effects of porin mutations on antibiotic permeability of the bacterial cell wall. **A.** Normal synthesis of porin with passage of the β-lactam into the periplasm. **B.** Decreased synthesis of wild-type porin with decreased passage of β-lactam into the periplasm. **C.** Normal synthesis of restricted-channel porin with restricted passage of β-lactam into the periplasm. **D.** Normal synthesis of mutated porin with decreased passage of β-lactam into the periplasm. **E.** Normal synthesis of wild-type porin with a channel blocker so that no drug crosses into the periplasm. (Adapted from reference 441.)
Schematic representation of various porin modifications that confer multidrug resistance to β-lactams. The thickness of the straight arrows reflects the level of β-lactam penetration through porin channels. The curved arrows illustrate the uptake failure that occurs with various porin modifications: (1) decreased porin expression, (2) restricted porin channel: An exchange in the type of porin that is expressed, and (3) mutated porin: mutation or modification that impairs the functional properties of a porin channel. Porin-blocking molecules may also decrease antibiotic access. (Reprinted with permission from Pages JM, James CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in gram-negative bacteria. Nat Rev Microbiol 2008;6:893–903.)

Complete porin exchange may also occur during antimicrobial therapy to an altered porin phenotype that confers resistance. One study of *K. pneumoniae* strains collected during patient treatment showed that the OmpF porin type (OmpK35) had been replaced with an OmpC porin type (OmpK36), which has a small channel size.[240]

Because strains expressing OmpK35 have a much higher susceptibility to β-lactams (cefepime, cefotetan, cefotaxime, and cefpirome), the porin exchange to OmpK36 in these isolates conferred differential susceptibility to these drugs, and in most cases severely decreased the intracellular drug concentrations.[240] Development of resistance to β-lactams (cephalosporin) and imipenem has also been reported for *E. aerogenes* during the course of imipenem therapy. Resistance emerged within 5 days of starting antibacterial therapy and the posttherapy isolate was shown to have undergone a porin exchange to OmpK36.[53] These studies demonstrate that a resistant phenotype due to porin loss or exchange may occur during a course of antibiotic treatment. Porins may also undergo successive mutations leading to progressively increased levels of antibiotic resistance. A longitudinal 2-year study of *E. coli* strains recovered from patients on long-term complex antibiotic regimens showed that fluoroquinolones, cephalosporin, and carbapenem agents were clearly affected by this mechanism.[362] Liver and blood culture isolates demonstrated longitudinal increased levels of antibiotic resistance, and all of these strains had two mutations (D18E and S274F) in the OmpC porin that likely resulted in decreased drug influx. Porin mutations have also been reported in *E. aerogenes* clinical isolates shown to have high-level resistance to β-lactams.[368]

Antimicrobial agents other than β-lactam compounds may also depend on porin channels for access to the cell. Resistance to chloramphenicol, which is usually caused by enzymatic degradation, may also be mediated by altered porin proteins in enteric bacteria[620] and *Haemophilus influenzae*.[83] Similarly, resistance to aminoglycosides in *Serratia marcescens* may be mediated by alterations in porin proteins, although the primary mechanism for resistance is enzymatic degradation.[220] Resistance to fluoroquinolones, which is principally mediated by changes in the structure of the target enzyme, may also be caused by alterations in membrane proteins.[541]

A second permeability barrier also exists for some bacteria for agents that must gain entrance to the bacterial cytoplasm. Crossing the second membrane is accomplished by a process that requires expenditure of energy and a minimal negative charge in the cytoplasm, the proton motive force, in order to "pull" aminoglycoside antibiotics into the cytoplasm. This transport mechanism has been demonstrated in both gram-positive[378] and gram-negative bacteria.[77] Mutant *Enterobacteriaceae* have been described that were resistant to antimicrobial agents in vitro because of deficiencies in this transport mechanism, but their clinical significance was

unclear.[534] These variants appeared as small colonies on agar media, but they may revert to normal colony morphology.

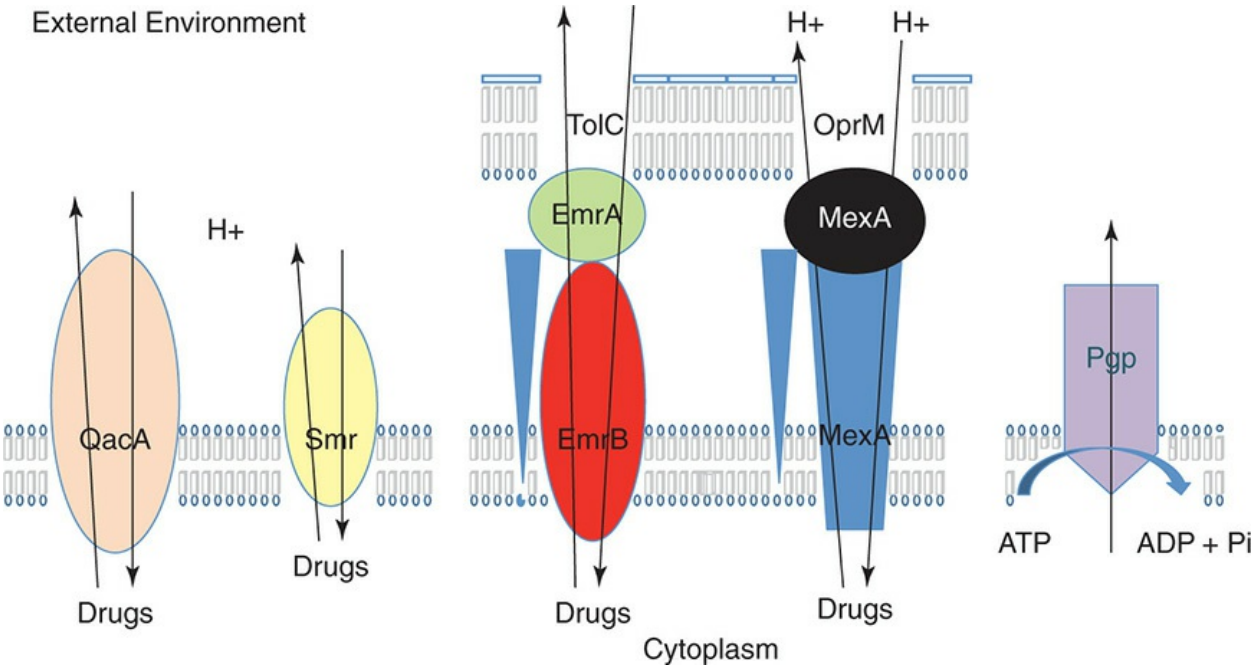
Efflux Pumps. Efflux pumps are a naturally occurring mechanism that bacteria use to excrete a solute out of the cell. Active transport mechanisms are biologically useful for removal of a variety of potentially toxic substances, of which antimicrobial agents may not even be the most important.[468,545] However, in some susceptible bacterial strains, efflux pumps can be induced by an antibiotic substrate, and subverted from natural functions to expeditiously pump the drug out of the cell; thereby, the bacterium becomes resistant to its intracellular action of the drug. Antimicrobial resistance in efflux mutants may be due to either increased expression of the efflux pump protein or mutation in the protein due to amino acid substitutions that make a substrate more efficiently exported.[342,426,467,468,545,680] Specific efflux pumps export one substrate, but others may transport a variety of dissimilar substrates, including antibiotics of multiple classes. Efflux pumps of the later type are associated with multiple-drug resistance (MDR) for antibiotics in several major classes.[426,468,545] In either case, the effect is to lower the intracellular concentration of antimicrobial agents so that drug concentrations never reach a sufficiently high level to exert effective antimicrobial activity. Efflux pumps are encoded by chromosomal genes or on plasmids (e.g., *tet* gene for tetracycline resistance and *qac* gene for resistance to quaternary ammonium compounds).[467] Structurally efflux pump proteins have 12 transmembrane-spanning regions, and this along with genomic studies has been used to identify proteins involved in multidrug efflux.[467,680] Efflux pumps may also work in concert with other resistance mechanisms in the bacterial cell to confer complete resistance to a particular class of antibiotics, most notably the fluoroquinolones.[436]

Microorganisms with larger genome size possess more genes for efflux pumps, and multiple MDR efflux pumps can be found in the same organism such as the Acr systems of *Enterobacteriaceae* or the Mex systems in *P. aeruginosa*.[545,467] Five families of efflux pump proteins have been described (Fig. 17-9).[342,426,467,468,545,680] Each of these families encodes bacterial MDR efflux and includes the multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS), staphylococcal multiresistance (SMR), resistance nodulation division (RND), and ATP binding cassette (ABC) superfamily. Although ABC transporters are located in the genomes of pathogenic bacteria, to date, *Lactococcus lactis* LmrA is the only organism so far shown to develop MDR resistance by this efflux pump superfamily.[638]

The RND family is the most important efflux pump systems in *E. coli* and other gram-negative bacteria. It has a tripartite structure. The three components include a transporter (efflux) protein in the inner membrane (AcrB), a periplasmic accessory protein (AcrA), and an outer membrane protein (OMP) channel (TolC).[322] Genomic studies have shown that efflux pump genes among different bacterial species have a high degree of similarity, including *E. coli* AcrB/AcrB, *P. aeruginosa* MexB/MexB,*Campylobacter jejuni* CmeB/CmeB, and *Neisseria gonorrhoeae*

MtrD/MtrD.[467] The RND family efflux pumps have a similar function in other gram-negative species to that shown in *E. coli*. In *E. coli*, the AcrB transporter takes its substrate from within either the phospholipid bilayer or the cytoplasm and transports them into the external cellular medium by cooperation with TolC.[183,322,342] The cooperative interaction of AcrB and TolC is facilitated by AcrA.[322] RND efflux pumps have subsequently been characterized in many other gram-negative species, including *P. aeruginosa* (i.e., MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN),[467,486,545] *S. enterica* serovar Typhimurium,[80] and other *Enterobacteriaceae,* including *E. aerogenes*,[491]*Klebsiella* spp.,[383] *Serratia marcescens*,[328] *Morganella morganii*,[535] *H. influenzae*,[540] *Proteus mirabilis*, and *Helicobacter pylori*.[633] The RND pumps use the proton gradient across

the bacterial cell membrane to export one drug molecule per hydrogen ion used.[452] MATE MDR efflux pumps use both proton motive force and sodium ion gradients to export substrates. This type of transporter has been described in many different bacterial pathogens, including *P. aeruginosa* (PmpM),[244] *E. cloacae*,[245] *A. baumannii*, *Clostridium difficile* (CdeA),[172] *S. aureus* (MepA),[384] *Bacteroides thetaiotaomicron* (BexA),[396] *Brucella melitensis* (NorMI),[65] *Haemophilus influenzae* (HmrM),[673] and both *Vibrio cholerae* (McrM and VcnA)[35] and *Vibrio parahaemolyticus* (NorM).[108] Several efflux pumps also operate in gram-positive and gram-negative bacteria that are important in the development of fluoroquinolone resistance, and these transporters are members of the MFS family. NorA in *Staphylococcus aureus* has been shown to cause fluoroquinolone resistance.[213] The efflux pump pmrA has also been shown to confer fluoroquinolone resistance in clinical isolates of *S. pneumoniae*.[465,466] QepA is a plasmid-mediated efflux pump that has recently been characterized that confers high-level fluoroquinolone resistance in *E. coli* and other gram-negative bacteria.[99,100] Efflux in both of these transporters also uses the proton motive force across the cell membrane.



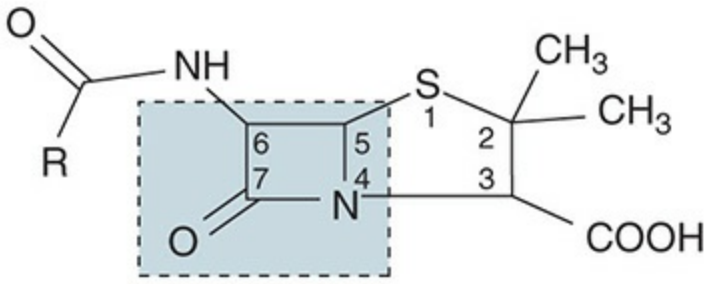
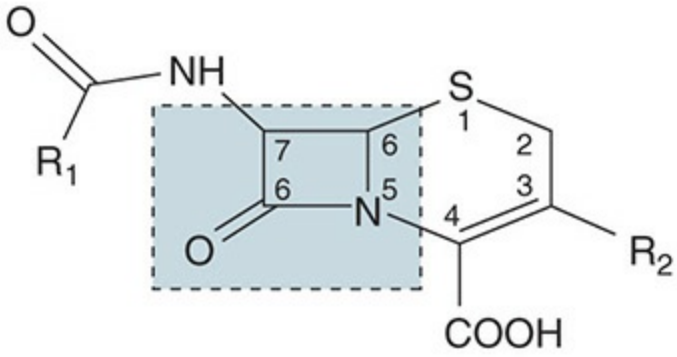
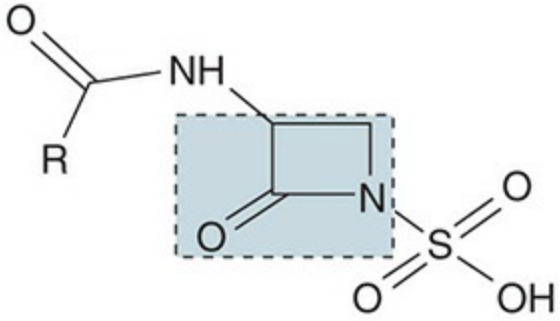
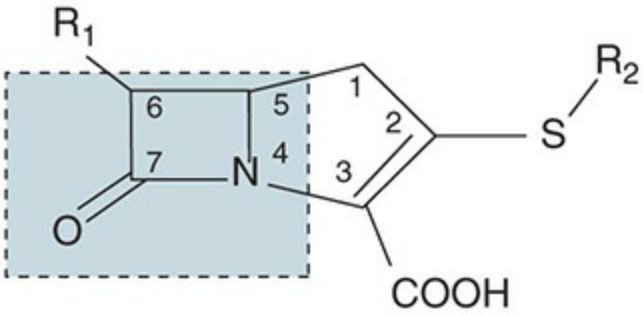
■ **FIGURE 17-9** Schematic illustrations of efflux pumps. Qac and Smr represent pumps found in gram-positive bacteria. EmrAB and MexAB-OprM represent pumps found in gram-negative bacteria. P-glycoprotein (Pgp) is found in mammalian cells and represents an ATP-binding cassette superfamily of pumps. ADP, adenosine diphosphate; Pi, inorganic phosphate. (Adapted from references 467 and 545.)

The energy-dependent efflux mechanism is a prime defense for bacteria against tetracyclines,[387] newer glycylcyclines,[151] and macrolides,[219] three groups of antibiotics that interfere with protein synthesis at the ribosomal level. Similarly, removal of the antibiotic is a resistance mechanism of staphylococci against the fluoroquinolones, which interfere with DNA gyrase.[213] Aminoglycosides have also been shown to be substrates for a number of multidrug efflux pumps, including members of the five superfamilies of bacterial transporters.[286] The resistance of gram-negative bacilli to many antibiotics was originally attributed to poor entry of the antibiotic into the bacterial cell because of size, hydrophobicity or charge. Active efflux of these antibiotics from the bacterial cell has been shown to be equally important because the pumps can excrete chemical compounds, including antimicrobial agents, directly into the extracellular medium, where their reentry is restricted.[342,426,467,468,545,680]

Antibiotic Inactivation by Destruction or Modification

β-Lactamases. β-lactamases are a family of enzymes produced by bacteria that vary in their spectrum of activity against the β-lactam class of antibiotics. β-lactamases are

the primary cause of bacterial resistance to β-lactam antibiotics. β-lactamases have now been described that can inactivate one or more β-lactam antibiotics or the entire group of antibiotics. These bacterial enzymes inactivate this major class of antibiotics by cleaving the amide bond in the four-atom β-lactam ring structure (Fig. 17-10).[90] One of the earliest β-lactamases found is a penicillinase that confers staphylococcal resistance to penicillin,[511] but more recently extended-spectrum β-lactamases have been characterized in enteric bacteria that can inactivate a wide spectrum of penicillin and cephalosporin drugs.[90,285,450] Due to molecular techniques, the genes encoding β-lactamases in many β-lactam-resistant clinical isolates have been sequenced and their amino acid sequences deduced (G. Jacoby and K. Bush, http://www.lahey.org/Studies/). By 2009, the number of unique protein sequences for β-lactamases exceeded 890, and this growth will likely continue as more bacterial strains are studied.[90,281,285,450,497] The specificity of a β-lactamase for a particular β-lactam antibiotic is an important determinant in the efficiency with which the enzyme hydrolyzes and inactivates the compound. A point mutation in one or more amino acids can change the specificity of the molecule if it occurs in a structurally critical area of the enzyme,[49,88,90] causing the rapid development of β-lactam resistance, even to more recently marketed drugs.

Group	Structure
Penicillins	
Cephalosporins	
Monobactams	
Carbapenems	

■ **FIGURE 17-10** Structure of the main groups ofβ-lactam antibiotics—the β-lactam ring is designated by the dotted line. (Reprinted with permission from Robtsovam MY, Ulyashova MM, Bachmann TT, et al. Multiparametric determination of genes and their point mutations for identification of β-lactamases. Biochemistry (Moscow) 2010;75:1628–1649.)

Bacteria naturally produced β-lactamase enzymes prior to the introduction of a relevant antibiotic substrate. Two groups of investigators have presented evidence to support the concept that the physiologic role of β-lactamases is to restructure the peptidoglycan during bacterial cell growth.[43,624] They found that synthesis of β-lactamase was induced both by the presence of β-lactam antibiotics and cell wall precursors in the extracellular environment, recalling the structural similarity of penicillin and the D-alanine-D-alanine dipeptide terminus of peptidoglycan chains. It is now evident that the β-lactamases and the PBPs have a common, albeit distant

evolutionary origin.[566] Both classes of compounds must interact with β-lactam antibiotics in order to perform their function. Similar amino acid sequences have been demonstrated in PBPs of high and low molecular weight and certain types of β-lactamase. Similarities in conformation and three-dimensional structure between β-lactamases and PBPs have also been documented, and some PBPs have weak β-lactamase function.[388,423] Furthermore, the mechanism of action of both β-lactamases and the transpeptidase PBP is cleavage of an amide bond by an acyl-enzyme mechanism.[88]

Functional β-lactamase classification schemes also include the ability of various β-lactamases to be inhibited by inhibitors.[85,87,90,353] The three inhibitors of β-lactamase activity that are used in clinical medicine are clavulanic acid, sulbactam and tazobactam. These compounds resemble β-lactam antibiotics and can bind to the β-lactamase, either reversibly or irreversibly, protecting the antibiotic from destruction.[353] Although these enzyme inhibitors must mimic β-lactams in order to function, they have limited antibacterial activity in their own right.[85] However, when a β-lactamase inhibitor is combined with a potent β-lactam antibiotic, the presence of the inhibitor either partially or completely restores the bactericidal activity of the antibiotic. All three inhibitors are effective against staphylococcal penicillinase but have variable effectiveness against the chromosomal enzymes of gram-negative bacteria. Clavulanate and tazobactam are superior to sulbactam in activity against plasmid-mediatedβ-lactamases of gram-negative bacteria, including the extended-spectrum β-lactamases,[453] but there is no significant difference between the inhibitory activities of

clavulanate and tazobactam (Table 17-5). Some β-lactamases such as the class A carbapenemases (e.g., KPC-2) are resistant or only weakly inhibited by the activity of all three compounds.[310] Clavulanate is a more efficient inducer of class C/group 1 AmpC β-lactamases than is either tazobactam or sulbactam.

A variety of β-lactamase classification schemes has been developed to group these complex and varied enzymes, leading to some confusion in nomenclature. A useful early classification scheme was provided by Richmond and Sykes,[512] but the number and variety of enzymes have proliferated beyond the scope of their scheme. A more modern scheme, based on molecular structure proposed by Ambler,[9] includes, of necessity, only enzymes that have been characterized. Subsequently, Bush–Jacoby–Medeiros developed a β-lactamase classification scheme that integrates functional and molecular characteristics.[91] Recently, Bush and Jacoby have provided an updated classification scheme for β-lactamases based on alignment by structural and functional classifications which is summarized and compared to the Ambler molecular scheme in Table 17-5.[90] New functional subgroups have also had to be added to the scheme because of the identification and expansion of major β-lactamase families, and variants will continue to be identified on a regular basis.[90] As in previous functional classification schemes, enzymes have been grouped based on their ability to hydrolyze specific β-lactam classes and on the inactivation properties of the β-lactamase inhibitors clavulanic acid, sulbactam and tazobactam. Because many β-lactamases have only been described on the basis of a protein sequence with little functional description, a set of structural and functional criteria has been proposed by Bush and Jacoby for the scientific characterization of newly discovered enzymes.[90] Box 17-1 outlines some key educational points about β-lactamases. A brief description is provided of each of the three functional enzyme groups, as well as their subgroups.

Group 1 Cephalosporinases. Ambler Class C/Bush–Jacoby Group 1 enzymes are primarily cephalosporinases, either constitutive or inducible, that are found on the chromosomes of many *Enterobacteriaceae* and a few other gram-negative bacteria.[281]Plasmid-mediated group 1 enzymes such as CMY, FOX, MIR, ACT, and DHA have been known for the past two decades, but CMY enzymes (1 to 50) are most commonly found in clinical isolates.[281] AmpC β-lactamases primarily have activity on the cephalosporins and are usually resistant to inhibition by clavulanic acid and active on cephamycins, such as cefoxitin and cefotetan (i.e., these drugs are resistant). They also have a high affinity for aztreonam (Ki values as low as 1 to 2 nM), which distinguish these enzymes from the class A cephalosporinases.[89,281] Exceptions to the usual activity profile of AmpC enzymes have also been described, including a novel AAC-1 enzyme in a *K. pneumoniae* strain that was not active on cefoxitin,[32] a novel enzyme that demonstrated inhibition by clavulanic acid or

tazobactam in *E. coli* and *K. pneumoniae* isolates,[23,666] and a novel enzyme in an *E. coli* isolate that demonstrated resistance to cefotaxime but not ceftazidime.[683] Carbapenem resistance may also occur, particularly to ertapenem, in gram-negative strains that produce a high level of AmpC β-lactamase in combination with loss of OM porins.[64,284,494] AmpC expression in many organisms including *Serratia marcescens, Citrobacter freundii, Enterobacter* spp., *Morganella* spp., *Providencia* spp., *Acinetobacter baumannii,* and *Pseudomonas aeruginosa* is constitutive at a low level, but induced on exposure to certain β-lactams (amoxicillin, ampicillin, imipenem, and clavulanic acid).[281,352,653]

Subgroup 1e enzymes are newly described variants of group 1 enzymes called extended-spectrum cephalosporinases that have increased activity against ceftazidime and other oxyimino-β-lactams because of amino acid substitutions caused by mutations.[433] Production of high levels of this type of enzyme in combination with a porin mutation may confer clinically significant carbapenem resistance.[369] Several new enzymes in this subgroup have been described recently, including CMY-37 in *Citrobacter freundii*, CMY-10 in *Enterobacter cloacae,* and CMY-19 in *E. coli*.[90]

Group 2 Serine β-Lactamases. This is the largest group of β-lactamases and includes molecular classes A and D. The dramatic increase in the number of enzymes within functional group 2 in the last two decades is due to the discovery and increasing identification worldwide of extended-spectrum β-lactamases (ESBLs). Subgroup 2a penicillinases have a limited spectrum of activity for benzylpenicillin and penicillin derivatives, and are the primary β-lactamases in gram-positive cocci such as staphylococci and less commonly enterococci.[90] These enzymes have poor hydrolytic activity for cephalosporin, carbapenem or monobactam compounds, except that they readily hydrolyze nitrocefin. Most of these enzymes are chromosomal although some staphylococcal penicillinases may be carried on a plasmid, and all subgroup 2a enzymes are inhibited by clavulanic acid and tazobactam.

Subgroup 2b β-lactamases include TEM-1, TEM-2, and SHV-1 enzymes, some of the most common plasmid-mediated β-lactamases identified several decades ago.[90] These enzymes readily hydrolyze penicillins and early cephalosporins including cephaloridine and cephalothin, and they are strongly inhibited by clavulanic acid and tazobactam. Subgroup 2be β-lactamases are ESBLs. Characteristics of these ESBL "wide-spectrum β-lactamases" are summarized in Table 17-5. These broad-spectrum enzymes not only retain activity against penicillins and cephalosporins similar to 2b enzymes, but also hydrolyze one or more oxyimino-β-lactams, such as ceftotaxime, ceftazidime, and aztreonam. Initially, ESBLs developed because of amino acid substitutions in TEM-1, TEM-2, and SHV-1 enzymes, which are inhibited by clavulanic acid.[90,496] A rapidly evolving group of ESBLs, the CTX-M enzymes have been more recently described, which most likely originated from and are related to

chromosomally determined β-lactamases in *Kluyvera*.[51] Most CTX-M enzymes hydrolyze cefotaxime more efficiently than ceftazidime, and many are able to also hydrolyze other β-lactam substrates including cefepime.[450] CTX-M enzymes are inhibited by tazobactam and to a lesser extent by clavulanic acid.[90] Other less commonly encountered ESBLs are members of the PER, VEB, and other families (Table 17-5).[217,477,489] Subgroup 2br enzymes are broad-spectrum β-lactamases that retain subgroup 2b activity but have developed resistance to clavulanic acid and related β-lactamase inhibitors. Although TEM enzymes (TEM 30 and 31) and some SHV enzymes (SHV-10 and others) have been functionally characterized within this group, no CTX-M enzymes have so far been found with this activity profile.[90] Another subgroup of 2b enzymes, the 2ber β-lactamases have been called CMT (complex mutant TEM) (i.e., TEM-50), and are all classified within the TEM family. CMT enzymes combine an extended-spectrum activity with modest resistance to clavulanic acid inhibition.[90]

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TABLE 17-5 Bush–Jacoby β-Lactamase Classification Compared to the Ambler Molecular Class System[a]

Bush–Jacoby (2010)[a]	Ambler Molecular Class	Enzymes	Active Site	Antibiotic Substrate(s)	Enzyme Inhibitors[b]	Found in
Group 1 Cephalosporinase	C	AmpC, ACT-1, CMY-2, FOX-1, MIR-1	Serine	Hydrolyzes cephalosporins including cephamycins	PBA, DPA, Cloxacillin	*Enterobacteriaceae, Acinetobacter* spp.
Group 1e Cephalosporinase	C	GC1, CMY-37	Serine	Increased hydrolysis of ceftazidime and other oxymino-β-lactams[c]	Not inhibited by CV of PTZ	*Enterobacteriaceae*
Group 2a Penicillinases	A	PC1	Serine	Increased hydrolysis of penicillin	CV or PTZ	*S. aureus*
Group 2b Penicillinases	A	TEM-1, TEM-2, SHV-1	Serine	Penicillin, first-generation cephalosporins	CV or PTZ	*Enterobacteriaceae*
Group 2be ESBLs	A	TEM-3, SHV-2, CTX-Ms, PER-1, VEB-1	Serine	Increased hydrolysis of oxymino-β-lactams[c]	CV or PTZ	*E. coli, Klebsiella pneumoniae, K. oxytoca, P. mirabilis, Salmonella* spp., *Kluyvera* spp. (*CTX-Ms*)
Group 2ber ESBLs	A	TEM-50	Serine	Increased hydrolysis of oxymino-β-lactams[c] + resistance to inhibitors	Not inhibited by CV of PTZ	*Enterobacteriaceae*
Group 2d	D	OXA-01, OXA-10		Hydrolysis of cloxacillin or oxacillin	Variable with CV or PTZ	*Enterobacteriaceae*
Group 2de ESBLs	D	OXA-11, OXA-15	Serine	Hydrolysis of cloxacillin or oxacillin + oxymino-β-lactams[c]	Variable with CV or PTZ	*P. aeruginosa*
Group 2df Carbepenemase	D	OXA-23, OXA-48	Serine	Hydrolyzes carbapenems	Variable with CV or PTZ	*A. baumannii, Enterobacteriaceae*
Group 2e ESBL	A	CepA	Serine	Hydrolyzes cephalosporins	CV but not aztreonam	*Proteae*
Group 2f Carbapenemases	A	KPC-2, SME-1, IMI-1	Serine	Hydrolyzes carbapenems, oxymino-cephalosporins[c] and cephamycins[d]	Variable with CV or PTZ	*Enterobacteriaceae*
Group 3 Metallo-Carbapenemases	B	IMP-1. VIM-2, IND-1, L1	Zinc	Hydrolysis of carbapenems but not monobactams	EDTA	*P. aeruginosa, A. baumannii*
Group 4	Not included	Unknown				

[a] Updated Bush–Jacoby classification adapted from reference 90.
[b] β-Lactamase inhibitors: PBA, phenylboronic acid; DPA, dipicolinic acid; CV, clavulanic acid; TZ, tazobactam; EDTA, ethylenediaminetetraacetic acid.
[c] Oxymino-cephalosporins = ceftotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam.
[d] Cephamycins = cefoxitin and cefotetan.

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BOX 17-1

Key Educational Points About β-Lactamases

- Each enzyme has different affinities for variousβ-lactam antibiotics:
  - Penicillinases break down penicillin drugs.
  - Cephalosporinases break down cephalosporin drugs.
  - Extended-spectrumβ-lactamases hydrolyze most β-lactams except carbapenems.

- Carbapenemases hydrolyze carbapenems.
- Different β-lactamase classification systems differentiate these enzymes based on their structure (Ambler) or structure and function (Bush).
- Multiple β-lactamases of different types may be present and expressed in the same isolate, especially among gram-negative bacteria:
  - *Enterobacteriaceae*—strains with BOTH an AmpC β-lactamase + an ESBL.
  - *P. aeruginosa*—strains with BOTH an AmpC β-lactamase + an MBL.
  - *Acinetobacter baumannii*—strains with BOTH an AmpC β-lactamase + MBL + OXA enzyme(s).
- Acquisition of an integron gene cassette with one or moreβ-lactamase genes often results in multidrug resistance because these determinants carry other antibiotic resistance genes (e.g., fluoroquinolones, aminoglycosides, etc.).
- Multidrug-resistant bacteria often have multiple mechanisms of resistance operative that confer the final resistance phenotype.
  - β-Lactamase + porin mutation that decreased cell wall permeability.
  - β-Lactamase + efflux system that pumps the antibiotic out of the cell.

Subgroup 2c penicillinases hydrolyze carbenicillin or ticarcillin almost as rapidly as benzylpenicillin, but have weaker hydrolysis rates for cloxacillin or oxacillin. They are readily inhibited by tazobactam and clavulanic acid. Few new enzymes in this subgroup have been recently described because both of these drugs are no longer available for clinical use. Extended-spectrum carbenicillinase RTG-4(CARB-10) have more recently been described from *Acinetobacter baumannii*, which has activity against cefepime and cefpirome.[488]

Subgroup 2d β-lactamases hydrolyze cloxacillin or oxacillin and are therefore called OXA enzymes. These enzymes are in molecular class D, and may or may not be inhibited by clavulanic acid or tazobactam. OXA enzymes are currently the second largest family of β-lactamases (Table 17-5). A new subgroup 2de of OXA enzymes has recently been described in *P. aeruginosa* isolates in France and Turkey.[90,450] These enzymes (e.g., OXA-11 and -15) evolved from OXA-10 due to amino acid substitutions, and have retained 2d activity with an extended spectrum that includes oxyimino-β-lactams such as ceftazidime but not carbapenems.[90] Another new subgroup of OXA enzymes designated as 2df has carbapenem-hydrolyzing activity, albeit weak for some carbapenem substrates.[648] Most of these enzymes are chromosomal and have been characterized from *A. baumannii*,[648] but some plasmid-encoded OXA enzymes have been described in *Enterobacteriaceae* (e.g., OXA-23 and -48).[478]Typically, 2df OXA β-lactamases are not inhibited by clavulanic acid.

Subgroup 2e cephalosporinases may be confused in the clinical laboratory as group

1 AmpC enzymes or as ESBLs because they are found in similar gram-negative organisms and have similar resistance profiles. However, this group now mainly refers to the inducible, chromosomal cephalosporinases in the *Proteus*. These enzymes can be differentiated by demonstrating poor affinity for aztreonam and inhibition by clavulanic acid.[86]

Subgroup 2f β-lactamases are serine carbapenemases from molecular class A, which primarily hydrolyze carbapenems, and are better inhibited by tazobactam than clavulanic acid. Most of these enzymes are chromosomal and include the SME family as well as IMI-1 and NMC-1 β-lactamases.[430,495,498] More recently, plasmid-encoded serine carbapenemases including KPC and some GES enzymes have emerged in this subgroup and have rapidly spread worldwide.[63] KPC carbapenemases carried in *Klebsiella pneumoniae* have caused major outbreaks in hospitals, particularly in the eastern part of the United States.[63,495]

Group 3 Metallo-β-Lactamases (MBLs). MBLs are a unique group of β-lactamases that have a zinc ion at the active site (Table 17-5). These metalloenzymes hydrolyze carbapenems but have poor ability to hydrolyze monobactams.[647] They are not inhibited by tazobactam or clavulanic acid, but are readily inhibited by EDTA, dipicolinic acid or 1, 10-o-phenanthroline.[373] Two functional subgroups are now proposed, the major one being subgroup 3a that includes the major plasmid-encoded IMP and VIM family of enzymes (subclass B1), which have been described worldwide in *P. aeruginosa* as well as *Enterobacteriaceae*.[498,647] These enzymes have similar amino acid sequences that act as ligands for the two zinc ions in their active site, which provide broad hydrolyzing activity.[90] Several other MBL enzymes have been added to subgroup 3a including the L1 MBL from Stenotrophomonas maltophilia and subclass B3

MBLs (i.e., CAU-1, GOB-1 and FEZ-1) because they have similar broad-spectrum substrate profiles (i.e., high hydrolysis rates for penicillin, cephalosporin and carbapenem but not monobactam compounds); these enzymes, however, differ in the amino acid sequence involved in zinc ion binding at their active site.[90] In contrast, the subgroup 3b MBLs preferentially hydrolyze carbapenems, so that they are not detected by using a chromogenic cephalosporin substrate (i.e., nitrocephin) to test for β-lactamase activity.[495] These enzymes also work best if only one zinc binding site is occupied. The chromosomal MBLs in *Aeromonas* spp. are an example of a subgroup 3b metalloenzyme.[678]

Aminoglycoside Modifying Enzymes. The aminoglycoside drugs contain a nucleus

that is an aminocyclitol ring that can be either streptidine or 2-deoxystreptamine, and two or more sugars linked by glycoside bonds to the nucleus. Aminoglycosides are naturally occurring molecules produced by actinomycetes. Semisynthetic derivatives produced from *Streptomyces* are labeled -"cins" and include netilmicin, sisomicin, gentamicin, isepamicin, dibekacin, and amikacin.[503] *Micromonospora* derivatives are labeled -"mycins" and include streptomycin, kanamycin, neomycin, tobramycin, and paramomycin.[503] These compounds bind to the bacterial 30S ribosomal subunit rendering the ribosome unavailable for translation, resulting in inhibition of protein synthesis and cell death.[324,503] Most resistance to aminoglycosides results from inactivation of the compound by intracellular bacterial modifying enzymes, although efflux pumps and target site alterations also contribute to a lesser extent.[286,503] Amikacin is not inactivated by the common enzymes that inactivate gentamicin and tobramycin because of structural differences between these drugs. However, all aminoglycoside antibiotics are at risk for inactivation by one of these enzymes.[286,503,618] Aminoglycoside modifying enzymes catalyze the modification at –OH or $–NH_2$ groups of the 2-deoxystreptamine nucleus or the sugar moieties and can be either phosphotransferases (APHs), acetyltransferases (AACs), or nucleotidyltransferases (ANTs) (Fig. 17-11).[359,503,618] These enzymes are present in virtually all bacterial types due to the continuous emergence of new enzyme variants that can utilize a growing number of antibiotic substrates along with the fact that their encoding genes can undergo horizontal transfer as part of integrons, gene cassettes, transposons or conjugative plasmids.[618] Aminoglycoside nomenclature for the various modifying enzymes has been confusing because the original system used only letters.[435] Most scientists now use a three-letter identifier of the activity followed by the site of modification between parenthesis **(class)**, a roman number for the resistant profile **(subclass)** and a lowercase letter as the individual identifier (i.e., **AAC** (6′)-I enzymes are designated **AAC** (6′)-Ib through to **AAC** (6′)-Iaf).[550]The number of aminoglycoside modifying enzymes described to date is extensive and beyond the scope of this chapter, but interested readers are referred to several excellent reviews.[286,503,618]

Aminoglycoside acetyltransferases (AACs) belong to the GCN5-related *N*-acetyltransferases (GNAT) superfamily of proteins, and catalyze the acetylating of one of the four amino groups ($–NH_2$) of the compound. AACs have been classified based on their specificity of acetyl transfer on the aminoglycoside molecule, and catalyze acetylating at the 1[AAC (1)], 3[AAC (3)], 2′ [AAC (2′)], or 6′ [AAC (6′)] positions (Fig. 17-11). All types of AAC enzymes have mainly been found in gram-negative bacteria, although some types have also been found in actinomycetes [AAC (1)], mycobacteria [AAC (2′)], and gram-positives [AAC (6′)].[503] AAC (6′) enzymes are

the most commonly found enzymes in gram-negative as well as gram-positive bacteria, and the genes have been found encoded in chromosomes and plasmids, often as part of mobile genetic elements. The two main subclasses of AAC (6′) enzymes differ in their activity against amikacin and gentamicin C1. AAC (6′)-I enzymes have high activity against amikacin and gentamicin C1a and C2 but low activity against gentamicin C1. The AAC (6′)-II enzymes catalyze acetylating of all three forms of gentamicin but have no activity against amikacin. AAC(6′)-Ib is the most clinically relevant acetyltransferase and is responsible for the resistance to amikacin and other aminoglycosides in several gram-negatives, including *Acinetobacter*, *Enterobacteriaceae*, *Pseudomonadaceae,* and *Vibrionaceae*.[503] A novel class of AAC enzyme has also recently been described that also uses fluoroquinolones as substrates, and has been designated AAC (6′)-Ib-cr because it likely originated from AAC(6′)-Ib by modification of only two amino acids (Trp102Arg and Asp179Tyr). Horizontal transfer of the AAC (6′)-Ib-cr determinant, will therefore confer high-level resistance to both aminoglycosides and fluoroquinolone antibiotics.[523,576] Given the rapid pace of new discovery of aminoglycoside modifying enzymes of the AAC type, many more will likely be described in the future. ANTs catalyze the transfer of an AMP group from ATP to a –OH group in the aminoglycoside molecule, thereby inactivating the compound. Five classes of ANTs have been described that catalyze adenylation at the 6[ANT(6)], 9[ANT(9)], 4′ [ANT(4′)], 2″ [ANT(2″], and 3″ [ANT(3″)] positions, but only ANT(4′) contains two subclasses, I and II.[678] ANT enzymes of various types have more commonly been found in gram-positive bacteria species, including *Enterococcus*, *Staphylococcus,* and *Bacillus*.[107,503,631] However, ANT(4′)-IIa was identified in plasmids of *Pseudomonas* and *Enterobacteriaceae*,[282] and a transposon carrying ANT(4′)-IIb has been described in a *P. aeruginosa* isolate.[136] APHs catalyze the transfer of a phosphate group to the aminoglycoside molecule. The classes and subclasses within the APH enzymes include APH(4)-I, APH(6)-I, APH(9)-I, APH(3′)-I through VII, APH(2′)-I through IV, APH(3″)-I, and APH(7″)-I.[503] APH enzymes of various types have been mainly found in gram-negative species, including *P. aeruginosa*, *S. maltophilia*, *A. baumannii*, *K. pneumoniae*, *C. jejuni,* and *Legionella pneumophila*, but the APH(3′)-1c determinants have also been distributed into *Corynebacterium* spp.[136,282,437,503,632]

Although aminoglycosides have fallen into clinical disfavor due to their inherent toxicity profile and the availability of other potent agents that are active against gram-negative bacteria, increased antibiotic resistance to one or more of these drugs has also diminished their use. Aminoglycoside resistance has developed in epidemic proportions at hospitals around the world since their introduction into clinical practice.[203,204,676] Miller and colleagues, who summarized the results of multiple studies, found variations over time and among geographic areas.[395] There appeared to be a correlation of

resistance with patterns of antibiotic use in these and more recent studies.[5,644] Some aminoglycoside modifying enzymes in gram-negative bacteria have now become so widely distributed that certain drugs such as kanamycin are no longer used, and others such as streptomycin are reserved for specific clinical situations. Rate of resistance to other aminoglycosides such gentamicin and tobramycin also vary widely across different jurisdictions. Even amikacin which is least vulnerable to inactivation may be rendered ineffective by other mechanisms such as efflux, particularly in *Pseudomonas aeruginosa*.[400]

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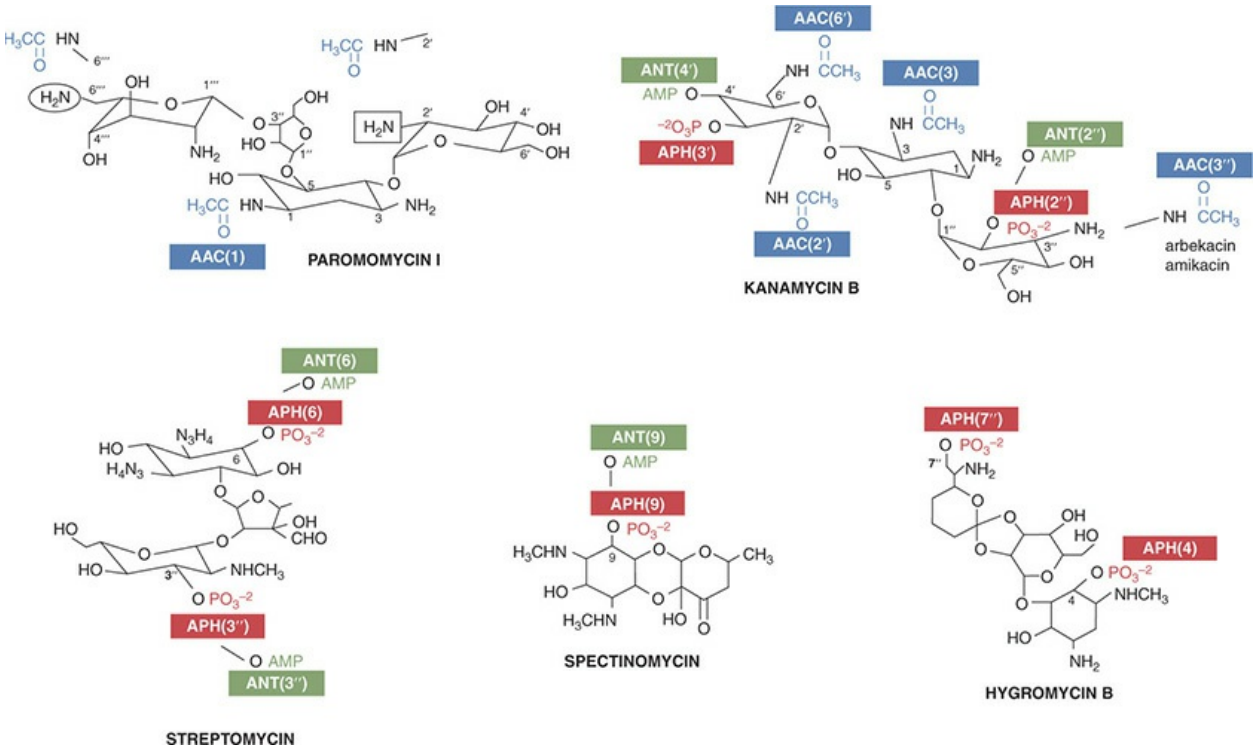

■ **FIGURE 17-11** Actions of aminoglycoside modifying enzymes on common agents representative of aminoglycosides and modification sites by AAC, ANT, and APH enzymes. An example of each kind of modification is shown on one of the substrates. The square and oval on positions 2′ and 6″ in paromomycin I indicate that this molecule is preferentially acetylated at the position 1. Kanamycin, streptomycin, spectinomycin, and hygromycin B can be modified in multiple positions. (Adapted from reference 503.)

Other Enzymes. Several other major classes of antibiotics may be inactivated by bacterial enzymes. In the case of chloramphenicol, the acetyltransferase enzyme is responsible for most clinical resistance.[201] A minor mechanism for resistance to the

tetracyclines is enzymatic inactivation.[602] Antibiotic resistance to both macrolides and lincosamides in gram-positive bacteria may be produced by several mechanisms alone or in combination including enzymatic inactivation, alteration of ribosomal targets or active efflux.[332] Many rRNA methylase genes (*erm*) have been identified that bring about methylation of the 23S rRNA component of the 50S ribosomal subunit involving key adenine bases in the peptidyl transferase functional domain.[522] The MLSB phenotype conveyed by the *erm* genes are widespread in gram-positive and gram-negative bacteria and confer resistance to all macrolides (including clarithromycin and azithromycin), lincosamides **(clindamycin),** and streptogramins (quinupristin-dalfopristin).

Target Modification

Alteration of the target sites where antibiotics act is a common mechanism of resistance. Most antibiotic target sites in bacterial cells also play essential roles in microbial growth and/or metabolic systems. Mutations in the target site must therefore occur such that the bacterium evades antimicrobial action but normal cellular function continues unabated. Frequently, bacterial mutations that result in decreased susceptibility to one or more major classes of antibiotic, not only result in permanent modification of the target structure but also invoke other changes in the cell to compensate for the altered target site (i.e., addition of new genes). This section provides a brief description of the most important types of target site modification that result in clinically significant resistance to one or more classes of antibiotics.

Penicillin-Binding Proteins. One of the most clinically relevant target site changes that occurred primarily in gram-positive bacteria is the acquisition of an altered transpeptidase (PBP) structure, so that β-lactams have reduced noncovalent binding with an acylation by their target site.[200] Methicillin-resistant *S. aureus* (MRSA) strains

have a high level of resistance to methicillin and other β-lactam antibiotics because of acquisition and expression of the *mec*A gene.[628] This gene encodes for an altered PBP 2a (i.e., also designated as PBP2′) that belongs to a group of transpeptidases involved in the synthesis of the bacterial cell wall.[237,239] Exposure of methicillin-susceptible staphylococci to methicillin induced an alteration of the PBP 2 to form a variant (PBP 2a); variants that lost resistance did not contain the variant binding protein.[239]Subsequently, it has been shown that the *mec*A gene is carried on a large genetic element called the staphylococcal cassette chromosome *mec* **(SCC*mec*)** that is carried on the chromosome and integrated near the origin of replication.[255] *S. aureus*

most likely acquired SCC*mec* via horizontal transfer from a coagulase-negative *Staphylococcus* species. To date, 11 different types of SCC*mec*, designated I to XI are currently recognized; along with several variants.[255,625] Different types of SCC*mec* encode other antibiotic resistance genes and range in size from 21 to 67 kb.

MRSA strains have also recently been described in Europe from animal and human sources that carry a novel *mec*A homologue that was originally called *mecALGA*$_{251}$ because it was discovered in strain LG251, but is now designated as the *mecC*gene.[205,553] The *mecC* gene has only 69% sequence identity to the *mec*A gene, and also has quite different homology to the PBP2a encoded by *mec*A.[276]

Resistance to β-lactam antibiotics in *S. pneumoniae* also occurs due to decreased affinity with PBPs. Penicillin resistance occurs because of alteration in PBP2b while resistance to third-generation cephalosporin drugs is due to altered structures of PBP1a and PBP2x.[225,323,412] The mechanism of alteration of the PBP structure in *S. pneumoniae* occurs in a different way than that described for *S. aureus*. Alterations of multiple PBPs result in a multistep process of increasing resistance due to recombination events between the PBP genes of *S. pneumoniae* and those from closely related *Streptococcus* spp. through a process of transformational exchanges of genetic information between these closely related bacteria.[125,170] Successful genetic recombination events result in a mosaic gene structure for the *PenA* and *PenB* genes that encode penicillin resistance in this organism by alterations in PBP.

Enterococci also develop resistance to glycopeptide antibiotics due to the development of intrinsically low-affinity PBPs.[17,334] Both *Enterococcus faecium* and *Enterococcus faecalis* use several PBPs as targets for binding β-lactams. Acquisition of one or two related gene clusters, called VanA and VanB, by enterococci confers high-level resistance to glycopeptide drugs due to the overproduction of low-affinity PBP 5 (PBP5fm) that has reduced binding capability for β-lactams.[20,319] The VanA and Van gene clusters are located on transposons and plasmids, and have been horizontally transferred from enterococci to other bacterial species. The Van gene cluster encodes enzymes that produce a modified peptidoglycan precursor terminating in D-Alanyl-D-Lactate (D-Ala-D-Lac) instead to D-Ala-D-Ala.[20,319,334,335] The peptide loop of mutant PBP 5fm of *Enterococcus* is more rigid than that of PBPs with high-affinity β-lactams binding, and the active site contains a hydrophobic Valine residue that could also decrease access to β-lactams. PBPs structure appears similar to that of low-affinity PBP2a in MRSA strains. The VanA and VanB gene cluster is made up of three genes thought to originate from glycopeptide producing bacteria including: the Van H or Van H_B genes genes that encode a dehydrogenase that reduces pyruvate to D-lactate, 2) the VanA or VanB genes encodes a ligase that synthesizes D-Ala-D-lac, and 3) Van X or VanX_B encodes D-dipeptidase that hydrolyzes preexisting D-Ala-D-

Ala.[334,634] Glycopeptide resistance in enterococci is inducible in the presence of vancomycin or teicoplanin, and a two-component regulatory system VanRS and $VanR_BS_B$ controls expression of the Van genes.[20,334,335] Other glycopeptide resistance gene clusters characterized in *E. faecium* include VanD, VanE, and VanM, while VanG and VanN may also occur in *E. faecalis*.[57,394,429,672] *Enterococcus casseliflavus* and *Enterococcus gallinarum* are inherently resistant due to the presence of a VanC gene cluster.[111,336]

S. aureus strains with reduced susceptibility to vancomycin, or vancomycin-intermediate *S. aureus*/vancomycin-resistant *S. aureus* (VISA/VRSA), were initially discovered in Japan in 1997 and had minimum inhibitory concentrations (MICs) = 8 μg/mL (i.e., also called glycopeptide-intermediate *S. aureus* or GISA).[253,567] These strains demonstrated several cell wall changes compared to control isolates including an increase in cell wall thickness. An increased proportion of peptidoglycan stems from peptides that contain nonamidated glutamine residues that result in decreased peptidoglycan cross-linking.[253,607] Therefore, a decreased amount of the glycopeptide drug reaches the cell wall targets in VISA/VRSA strains because the thicker cell wall entraps the antibiotic molecules before it reaches the cytoplasm membrane.[140] In 2002, a new mechanism for high-level resistance to vancomycin was found in an MRSA strain with an MIC = 32 μg/mL.[235] The strain had acquired the VanA gene cluster from enterococci, but it also had an SCC*mec*A gene cassette and together these genes encoded for multiple changes in the bacterial peptidoglycan composition bringing about high-level resistance to glycopeptides. To date, reports of vancomycin resistance in *S. aureus* and MRSA strains have remained isolated,[560,612] but the future dissemination of this type of resistance would have serious clinical consequences.

β-lactam resistance due to PBP changes in other organisms has also been described. In Japan, *H. influenzae* strains have been characterized that are β-lactam resistant due to acquisition of the *ftsI* gene that encoded mutations in PBP3.[241,379] Nontolerant group A streptococci have decreased binding of penicillin to PBP 3, increased binding to PBP 5, and replacement of PBP 2 with a new PBP 2a′ that has decreased affinity for penicillin-binding.[174] PBP 3 affinity for penicillin is also reduced in *Listeria monocytogenes* strains with decreased susceptibility to imipenem and penicillin.[635] Several gram-negative bacterial strains have been characterized that have decreased affinity of PBP 2 for imipenem, including *P. mirabilis*, *P. aeruginosa,* and *A. baumannii*.[36,212,420] Amoxicillin resistance in *Helicobacter pylori* has also been shown to be due to mutations in *pbp1*.[451]

DNA Gyrase. Resistance to the fluoroquinolones is mainly caused by chromosomal mutations in two enzymes involved in DNA synthesis, DNA gyrase and topoisomerase

IV. DNA gyrase produces negative super helical coils in the bacterial DNA in front of the replication fork. This enzyme complex is composed of two subunits including GyrA and two GryB

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subunits that are encoded by their respective *gyrA* and *gyrB* genes. Topoisomerase **IV** decatenates interlinked daughter chromosomes following replication. This enzyme complex in most bacteria is comprised to two ParC and two ParE subunits encoded by their respective *parC* and *parE* genes. In *S. aureus,* topoisomerase **IV** is encoded by two subunits called GrlA and GrlB.[543] Fluoroquinolones bind to the **DNA–DNA** gyrase or topoisomerase enzyme complexes bringing about conformational changes in these enzymes that block replication of **DNA** and introduce double-stranded **DNA** breaks.[262] Mutations in *gyrA* that alter the GyrA subunit of the **DNA** gyrase occur in a region of the protein that is highly conserved between different bacterial species called the fluoroquinolone resistance-determining region **(QRDR)**, and specifies the active site of the enzyme (i.e., between amino acids 67 and 106).[261] Two known mutations *nalC* and *nalD* in the **DNA** gyrase B subunit result in amino acid substitutions in the central part of the molecule between positions 426 and 447 that confer a moderate level of resistance to nalidixic acid, and these mutations have so far only been rarely detected in clinical isolates.[261] The level of resistance to fluoroquinolones may therefore differ according to the site of mutation, and the substituted amino acid in a particular clinical isolate.

In gram-negative bacteria, high-level fluoroquinolone resistance commonly occurs due to mutations in the GyrA subunit. Although GyrB subunit alterations (i.e., *gyrB* gene mutations) occur, they are much less common than GyrA mutations, and produce lower levels of fluoroquinolone resistance than mutations in *gyrA*. Mutations encoded by the *gyrA* gene result in alterations in the portion of the GyrA subunit that is bound to the DNA during replication (i.e., active site), and result in drug resistance in *Enterobacteriaceae* due to decreased affinity for the altered DNA gyrase–DNA complex.[262,551,654] Topoisomerase IV serves as a secondary target for fluoroquinolone drugs in gram-negative bacteria, and mutations are less commonly found in *ParC* or *ParE* genes.[262]

Although mutations in *ParC* or *ParE* genes that result in alteration of the topoisomerase IV enzyme–DNA complex have been described in *E. coli*, concomitant mutations in *gyrA* in some strains result in high-level fluoroquinolone resistance.[262,380,551]Topoisomerase IV enzyme mutations occur most often in gram-positive bacteria, particularly in *S. aureus* and *S. pneumoniae* where alterations in either subunit may result in high-level fluoroquinolone resistance. However, mutations

in *ParC* or *GrlA* in *S. aureus* occur more frequently than mutations in *ParE* or *GrlB* in *S. aureus*.[260,304,305] Similar mechanisms bring about fluoroquinolone resistance in gram-positive bacteria. Mutations in *ParC* result in reduced drug affinity for the DNA–topoisomerase IV complex.[68,260,405]

The development and identification of several mechanisms of plasmid-mediated fluoroquinolone resistance, primarily in gram-negative bacteria is cause for concern.[99,380,523] Plasmid-mediated fluoroquinolone resistance was initially reported in *K. pneumoniae* isolates due to acquisition of a *qnr* gene that conferred high-level ciprofloxacin resistance (i.e., MICs as high as 32 μg/mL).[524] Drug resistance was found to be due to a plasmid-mediated *qnrA1* gene which encoded a QnrA1 protein, a pentapeptide repeat protein that acts by protecting DNA gyrase from fluoroquinolone binding. Five other QnrA variant proteins (Qnr2 to Qnr6) have since been identified, and three other plasmid-mediated bacterial *qnr* determinants that share some similarity to *qnrA1* have been reported (i.e., *qnrB1*, *qnrC1,* and *qnrS1*).[99] The prevalence of *qnr* genes in gram-negative bacteria has been reported to range from 1% to 5%.[99] So far, two other major plasmid-mediated mechanisms have been described that also confer high-level fluoroquinolone resistance including the aminoglycoside modifying enzyme acetyltransferase AAC(6′)-Ib-cr (See Aminoglycoside Modifying Enzymes),[523] and the QepA efflux pump related to the MFS system (See Efflux Pumps).[99]

Other Targets. Resistance to the action of antibiotics that block protein synthesis by binding to the 50S ribosomal subunits such as the macrolides, lincosides, and streptogramin B may also occur by alteration in the target site. Mutation in the 23S rRNA component of the 50S subunit close to sites where methylation resistance occurs is also independently associated with resistance to the macrolides in many bacterial species.[332] Alteration in the L4 and L22 proteins of the 50S subunit as well as mutations in its 23 rRNA component has been described in *S. pneumoniae*.[61,93]

Oxazolidinones (i.e., Linezolid) also inhibit protein synthesis by binding to the 50S ribosomal subunit, as well as inhibition of the formation of the initiation complex and translocation of peptidyl-tRNA for the A to the P site.[60] Resistance to linezolid has recently been described in both *S. aureus* and vancomycin-resistant enterococci (VRE) due to mutation in the 23S rRNA subunit, which results in decreased antibiotic affinity for its target site.[391,492]

Rifampin is an important drug that is used to treat *Mycobacterium tuberculosis* and *H. pylori*, and as an adjuvant agent in combination with other antibiotics for serious invasive *S. aureus* infections. This drug acts on the RNA polymerase encoded by the *rpoB* gene. Point mutations, insertions, and deletions readily develop in the β-subunit

of this enzyme, particularly when patients are treated with rifampin alone.[11,248,249,519]

Trimethoprim resistance in *S. aureus* and *S. pneumoniae* occurs by mutation in the *dhfr* gene resulting in modification of the dihydrofolate reductase and decreased antibiotic affinity for the target enzyme.[270,271,544] Many different types of transferable trimethoprim-resistant genes located on integrons, transposons, and plasmids have also been described in gram-negative bacteria.[33,476,643,650]

Finally, antibiotic resistance to mupirocin and fusidic acid that are used to decrease the bacterial load at skin and mucosal surfaces due to *S. aureus* also occurs by target site modification. High-level resistance to mupirocin (i.e., MIC 512 μg/mL) in *S. aureus* involves acquisition of the *mupA* gene that results in changes in the target enzyme, isoleucyl-tRNA synthetase.[129] More recently, a novel *mupB* gene has been reported that conferred high-level mupirocin resistance in *S. aureus*, but the encoded class I tRNA synthetase had limited homology to either the *mupA* or *ileS* gene products.[547] In contrast, low-level mupirocin resistance results when point mutations occur in the chromosomal isoleucyl-tRNA synthetase gene,*ileS*.[10] Fusidic acid also blocks protein synthesis in staphylococci by inhibiting the function of elongation factor (EF-G). Fusidic acid-resistant isolates of *S. aureus* have

point mutations within the chromosomal *fusA* gene that encodes EF-G.[321,626]

Bypass as a Resistance Mechanism

Bacteria may also become resistant to the action of specific antibiotics by developing an alternate metabolic pathway for normal functions while bypassing the action of the drug. The most clinically important example of a bypass mechanism is the ability of naturally occurring strains of *Enterococcus* species to use compounds such as folinic acid for growth in vivo in the presence of trimethoprim-sulfamethoxazole.[221,685] Strains may appear susceptible in vitro, although they do not respond to chemotherapy in vivo. The in vitro result can be converted from susceptible to resistant by the addition of folinic acid to the testing medium. Enterococci should not, therefore, be tested against trimethoprim-sulfamethoxazole. Enterococci have also been described that have developed an alternate mechanism for cell wall synthesis that produces an alternate way of cross-linking components of peptidoglycan that bypasses the target sites for both β-lactam and glycopeptide antibiotics.[137]

Multiple Antibiotic Resistance Mechanisms in

Bacteria

Bacteria have developed multiple resistance mechanisms, and one or more similar or different types of resistance mechanism are found in multidrug-resistant pathogens. Several gram-negative bacteria are prime examples of MDR organisms including *P. aeruginosa*, *A. baumannii,* and *Salmonella* spp. where pan-resistance is now being found in some strains due to the presence of integron gene cassettes with multiple resistance genes that encode one or more β-lactamases, aminoglycoside modifying enzymes, fluoroquinolone resistance determinants, tetracycline resistance, and resistance to disinfectants.[476,487,643]

Another extremely effective synergistic defense mechanism in gram-negative bacteria is the combination of β-lactamase production in addition to decreased permeability of the OM or increased efflux out of drug out of the cell. *P. aeruginosa* is perhaps the best example of where this combination of resistance mechanisms may be encountered in the same strain.[619] *S. marcescans* is another example of an isolate that normally produces a low level of an AmpC β-lactamase but is usually susceptible to third-generation cephalosporins and carbapenems. When the organism overproduces β-lactamase in the periplasmic space and also has decreased OM permeability, then the isolate becomes highly resistant to the action of these important compounds.[246]

Laboratory Guidance of Antimicrobial Susceptibility Testing

Clinical microbiology laboratories play an essential role in guiding the prescription of antimicrobial agents for effective treatment of not only serious invasive infections in hospitalized patients, but also a wide range of infections in ambulatory patients. The primary goal of antimicrobial susceptibility testing is to provide accurate in vitro testing of a bacterial pathogen to a cadre of available antibiotics to determine its "antibiogram" or susceptibility profile in order to predict the in vivo effectiveness of a particular antibiotic or antibiotic regimen being used to treat a patient. A second function of antibiotic susceptibility tests is to monitor the effectiveness of therapy. Although antibiotic susceptibility results must be performed in such a way that an accurate in vitro result is achieved for each drug tested against the isolate. Testing must also be performed in such a manner that the results are available to clinicians in a timely fashion.[603] Rapid identification of cultured isolates facilitates production of timely antibiotic susceptibility results reporting, and may currently be done the same day using a rapid nucleic acid amplification method,[603] or one of the commercially

available automated biochemical systems[303,347,536,615,667] or matrix-assisted laser desorption/ionization time of flight (MALDI-TOF).[94,413,563] Immediate inoculation of an automated antibiotic susceptibility system can also provide expedited results allowing for the same day reporting of the susceptibility profile of an isolate.

Rapid reporting of antibiotic susceptibility results is important for all patients with infection, but essential for those who are critically ill. Box 17-2 outlines the improved clinical outcomes of prompt appropriate antibiotic therapy in patients with serious invasive infections. In contrast, inappropriate antibiotic prescriptions are associated with a decrease in quality of care and an increase in healthcare costs. For example, studies of patients admitted to ICU with septic shock due to bacteremia have shown that 20% initially received inappropriate empiric antibiotic regimens (i.e., antibiotics that are ineffective against the infecting bacteria due to either inherent or acquired resistance), and resulted in a fivefold decrease in survival (i.e., 52.0% of patients survived when prescribed the appropriate empiric antibiotic regimens versus 10.3% of patient who were prescribed the inappropriate empiric antibiotic regions [$p < 0.0001$]).[327] Delayed detection and reporting of bloodstream infections also has been shown to increase the length of hospitalization and hospital costs.[34] Similar findings have also been documented for

other types of serious invasive infections including bacterial peritonitis and pneumonia whether community-, hospital-, or ventilator-acquired.[147,208,422] Although clinicians must prescribe broad-spectrum therapy initially that covers all types of pathogens of concern, the clinical microbiology laboratory also must be able to expedite reporting of antibiotic susceptibility results to facilitate early treatment with the best therapeutic regimen.

BOX 17-2

Improved Clinical Outcomes From Rapid Reporting of Antimicrobial Susceptibility Results

- Improved patient outcomes
- Lower mortality rates
- Less diagnostic procedures ordered including:
  - Laboratory tests
  - Diagnostic imaging procedures
- Shortened ICU stays

- Decreased ventilator days
- Fewer prescriptions for inadequate antibiotic therapy
- Shortened length of stay
- Lower total cost of hospitalization

Several studies have clearly shown that the rapid reporting of antibiotic susceptibility results may influence the patient outcome from infection, shorten hospital stays, and allow improved tailoring of antibiotic regimens resulting in cost savings and potentially decreased development of antibiotic resistance due to inappropriate or sub-inhibitory drug exposure of the bacterium.[603] Doern and colleagues performed one of the earliest assessments of the clinical impact of "rapid" reporting.[161] For 1 year, bacterial isolates were tested using an expedited commercial method (rapid group) or traditional overnight methods (conventional group). The mean time to reporting results was 11.3 hours and 19.6 hours in the rapid and conventional groups, respectively. The length of hospitalization was comparable in the two groups, but there were significant improvements in the rapid group for the factors listed in Box 17-2.

A subsequent study of isolates from blood cultures demonstrated a statistical correlation of increased time from collection of blood cultures to initial reporting of results with increased length of hospital stay.[34] In this case more rapid detection of bacteremia isolates with instruments that continuously evaluate growth in blood culture bottles was responsible for the effect. More recent studies have confirmed that more rapid detection and more rapid reporting of susceptibility results produce additive, if not synergistic, results. Recent studies by clinical microbiology laboratories in Europe confirm and expand these early findings. Eveillard and colleagues demonstrated that provision of a 24 hours a day bacterial identification and antibiotic susceptibility testing service including nights led to earlier implementation of antibiotic therapy in 22.6% of the patients, and a change to narrower spectrum effective regimens in another 5.3% of patients.[184] Galar and colleagues compared two groups of hospitalized patients with bacterial infections including a control group where microbiology results were available the day following analysis, and an intervention group where results were available the same day of analysis.[202] Although the mortality rates were not significantly different between these two groups of patients, the faster reporting of identification and susceptibility results was associated with a significant reduction in hospital stay and in the overall costs of patient care for those with wound or urinary tract infections or abscesses, but not those with bacteremia.[202]

Antimicrobial test procedures are summarized in Table 17-6, which includes a brief description of each test and information regarding testing. The tests may be divided conveniently into two groups: (1) tests that *predict* the

effectiveness of therapy and (2) tests that *monitor* the effectiveness of therapy.

TABLE 17-6 Guidance of Antimicrobial Therapy

Procedure	Specimen	Definition	Interpretation
Tests That Predict the Effectiveness of Therapy			
Minimum inhibitory concentration (MIC)	Isolate	Lowest concentration of drug that inhibits visible growth	MIC
Disk diffusion	Isolate	Diameter of zone of inhibition around antibiotic impregnated disk	Zone diameter approximates MIC
Gradient diffusion (Etest strip)	Isolate	Read at point of intersection of zone of inhibition with the scale on the strip	MIC
Growth curve calculation (e.g., Vitek 2)	Isolate	Calculated MIC based on the growth of the organism in the presence of the antibiotic	Computer-generated MIC
Tests That Monitor the Effectiveness of Therapy			
Molecular detection	Isolate	Molecular detection of antibiotic resistance gene(s) or gene product(s) (i.e., *mecA* in *S. aureus*, Van genes in enterococci, etc.)	Strain positive or negative
Minimum bactericidal concentration	Isolate	Lowest concentration of antibiotic that kills 99.9% of the inoculum	Predictive of bactericidal activity level
Serum bactericidal titers	Peak serum, trough serum, Isolate	Dilution of serum that kills 99.9% of the inoculum Rarely performed; no indication for test except is some cases of endocarditis	Predictive of response to current antibiotic regimen
Antimicrobial levels	Peak serum, trough serum	Concentration (μg/mL) of antibiotic in serum just before the antibiotic is given (trough level) and 15–20 minutes after the dose is administered (peak level)	Peak level = therapeutic indicator Trough level = toxicity indicator
Antibiotic Synergy Test	Isolate	Predicts the antagonistic, indifferent or additive effects or two or more antibiotics in combination Rarely performed except for respiratory isolates from patients with cystic fibrosis	Additative activity predicts synergistic effect

Several types of antimicrobial susceptibility tests for the prediction of drug effectiveness have been devised. The two reference methods are the macroscopic broth dilution and agar dilution procedures. Both are designed to quantify the lowest concentration of an antimicrobial agent that inhibits visible in vitro growth of the microbe designated the MIC. The test used most widely in both small and large clinical microbiology laboratories to guide antimicrobial agent therapy is the disk diffusion procedure (Kirby–Bauer test), in which clinical interpretations are derived from correlations with the reference test (Chart 17-1). An increasing number of laboratories, however, routinely use a miniaturized broth test (microbroth dilution test) or an automated commercial system. The broth microdilution test has become so prevalent and so well studied that it has become the reference standard for many investigators (Chart 17-2).

A variety of options for antimicrobial susceptibility studies are now commercially available, including commercially available MIC plates (both frozen and dehydrated), computer-assisted growth analysis and gradient diffusion (Chart 17-3). For the

purposes of discussion in this chapter these approaches will be included in the designation "dilution tests" or "MIC tests."

The remaining supplemental tests used to monitor the bactericidal activity of a specific drug regimen are now rarely used, and thus a discussion of these procedures is beyond the scope of this chapter. The monitoring of the peak and trough levels of specific antibiotics (i.e., aminoglycosides and vancomycin) is usually performed in clinical chemistry laboratories. The interested reader is referred to several excellent references that detail methods for performing Minimum Bactericidal Tests (MBT), Antibiotic Synergy Tests (AST), and Serum Bactericidal Tests (SBT).[113,116,469] A brief description of the application of ASTs for multidrug-resistant pulmonary isolates from patients with cystic fibrosis is provided later in the chapter.

The most useful means for assessing the adequacy of antimicrobial therapy in many infections is to monitor the clinical outcomes listed in Box 17-2. Ultimately, the response of the patient to treatment and a "cure" is the aim of antibiotic therapy and, if requested, the laboratory may need to demonstrate by repeated culture that the infecting organism either has been eliminated (bacterial cure) or persists (bacterial failure). Unfortunately, a bacterial cure does not always ensure a successful clinical outcome.

A goal of microbiologists has been, and should continue to be, the provision of standardized in vitro tests that can be reproduced from day to day and from laboratory to laboratory. Without reproducibility, there is no scientific basis for reporting accurate antibiotic susceptibility results on which to base therapy. Unfortunately for patients, a high number of serious errors in laboratory performance of antibiotic susceptibility testing have been documented by surveillance studies either because of in-house errors in test performance or policy and procedure, or frequently to methodologic failure to accurately detect a particular resistance phenotype.[132,156,382,575,587,613] Although standardization of the variables outlined herein is required for the accurate reproducible susceptibility testing, the in vitro result is at best a prediction of a treatment response in a given patient.

The factors that determine the outcome of an infectious process are complex and, in many instances, are incompletely addressed by in vitro tests.[278,652] Pharmacokinetic (PK) and pharmacodynamics (PD) (PK/PD) thresholds for a particular antibiotic also influence the clinical outcome of infection treatment,[175,194] and increasingly clinically relevant antibiotic susceptibility breakpoints are based on the appropriate dosing of an antimicrobial agent calculated on available PK/PD data for a given antibiotic agent.[121,122,278,333] Based on PK/PD data, Clinical and Laboratory Standards Institute (CLSI) has introduced a "susceptible-dose dependent" or SDD interpretation for antibacterial susceptibility testing, although it has been applied for interpretation of antifungal susceptibility test results for several years.[122] The SDD category implies that susceptibility of an isolate is dependent on the dosing regimen that is used in the

patient. The SDD had previously been included in an intermediate result, but this concept was often not evident or understood by clinicians. If the laboratory reports the isolate as having an MIC or zone diameter that equates to an SDD result, it is necessary to use a dosing regimen that results in a higher drug exposure than the dose that was used to establish the susceptible breakpoint (i.e., clinicians should use the maximum approved dosage regimen in order to achieve adequate therapeutic effect against an SDD isolate. For example, CLSI has recently recommended SDD category reporting instead of "intermediate" when reporting cefepime results for *Enterobacteriaceae* isolates because there are multiple approved dosing options for cefepime. A report of SDD for an *Enterobacteriaceae* isolate indicates to the clinician that higher cefepime doses (i.e., greater than 1 g every 12 hours) should be used to treat infections caused by isolates when the cefipime MIC is 4 or 8 μg/mL or the zone is 19 to 24 mm.[122]

The inflammatory and immune defenses of the patient are also essential to a successful clinical outcome. It is clear that suboptimal concentrations of antimicrobial agents, below the concentration necessary to kill the bacterium, may enhance the ability of phagocytes to ingest and kill an infecting microorganism.[251,583] The penetration of antimicrobial agents into sites of infection is another important variable that cannot be addressed in vitro. High concentrations of antimicrobial agents may be achieved in sites where they are excreted from the body, usually the urine or the bile. In contrast, low concentrations relative to serum may be found in tissues such as prostatic fluid, bone or cerebrospinal fluid. In the case of chloramphenicol or clindamycin, excretion is primarily via the biliary tract, and a minimum amount of the drug is found in the urine. The reverse holds for other drugs such as nitrofurantoin and norfloxacin, which do not achieve effective levels in sites other than the urinary tract.

The ineffectiveness of many antimicrobial agents, such as the aminoglycosides, in the treatment of *Legionella* infections, despite their excellent activity in vitro, is probably caused by the poor penetration of the antibiotics into macrophages where the bacteria are growing.[4,234] In other situations, bacterial physiology is a significant determinant (e.g., metabolic bypass of trimethoprim and sulfonamide effects by enterococci). Many infections, particularly those caused by obligate anaerobic bacteria, are synergistic; that is, the bacteria depend on each other for survival.[377,528] Such an infection may be cured by drugs that are ineffective against

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some of the infecting bacteria if the essential participants are eliminated.[72,529] Considering all the factors that influence the outcome of an infection, it is imperative that the laboratory provide physicians with the "track record" of the antimicrobial agent to guide them in the selection of appropriate antimicrobial agent therapy. Well-

designed clinical studies have demonstrated a correlation of outcome with the appropriateness of therapy.[147,327] Physicians must, therefore, correlate the results of antimicrobial agent susceptibility tests and clinical experience when selecting therapeutic regimens for patients with similar infections.

The following section discusses the methods used to perform routine antibiotic susceptibility testing of commonly isolated bacterial pathogens. Insight into the limitations of current procedures, as well as bacteria for which no standardized testing or interpretation guidelines are highlighted, which serve to clarify the reasons that an absolute correlation should not be made in these cases between laboratory results and clinical outcome.

Antibiotic Susceptibility Testing Methods

Methods to test the inhibitory activity of antimicrobial agents have mainly been standardized for aerobic bacteria that grow well after overnight incubation in air and have unpredictable susceptibilities.[114,117,121,122] Decreased susceptibility trends and special procedures for reliable detection of particular types of resistance in commonly isolated aerobic bacteria are also outlined within this section. Fastidious bacteria, which grow more slowly or require nutritional or atmospheric supplements, require specialized test procedures, and should be tested with the careful use of control bacterial strains in order to demonstrate the absence of inhibitory effects of the method on the isolate.[114] The disk diffusion test may be modified for such organisms if the procedure has been validated by comparison to reference tests and to clinical experience.[114]

Microbiologists should resist requests from clinicians to extend the standardized procedures beyond their established limits. Laboratories may be requested to perform unwarranted susceptibility tests that will not provide a valid result (e.g., trimethoprim-sulfamethoxazole testing on enterococci), or susceptibility testing on isolates for which there are no established interpretation guidelines. Table 17-7 summarizes a variety of situations where antibiotic susceptibility tests should be performed but the results may or may not be reported. Some of these issues are considered more thoroughly in appropriate sections of this chapter.

Only isolates producing an infection should be tested. Bacteria recovered from a normally sterile body fluid are usually pathogenic. If the potentially pathogenic bacterium is isolated from a site that contains colonizing flora, such as the upper respiratory tract or the skin, the culture should be scrutinized more closely before a susceptibility test is performed, particularly if multiple species of organisms are present. Examination of a Gram-stained smear may document the inclusion of squamous epithelial cells, which suggests contamination by colonized secretions, or the

absence of segmented neutrophils, which indicates the lack of an inflammatory response. In situations in which the microbiologist cannot determine the suitability of a susceptibility test, consultation with the clinician is appropriate.

The list of bacteria that have consistently predictable susceptibilities is, unfortunately, becoming shorter, which necessitates changing CLSI recommendations about the use of specific antibiotic agents as surrogates to predict susceptibility within a class of drugs. The prevalence of β-lactamase-producing strains of *Haemophilus influenzae* and *Neisseria gonorrhoeae* is sufficiently high that susceptibility to penicillin analogs cannot be predicted.[256,601] β-lactam, aminoglycoside and fluoroquinolone drug resistance has also become widespread in other types of gram-negatives including *Enterobacteriaceae*, *Acinetobacter* and *Pseudomonas*.[380,400,487,619] CLSI recently changed its recommendation for surrogate testing of urine isolates in patients with uncomplicated urinary tract infections because of changing cephalosporin resistance profiles in *Enterobacteriaceae*.[122] CLSI now recommends using cefazolin instead of cephalothin as the surrogate first-generation drug to more reliably predict the susceptibility of *Enterobacteriaceae* urine isolates to oral agents (i.e., cefadroxil, cephalexin, cefpodoxime, and loracarbef). Other cephalosporins including cefpodoxime, cefdinir, and cefuroxime axetil may also be tested individually as required because some isolates may be susceptible to these agents while testing resistant to cefazolin. Pneumococci that are relatively resistant to penicillin (i.e., MIC, 0.12 to 1.0 μg/mL) or have high-level resistance (i.e., MIC > 1.0 μg/mL) are increasingly common in the United States.[160,290] Pneumococcal strains isolated from serious infections should be tested for resistance because *Streptococcus pneumoniae* with multidrug resistance to β-lactams, macrolides, and fluoroquinolones is also increasingly isolated.[193,490] Although clinical *Streptococcus pyogenes* and *Streptococcus agalactiae* isolates remain susceptible to penicillin, resistance to macrolide antibiotics has become common in these two important genera.[269,314,444] Vancomycin has become the mainstay of treatment against serious invasive gram-positive infections due to the nosocomial transmission, and the emergence of community-acquired MRSA strains,[148,623] while the increased use of vancomycin that has subsequently resulted in VRE also becoming a major nosocomial problem.[17] Vancomycin resistance in staphylococci has also emerged.[253] Increasing ampicillin resistance and high-level aminoglycoside resistance with a loss of synergy between a cell wall agent and the aminoglycoside have been reported from many jurisdictions.[16] These trends in antimicrobial resistance in many common bacterial pathogens has meant that clinical laboratories must perform much more routine susceptibility testing in order for clinicians to be able to accurately prescribe therapy even for common types of ambulatory infections.

There remain a few clinical situations where susceptibility or resistance is still

predictable and routine testing is not indicated. Although clinical and/or bacteriologic infection relapses may occur for reasons other than antibiotic resistance including inadequate antibiotic penetration into the site of infection and reinoculation, antibiotic resistance should be ruled out by testing the isolate when the clinical response is not as expected (i.e., potentially treatment failure) or when the patient has one or more listed antibiotic allergies. Although *Streptococcus pyogenes* remains susceptible to penicillin, treatment failure has been documented due to treatment of acute bacterial pharyngitis with a macrolide when the strain was resistant.[41] Treatment failure has also been recently reported for routine intrapartum antibiotic prophylaxis due to macrolide-resistant group B *Streptococcus,* which resulted in neonatal infection.[110] Microbiologists must be cognizant of trends in antibiotic resistance in pathogens not only in their respective geographic location, but globally so that antibiotic testing of previously susceptible bacterial species can be routinely implemented when appropriate.

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TABLE 17-7 Situations in Which Antimicrobial Susceptibility Testing (AST) Should Not Be Performed and/or the Results Not Reported

Category	Antibiotic(s)	Organism(s)	Site	Action	Rationale
Susceptibility predictable	Penicillin/oxacillin	*S. aureus* *S. pneumoniae* β-Hemolytic streptococci (*S. pyogenes*, *S. agalactiae,* etc.)	All	Do not test other β-lactams	Results of these primary agents predict susceptibility to other β-lactams.
Organisms identification and AST confirmation required	Varies by organism	See M100-S24 appendix A	All	Confirm ID and AST for the listed resistance phenotype according to organism	Uncommon resistance phenotype in most institutions (e.g., *S. aureus* that are Vancomycin I or R, *Enterobacteriaceae* that are carbapenem I or R etc.)
Intrinsic resistance[a]	Varies by organism	See M100-S24 appendix B	All	Do not test listed drugs according to species	Some drugs may be reported as R depending on organism.
In vitro resistance not predictable	Cephalosporins	*Enterococcus* spp.; *Listeria monocytogenes*	All	Do not test	Results may be misleading.
	Aminoglycosides (low dose)	*Enterococcus* spp.	All	Do not test	High-dose testing for synergy; results may be misleading.
	Clindamycin; trimethoprim; trimethoprim-sulfamethoxazole	*Enterococcus* spp.	All	Do not test	Results may be misleading.
	All β-lactam antibiotics	*Staphylococcus* spp. resistant to oxacillin	All	Do not test or report as R	AST results do not predict clinical efficacy and will be misleading.
	Salmonella spp.; *Shigella* spp.	All	All		
Antimicrobial agent does not accumulate at site of infection	First- and second-generation cephalosporins; clindamycin; macrolides; tetracyclines; fluoroquinolones; antibiotics administered orally	All; including *S. pneumoniae*	CSF	Do not test	Antimicrobial agents do not penetrate into CSF or do not accumulate in therapeutic concentrations across the blood–brain barrier.
	Chloramphenicol; tigecycline	All	Urinary tract	Do not test	Antibiotic not excreted in urine
	Daptomycin	All	Lower respiratory tract	Do not test	Antibiotic inhibited by surfactant in lungs
	Norfloxacin; nitrofurantoin	All	Non-urine sites	Do not test	Antibiotic does not reach therapeutic levels in sites other than the urinary tract.
Infants and children	Fluoroquinolones tetracyclines	All	All	Test but do not report unless requested	These agents may be contraindicated in infants and young children.[b]
Pregnant of lactating women	All antimicrobial agents that are contraindicated	All	All	Test and report antimicrobial agents that are safe to administer[b]	

[a] See M100-S4 appendix B. Intrinsic resistance is defined as inherent or innate (not acquired) antimicrobial resistance, which is reflected in wild-type antimicrobial patterns of all or almost all representatives of a species. Intrinsic resistance is so common that susceptibility testing is unnecessary. For example, *Citrobacter* species and many other *Enterobacteriaceae* are intrinsically resistant to ampicillin.[122]
[b] See M100-S24 Table 1A, 1B, 1C drugs recommended for testing and reporting.
Adapted from reference 122.

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Standardization of Antibiotic Susceptibility Testing Methods

The most important improvement in laboratory guidance of susceptibility testing over the past several decades has come from the development of standardized published procedures by the CLSI (www.clsi.org) in the United States, the European Committee Consensus on Antimicrobial Susceptibility Testing (EUCAST), and other groups that have been widely adopted. The methods outlined in this chapter are compliant with CLSI recommendations,[114,117,121,122] but readers can go to the websites for EUCAST (www.eucast.org), the British Society for Antimicrobial Chemotherapy (BSAC) (www.bsac.org.uk), Deutsches Institut für Normung (German Institut for Standardization) (www.DIN.de), and the Committé Antiobiogramme—Société Française de Microbiologie (CA-SFM) (www.ca-sfm.org)—to compare methods published by these other groups. It is important that revised procedures and current recommendations be promptly promulgated and implemented into practice in all clinical laboratories. An institutional membership in the CLSI ensures the timely receipt of all new and revised recommendations. The following parameters are some of the important facets of susceptibility testing that have been standardized.

Growth Medium. Müeller–Hinton broth (MHB) and agar (MHA) have been selected for testing aerobic and facultative anaerobic isolates. These formulations have been standardized in order to produce highly reproducible susceptibility results, and typically contain dehydrated beef infusion, acid digest of casein, and cornstarch.[122,480] Cooled molten agar should routinely be poured into plates as soon as possible after mixing, and generally 20 to 25 mL of molten agar in a 90-mm plate is used to achieve an agar depth of no more than 3 to 4 mm.

Müeller–Hinton medium, however, also contains agar, a natural compound that is prepared from red seaweed of the *Rhodophyceae* class.[331] Agar is mainly composed of polysaccharides, agarose and agaropectin. Variation initially occurred in the composition of agar among manufacturers and even among lots produced by a single company, depending on the source of the seaweed. Diffusion of chemicals can also be affected by the presence of sulfate ions that change the charge of the polysaccharides. Variable cation concentration also had a significant effect on the activity of aminoglycosides against *P. aeruginosa*.[141,508] Most of the problems in the variation among lots of MHA were overcome by the development of a reference standard for manufacturers to provide greater reproducibility.[480]

Limitations. Most pathogens grow satisfactorily on MHA, and the medium has minimal inhibitory effects on sulfonamides, trimethoprim and tetracycline. Large quantities of thymidine are present in some lots of media. Some organisms can use the thymidine to bypass the mechanism of action of trimethoprim and grow, even though they are innately resistant to the antibiotic. Enterococci are particularly affected; isolated colonies may appear within the established zone of inhibition around disks that contain trimethoprim.

Agar depth may also effect antibiotic susceptibility results, particularly too thick an agar depth.[298]

Specialized Medium and Additives. Some bacteria require the use of specialized media or the use of additives to ensure adequate growth. Most commonly blood, usually sheep of horse blood, is added to MHA in a 5% concentration in order to get adequate growth of *Streptococcus* spp., including *S. pneumoniae*, β-hemolytic streptococci, *Neisseria meningitidis*, *Campylobacter* spp., and *Helicobacter pylori*.[114,121,122,302] However, when testing sulfonamide activity, the use of horse blood–supplemented MHA is preferred because of its low levels of antagonists. *Haemophilus* organisms require hemin (factor X) and/or nicotinamide adenine dinucleotide (NAD) (factor V) for growth.[301] A specially formulated medium, *Haemophilus* Test Medium **(HTM)** which contains hematin, **NAD** and yeast extract supplements has been developed and recommended by the CLSI for routine testing of *Haemophilus influenzae*.[121,122] The advantages of HTM include visual clarity and reliability of testing with trimethoprim-sulfamethoxazole.[301] Some investigators, however, have experienced problems with the performance of both homemade and commercially prepared *Haemophilus* test medium.[392] Chocolate agar or HTM can also be used for *Haemophilus* spp. and other fastidious bacteria in the "HACEK" group (i.e., *Aggregatibacter, Cardiobacterium, Eikenella*, or *Kingella*) as well as *Pasteurella* and *Moraxella* if adequate growth is not achieved using CAMHA supplemented with 5% sheep blood.[114,121,122]

The bacteria previously known of as "nutritionally deficient streptococci," *Abiotrophia* and *Granulicatella,* require pyridoxal for growth. MHA with 5% horse blood plus 0.001% pyridoxal added allows for growth of these genera so that results can be interpreted.[114,298] *Neisseria gonorrhoeae* should be tested using GC agar to which a 1% cysteine-free defined growth supplement is added after autoclaving.[121,122]

Plain MHA does not contain sodium chloride (NaCl). Detection of resistance of certain strains of staphylococci to semisynthetic penicillins (i.e., oxacillin, nafcillin, methicillin) is improved by including 2% NaCl in the media for dilution susceptibility tests.[121,122]

pH. Microbial susceptibility tests are performed using media for which the pH has been standardized at physiologic levels. Media pH should be between 7.2 and 7.4 at room temperature. The pH of broth media may be tested directly with a pH electrode, and agar media may be tested by macerating enough of the agar so that the tip of the electrode can be submerged, by allowing a portion of agar to solidify around the electrode, or by using a properly calibrated surface electrode.

Limitations. Major variation in pH of the test medium can change the activity of aminoglycoside, macrolide and tetracycline compounds. Tetracyclines are more active in acidic environments but some antimicrobial agents (e.g., aminoglycosides and macrolides, such as erythromycin) are less effective in acidic environments than at neutral pH. In contrast, β-lactams such as the penicillin and the cephalosporins function well at a wide range of pH. Nonphysiologic levels of pH often develop at the sites of purulent infections such as bacterial meningitis[578] or abscesses,[243] and the pH of urine may also range from alkaline to acidic. Ideally then, in vitro susceptibility test conditions should perhaps reflect the in vivo conditions encountered by antibiotics in specific clinical situations (i.e., acidic inflammatory exudates, urine etc.) rather than those in laboratory culture media.

Serum. Antibiotics differ greatly in the degree to which they bind to proteins. In the bloodstream, free antibiotic is in equilibrium with serum protein-bound antibiotic. Free and protein-bound antibiotic can be measured, but it is not clear which is the more useful result. The **CLSI** method does not include added serum because of the difficulty in standardization of the product and uncertainty about how to interpret the results.

Limitations. In the laboratory, different values can be obtained for highly protein-bound antibiotics if serum is added to the medium. Perl and colleagues studied the effect of serum on 11 broad-spectrum antibiotics used to treat nosocomial gram-negative bacillary infections.[458] The results were identical with 9 of the 11 antibiotics. Only in the case of ceftriaxone (>95% protein-bound) and cefoperazone (90% protein-bound) were there substantial differences when serum was incorporated into the reference procedure.

Cation Concentration. Agar and broth media vary greatly in the concentration of divalent cations. By convention, antibiotic susceptibility testing is done under physiologic conditions. **MHA** has very low concentrations of divalent cations, but is adjusted to physiologic concentrations (20 to 35 mg/L $Mg^{2}+$ and 50 to 100 mg/L $Ca^{2}+$) during production. Some batches of **MHA** may actually have an abnormally high cation concentration, so that small inhibitory zones are produced when *P. aeruginosa* is tested against aminoglycosides. Such lots, which can be identified by testing with reference strains of known reactivity, should be discarded.

Limitations. The concentration of the divalent cations $Ca^{2}+$ and $Mg^{2}+$ affects the susceptibility results when certain combinations of bacterial species and antibiotics are tested.[508] With certain combinations of bacterium and antimicrobial agent, most notably *Pseudomonas aeruginosa* and aminoglycosides, the concentration of divalent

cations, particularly calcium (Ca^2+) and magnesium (Mg^2+), has a dramatic effect on the apparent in vitro susceptibility. A result that ranges from susceptible to resistant can be achieved by varying the cation concentration,[141,508] because of changes in transport of the antimicrobial agent across the cell membrane. The mechanism by which cation concentration affects the activity of *Pseudomonas aeruginosa*, for example, may involve the permeability of the bacterial cell wall.[78] The LPS in the cell wall of *P. aeruginosa* are cross-linked with divalent cations, providing stability. When the organisms are grown in media deficient in cations, cell wall permeability to the aminoglycoside antibiotics and other compounds is increased. The organisms, therefore, are more sensitive to the action of the aminoglycosides, producing falsely low MIC results or large inhibitory zone sizes.

Daptomycin activity also varies greatly with alterations in the calcium concentration in test media, and physiologic levels of this cation are required for accurate interpretation of test results on solid media.[30,199,292]

High levels of zinc ions can also reduce the activity of carbapenems, and again, the effect is greatest for gram-negative bacteria, particularly *P. aeruginosa*.[21,145]

Environmental Conditions. The conditions and duration of test incubation must be performed correctly in order to get accurate results. Antibiotic susceptibility tests are routinely incubated in ambient air at 35°C. Agar or broth is incubated in an ambient air incubator. A CO_2 incubator should not be used for routine tests. The carbonic acid formed on the surface of the agar or in the broth can cause a decrease in pH, which can affect the antibacterial activity of certain antibiotics, as previously discussed. In smaller laboratories where only a CO_2 incubator is available, it is acceptable to place the susceptibility plates or tubes in a sealed jar to prevent access of the CO_2 from the incubation.

Plates and tubes should be incubated routinely at 35°C. At higher temperatures, the detection of oxacillin-resistant staphylococci is compromised. If oxacillin resistance is suspected and not manifested at 35°C, the plates or tubes may be incubated at 30°C.

Incubation times vary depending on the test system used. The recommended incubation time for conventional disk diffusion systems is 16 to 18 hours, while that for dilution tests is longer at 16 to 20 hours. However, tests to determine the MICs of staphylococci for oxacillin and vancomycin, and the MIC of enterococci for vancomycin should be incubated a full 24 hours.

Inoculum. The inoculum is usually prepared from a broth culture that has been incubated for 4 to 6 hours, when growth is considered to be in the logarithmic phase. Several similar-appearing colonies should be sampled to minimize variation in the bacterial population. The density of the suspension is adjusted to approximately 10^8

colony-forming units (CFUs) per milliliter by comparing its turbidity to a McFarland 0.5 $BaSO_4$ standard. The standard is prepared by adding 0.5 mL of 0.048 M $BaCl_2$ (1.175% w/v $BaCl_2.H_2O$) to 99.5 mL of 0.36 N H_2SO_4. Aliquots of 4 to 6 mL of the barium sulfate turbidity standard are distributed to screw-capped tubes of the same size, sealed tightly and stored in the dark at room temperature. Alternatively, a 0.5 MacFarland standard prepared using latex particles can be purchased to calibrate turbidity from several commercial suppliers (i.e., ThermoScientific (Remel), KS, Lenexa). Nephelometers may be used to determine turbidity. Commonly, the degree of cloudiness in the broth is compared with the standard, visualizing the two against a white background on which black lines have been drawn. Further adjustments to the inoculum depend on the type of test used. Alternatively, commercially available devices for preparing a standardized inoculum have worked well.[24,29]

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If the organism is fastidious or time does not permit incubation for 4 to 6 hours, the direct colony suspension method can be used to calibrate the inoculum. Briefly, young colonies may be removed from the surface of an agar plate that has been incubated overnight and diluted to the proper density. This method is recommended when staphylococci are tested for resistance to methicillin and when testing isolates of *Streptococcus pneumoniae* and *Haemophilus influenzae*.

If a McFarland standard is used to prepare the inoculum, the density of the standard should be checked, using a spectrophotometer with a 1-cm light path and matched cuvettes. The 0.5 McFarland standard absorbance at 625 nm should be 0.08 to 0.10. The standard should be replaced or checked for adequacy monthly.[117] It is useful, but not essential, to document the number of organisms in the inoculum periodically by inoculating serial dilutions of the suspension onto agar plates.

Limitations. The number of bacteria in infected patients varies greatly, so the standardized inoculum used in the laboratory represent a reasonable compromise rather than a reproduction of in vivo conditions. For some combinations of bacterium and antimicrobial agent, the inoculum is of great importance in determining the in vitro susceptibility; called the inoculum effect.[181,397,496,594] Enzymatic inactivation of β-lactam antibiotics, such as the penicillins and the cephalosporins, is an important mechanism of bacterial resistance. These enzymes are always expressed in some bacteria, but under the right conditions enzyme production may be induced by the presence of the antibiotic. If small numbers of *Staphylococcus aureus* that contain an inducible β-lactamase are incubated in vitro with penicillin, the inoculum may be killed before the enzyme is produced in a sufficient amount to be detected.[418,629] Whereas in vivo the large numbers of bacteria present at the site of infection are producing the β-lactamase that inactivates the antibiotic. Inducible β-lactamases may also not be

detected in *Enterobacteriaceae*, if a small inoculum is used due to interactions between the gram-negative membrane and the enzyme.[351,496] Heteroresistance also occurs in staphylococci, whereby only a small fraction of bacterial cells in a colony express β-lactamase, particularly in the presence of a semisynthetic penicillin such as methicillin. Vancomycin-intermediate-resistant MRSA is also heteroresistant, and the ability to detect this type of resistance is greatly affected by inoculum.[526] The use of a small inoculum may therefore "miss" this type of resistance, because cells carrying the resistance phenotype may not be selected.

Antimicrobial Agents. Reference antimicrobial agent powders for use in dilution tests need to be "pure" and should be obtained from the manufacturer (Sigma Chemical Co.), or from the US Pharmacopeia in Rockville, MD. These reference powders are documented with an assay of antimicrobial activity. For example, the label may indicate that the powder contains 1,075 mg of active chemical in each 1,000 mg of powder. The amount of powder weighed must be adjusted for the activity of each lot. Vials should not be obtained from the hospital pharmacy because they may contain fillers and are not assayed for biologic activity. The antimicrobial agents should be stored in a desiccator as indicated for each agent. Many antimicrobial agents, especially those of the β-lactam class, are more stable at temperatures below −20°C. Suspensions of antimicrobial agent should be stored at −20°C or less, preferably at −70°C; they should not be refrozen after dispensing. Imipenem, which is particularly affected by freezing and thawing,[661] should be reconstituted each time a batch of plates or tubes is prepared. A frost-free freezer should not be used because repeated cycles of freezing and thawing occur.

Antimicrobial-impregnated disks should be stored at −20°C or lower in an anhydrous condition. Under guidelines established by the US Food and Drug Administration (FDA), manufacturers of antimicrobial agent disks must carefully control the concentration of antimicrobial agents in the disks to within 60% to 120% of the stated content; the actual variation is usually considerably less. A small working supply may be maintained at refrigerator temperatures in a desiccator. Disks should always be allowed to warm to room temperature before opening the desiccator, so that condensation of moisture from the air does not partially rehydrate the disks. The reporting of erroneous, spurious resistance of *Staphylococcus aureus* to oxacillin has resulted from use of a defective lot of disks.[56]

Selection of Antimicrobial Agents. The final selection of antimicrobial agents for the hospital formulary should be decided in consultation with members of the medical staff. However, laboratories should use the **CLSI** guidelines for deciding which antibiotics should be primarily tested and reported, or only selectively reported according to the definitions outlined in Table 17-8 for Group A, B, C, or U agents.

Specific assignment of different drug classes according to these testing and reporting groups are outlined in the specific organism tables published by **CLSI**,[121,122] and summarized in standards M100-S23 and M100-S24 for nonfastidious bacteria in Table 1A, fastidious bacteria in Table 1B, and anaerobic bacteria in Table 1C.[121,122] The **CLSI** recommendations for cascade **(selective)** testing and reporting are briefly outlined later in the chapter recognizing that the issues are complex and vary from institution to institution. It is not necessary to test each antimicrobial agent in the list, provided the organism is susceptible to all or most of the Group A and B agents. However, the patterns of antimicrobial agent usage and incidence of important types of bacterial resistance in each community should be known and considered when selecting antimicrobial agents for testing. Distinctions may be made between antimicrobial agents that are tested routinely and those for which results are reported routinely to physicians. Because automated systems allow susceptibility testing of many more drugs for most pathogens than those listed by **CLSI** as Group A and B agents, laboratory policy and procedures for routine cascade reporting within a particular drug class should be in place.[121,122]

Several antimicrobial agents may be therapeutically equivalent in some cases because they provide a similar spectrum of antimicrobial activity. The results of in vitro testing of a specific isolate for one agent, however, may not predict the in vitro result for other antimicrobial agents in the group; in effect, these antimicrobial agents are equivalent but not necessarily equally effective against every isolate. Each agent in the group that is considered for therapeutic use must be tested. Laboratories also need to test more antibiotics within a class of drugs in order to establish the resistance phenotype of an organism (e.g., *E. coli* that is ertapenem resistant but susceptible to all other carbapenems). In contrast the results of in vitro testing of some antimicrobial agents may be applied to close relatives so that it is not necessary to test each agent in the group; some of these situations are detailed in Tables 17-7 and 17-21.[114,117,121,122]

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TABLE 17-8 CLSI Grouping of Recommended Antimicrobial Agents for Testing and Reporting

Group	Category	Type of Antimicrobial Agent
A	Primary test and report	All agents should be tested and reported
		All agents should be tested but only selectively reported: 1. Primary agent in the same class is resistant 2. Patient has reported allergy to one or more primary

B	Primary test, selectively report	agents 3. Always reported on specific sites/sources infection (i.e., a third-generation cephalosporin for CSF isolates) 4. Failure of primary therapy 5. Polymicrobial or disseminated infection involving multiple body sites 6. Epidemiologic aid to infection control
C	Supplemental testing, selectively report	Test and report for specific resistance problems, or in patients with reported allergy to Group A and/or B agents, or in unusual infections
U	Supplemental	Testing of urine isolates only
O	Other	Antimicrobial agents that are indicated for the organism group but are not candidates for routine testing and reporting in the United States
Inv.	Investigational	Antimicrobial agents that are investigational for the organisms group that have not yet been approved by the FDA for use in the United States

Adapted from reference 122.

Reference Strains. The clinical microbiology laboratory must acquire and stock appropriate positive and negative quality control organisms in order to monitor the performance of antibiotic susceptibility tests. Reference strains for quality control (QC) have been selected and standardized for quality control of susceptibility tests that give reproducible reactions of expected test performance (Table 17-9). The ideal control strains have susceptibility end points in the midrange of antimicrobial concentrations tested and have minimal tendencies to change susceptibility patterns over time. These reference strains must be stored in a condition that minimizes the possibility of mutation. They may be stored frozen (below −20°C or preferably below −60°C) after suspension in a stabilizer, such as defibrinated whole blood, 50% fetal-calf serum in bacteriologic broth or 10% glycerol in broth. Alternatively, the strains may be lyophilized. For short-term storage, the bacteria may be grown on soybean–casein digest agar and stored at 2°C to 8°C. Fresh slants should be prepared every 2 weeks, and a new stock culture should be obtained when aberrant results are noted. A fresh subculture should be prepared each day the control strain is used. Procedures for reconstituting stock cultures have been published.[117]

QC organisms can be obtained from the American Type Culture Collection (ATCC; 12301 Parklawn Drive, Rockville, MD 20852) in the form of freeze-dried vials, and specific QC testing should be done with the recommended ATCC control strain(s). ThermoScientific (Remel), Lenexa, Kansas (Bacti-Disks) and Difco Laboratories, Detroit, Michigan (Bactrol Disks) supply cultures dried on paper disks, which are stable at 4°C for up to a year. Paper disk cultures can be easily removed from a vial and grown in broth for 4 to 6 hours before plating onto solid media for QC tests.

Quality Control. Rigorous QC is important for antimicrobial susceptibility testing because of the large numbers of variables that may affect the results. Several methods are commonly used for testing the susceptibility of bacterial isolates against a wide range of antimicrobial agents including disk diffusion, agar dilution, and macrobroth and microbroth dilution methods. Extensive QC guidelines have been published by the CLSI that describe disk diffusion[118,121,122] and broth agar dilution MIC procedures.[117,121,122] Laboratories should follow these guidelines so that reliable antimicrobial susceptibility results are obtained, and procedures should be updated as CLSI standards are revised. Rules for the frequency of a 20- to 30-day QC plan for antimicrobial susceptibility testing have been established by the CLSI (Table 17-10).[120] As of 2013, however,[121] CLSI changed the recommended schedule for performing routine daily QC from 20 to 30 consecutive days of testing to a new 3 × 5 day QC plan before implementing to a weekly QC testing schedule provided the results of daily tests are within the recommended limits for a particular drug/organism (i.e., drug/bug) combination and test method (Table 17-11). In the initial phase of a 3 × 5 day QC plan, 3 replicates are tested for 5 days (15 results), and testing is passed if 0 to 1/15 results are out of range and a weekly QC schedule can then be used. However, if 2 to 3/15 results are out of range during the initial phase of testing, then a second phase of daily QC testing should be done using another three replicates tested for 5 days (15 results). Daily QC is passed if 2 to 3/30 results in the second test phase are out of range. The advantages of implementing a 3 × 5 day QC plan compared to the previously recommended 20- to 30-day consecutive test plan daily QC testing is more efficient and completed in a shorter period of time, and QC problems may be more rapidly identified. Weekly QC of antibiotic susceptibility testing for each drug/organism combination should be done whenever a new test system is implemented, whenever a new drug is tested, and whenever certain test variables are modified as outlined in Tables 3C and 4F in CLSI M100-S23.[121] To date, this change to QC test frequency has not been published in CLIA regulations and therefore has not been adopted by laboratory accrediting agencies (i.e., CMS, CAP) in the United States. As of 2013,[121] CLSI also changed the frequency of testing a known negative (susceptible) and positive (resistant) strain for disks, agar plates used for agar dilution or single wells or tubes used in broth dilution methods from routine for both control

strains to only having to test a positive strain with each new batch/lot/shipment of test materials. Table 17-12 outlines the frequency of QC when modifications are made to antimicrobial test systems for both a 20- to 30-day and a 3 × 5 day QC plan. CLSI has also outlined recommendation for troubleshooting problems with QC testing (Box 17-3), and appropriate corrective actions to take when QC parameters are not met (Table 17-13).

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TABLE 17-9 Quality Control Strains for Antibiotic Susceptibility Testing (AST)

Type of Test	Purpose	Strain[a]
Disk diffusion	• General testing; susceptible strain for VAN screen agar • Monitoring batches of Müeller–Hinton agar for inhibitory compounds to sulfa drugs, TMP, or SXT	• *Staphylococcus aureus* (ATCC 25923) • *Enterococcus faecalis* (ATCC 29212 or ATCC 33186)
Microbroth dilution	• General testing • Used in conjunction with *S. aureus* (ATCC 29213) to control OXA salt agar screening tests • Reference laboratory testing of isolates	• *Staphylococcus aureus* (ATCC 29213) • *Staphylococcus aureus* (ATCC 43300) • *Helicobacter pylori* (ATCC 43504)
Disk diffusion or microbroth dilution	• General testing; VAN susceptibility and high-dose aminoglycoside (HDA) synergy • General testing and quality control including β-lactams • Control of β-lactamase inhibitors; used in conjunction with *E. coli* (ATCC 25922) for β-lactam/β-lactamase inhibitor combinations • Control of extended-spectrum β-lactamase tests • General testing • General testing	• *E. faecalis* (ATCC 29212) (VAN S and HDA S) + *E. faecalis* (ATCC51299) (VAN R and HDA R) • *Escherichia coli* (ATCC 25922) • *Escherichia coli* (ATCC 35218) • *Klebsiella pneumoniae* (ATCC 700603) • *Pseudomonas aeruginosa* (ATCC 27853) • *Haemophilus influenzae* (ATCC 49247) (AMP R and β-lactamase negative) + *H. influenzae* (ATCC 49766) (AMP S and more reproducible for some β-lactams

	• General testing	that ATCC 49247) • *Streptococcus pneumoniae* (ATCC 49619)
Agar dilution and disk diffusion	• General testing	• *Neisseria gonorrhoeae* (ATCC 49226)

[a] ATCC, American Type Culture Collection; VAN, vancomycin; OXA, oxacillin; AMP, ampicillin; TMP, trimethoprim; SXT, trimethoprim–sulfamethoxazole.
Adapted from CLSI M100 S24,[122] M07-A9,[117] and M2-A11[118].

TABLE 17-10 Performance and Frequency of 20 to 30 Daily Quality Control Plan for Antimicrobial Susceptibility Testing

Type of Test	Approach	Frequency	Initial Validation Testing	Criteria for Corrective Action	Comments
Disk diffusion or dilution	Daily QC	Daily	None	>1 result in 20 consecutive tests out of control	Conversion to weekly testing if <2 of 20 or <4 of 30 consecutive tests for each drug is out of control
Disk diffusion or dilution	Weekly	Weekly or with each change in reagent components or method	Test all control strains for 20–30 consecutive test days; conversion to weekly testing when results meet the criteria outlined for daily QC	Any result out of control	New antimicrobial agents must be validated. Weekly testing is appropriate only for routinely tested drugs. Infrequently tested agents must be tested on the day of use.
Agar screen tests	Daily	Daily	None	>1 result in 10 consecutive tests out of control	Daily testing must be done if the screening is performed less frequently than weekly or the agent being tested is labile (e.g., OXA screen test for *S. aureus*).
Agar screen tests	Weekly	Weekly or with each change in reagent components or method	Test all control strains for 20–30 consecutive test days; conversion to weekly testing when results meet the criteria outlined for daily QC or disk diffusion or dilution tests.	Any result out of control	New antimicrobial agents must be validated. Weekly testing is appropriate only for routinely tested drugs. Infrequently tested agents must be tested on the day of use.

QCP, quality control plan; QC, quality control; OXA, oxacillin.
Adapted from reference 120.

TABLE 17-11 Replicate (3 × 5 Day) Quality Control Plan for Daily Antimicrobial Susceptibility Testing for Each QC Strain and Drug Combination: Acceptance Criteria and Recommended Action

Number Out of Range With Initial Testing (Based on 15 Replicates)	Conclusion From Initial Testing (Based on 15 Replicates)	Number Out of Range After Repeat Testing (Based on all 30 Replicates)	Conclusion After Repeat Testing
0–1	QCP successful. Convert to weekly QC testing.	NA	NA
2–3	Test another three replicates for 5 days	2–3	QCP successful. Can convert to weekly QC testing.
≥4	QCP fails, investigate and take corrective action as appropriate. Continue QC each test day.	≥4	QCP fails. Investigate and take corrective action as appropriate. Continue QC each test day.

QCP, quality control plan; QC, quality control; NA, not applicable.
Adapted from CLSI M100-S23,[121] Table 3C.

Quality Assurance and Proficiency Testing. Laboratories should monitor errors in antibiotic susceptibility testing and categorize them as very major, major or minor. Erroneous results may be detected prior to reporting, either by an astute technologist or clinical microbiologist, or by the artificial intelligence rules built into an automated system that flag results that need verification. Comparison of erroneous result or those that are unexpected for a particular organism may alert the microbiologist to a potential problem with the identification and/or susceptibility results. Some of these clues are summarized in Table 17-7. Inadvertent testing of mixed cultures is a frequent cause of mistakes; inclusion of a "purity" plate with the susceptibility test is essential, but apparent purity of the tested isolate is not an absolute guarantee of purity.

All errors in either the testing, interpretation or reporting of antibiotic susceptibility results should be monitored as a major quality indicator of the entire complex process. All laboratories should aim to have an absolute minimum number of errors, but all errors should be investigated to identify and correct the root cause so that there is no recurrence. Technologists should be highly trained and skilled at not only performing the test procedures, but also identification of circumstances wherein a test should be repeated due to suspected erroneous results. Laboratory policy and procedure should also clearly identify specific antibiotic resistance profiles for major pathogens that require additional or confirmatory testing prior to reporting as outlined later in the chapter.

Laboratories performing antibiotic susceptibility testing must also participate in a proficiency testing program such as that offered by the College of American Pathologists (CAP), mandated by federal law, which improves performance and provides useful aggregate data for evaluating the status of susceptibility testing on a national basis.[464] Targeted proficiency programs have also been implemented successfully.[608]

Antimicrobial Susceptibility Test Procedures

Diffusion Susceptibility Methods

Disk Diffusion Susceptibility Test. Disk diffusion susceptibility tests became standardized in the United States based on the work of Bauer, Kirby, and coworkers (Chart 17-1).[118,121,122] This method is still widely used because of its ease of use and lower cost compared to other methods. Unlike dilution methods, an MIC value is not derived, but the diameter of the zone of drug inhibition is compared with the MIC values of the same strain to predict the category of susceptibility. Disk diffusion testing should be performed strictly according to the standard methods outlined by CLSI.[118,121,122] If the test is properly performed, the edges of the inhibitory zones should be clear and easy to measure.

Method Description. The basic principle of the standardized disk diffusion susceptibility test is illustrated in Figure 17-12. All disk diffusion methods rely on diffusion of drug released from an impregnated disk through agar. A detailed theoretical description of the principles of zone formation during this method has been previously described, and only a brief outline is given herein.[28,296] Disks are a standard 6 mm wide and are manufactured to contain a known amount of the drug being tested. CLSI recommends that disks be spaced on the agar plate so that there is 24 mm measured from the center of one disk to another in order to ensure no overlap in their inhibition zones. There should be a maximum of 15 minutes between inoculation of the agar with bacteria and application of the drug disk because diffusion starts shortly after its placement on the surface of the media. As soon as the antimicrobial agent-impregnated disk comes in contact with the moist agar surface, water is absorbed into the filter paper and the antimicrobial agent diffuses into the surrounding medium. The rate of extraction of the antimicrobial agent out of the disk is greater than its outward diffusion into the medium, so that the concentration immediately adjacent to the disk may exceed that in the disk itself. As the distance from the disk increases, however, there is a logarithmic reduction in the antimicrobial agent concentration. If the plate has been previously inoculated with a bacterial suspension, simultaneous growth of bacteria occurs on the surface of the agar. When a critical cell mass of bacteria is reached, the inhibitory activity of the antimicrobial agent is overcome and bacterial growth occurs. The time required to reach the critical cell mass (critical time; 4 to 10 hours for commonly tested bacteria) is characteristic of each species but is influenced by the composition of the medium and temperature of incubation. The lateral extent of antimicrobial diffusion before the critical time is reached will be affected by the depth of the agar because diffusion occurs in three dimensions. The points at which the critical cell mass is reached appear as a sharp circle with a margin of bacterial growth, with the middle of the disk forming the center of the circle if the test has been performed properly (Fig. 17-12). The concentration of diffused antimicrobial agent at this interface of growing and inhibited bacteria is known as the critical concentration and approximates the MIC obtained in dilution tests (See

Methods Using Liquid Media below). Although direct calculation of the inhibitory concentration is not done in practice, the MIC can actually be calculated with reasonable accuracy if the characteristics of antimicrobial diffusion and bacterial growth are known.[28]

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TABLE 17-12 Frequency of Quality Control When Modifications Are Made to Antimicrobial Susceptibility Test Systems

Required QC Frequency	Disks	Media (Prepared Agar Plates)	Inoculum Preparation	Measuring Zones	Instruments/Software (e.g., Automated Zone Reader)
1 Day	Use new shipment or lot number or new manufacturer	Use new shipment or lot number			Repair of instrument that affects AST results. Depending on the extent of repair (e.g., critical component such as the photographic device), additional testing may be appropriate for 5 days
5 Days		Use new manufacturer	Convert inoculum preparation/standardization to use of a device that has its own QC protocol (e.g., convert from visual adjustment of turbidity to use of a photometric device for which QC procedure is provided)		Software update that affects AST results. Monitoring all drugs, not just those implicated in software modification
15-Replicate QCP or 20–30 day QCP	Addition of new antimicrobial agent to existing system		Convert inoculum preparation/standardization to a method that depends on user technique (e.g., convert from visual adjustment of turbidity to another method that is not based on a photometric device)	Change method of measuring zones (e.g., convert from manual zone measurements to automated zone reader). In-house verification studies should also be done	

AST, antimicrobial susceptibility testing; QCP, quality control plan; QC, quality control.
Adapted from CLSI M100-S23, Table 3C[121].

BOX 17-3

Corrective Action for Antibiotic Susceptibility Tests That Are Out of Control

Source of Error

Obvious (e.g., wrong control strain, wrong temperature, contamination):

- Retest on day of QC failure until result is in control, then proceed as for an unknown error.

OR
Unknown:

- Possible explanations for the QC failure are summarized in Table 17-12.
- After implementing changes (i.e., new reagents) that could be problematic, proceed with validation.
- Test the failed antibiotic for five consecutive days.
- Proceed with additional testing if the results are out of control.
- Continue daily testing until the problem is resolved.
- Proceed with weekly testing once the results are in control.
- Suppress reporting of any antibiotics that are out of control until the problem is resolved.

TABLE 17-13 Troubleshooting Quality Control Problems in Antimicrobial Susceptibility Testing[a]

Observation	Potential Problem(s)	Corrective Actions[b]
MICs too large or zone sizes too small (isolates too resistant)	1. Inoculum too high 2. Deterioration of antimicrobial agent 3. Change in QC strain 4. Agar too deep 5. Incorrect reading of results	1. Check and adjust inoculums if necessary 2. Check potency of disks or powder; try new lot 3. Test new stock of QC strain 4. Check depth of agar 5. Repeat with multiple observers
MICs too small or zone sizes too large (isolates too susceptible)	1. Inoculum too low 2. Antimicrobial agent too potent 3. Change in QC strain 4. Agar too thin 5. Incorrect reading of results	1. Check and adjust inoculum if necessary 2. Check potency of disks or powder; try new lots 3. Test new stock of QC strain 4. Check depth of agar 5. Repeat with multiple observers
Results for		

Pseudomonas and aminoglycosides out of control	Cation content incorrect	Use cation-supplemented broth or try different lot of agar
Aminoglycosides and macrolides are resistant; tetracycline too susceptible	Medium too acid	Check pH of media
Aminoglycosides and macrolides are resistant; tetracycline too resistant	Medium too alkaline	Check pH of media
Trimethoprim MICs too large or zone sizes too small; results difficult to read	Excess thymidine in medium	Test medium with *Enterococcus feaecalis* (ATCC 29212); add thymidine phosphorylase or lysed horse blood
General	*QC Strain:* Use of wrong QC strain, improper storage, inadequate maintenance (e.g., use of the same working culture for >1 month), contamination, no viability, changes in the organism (e.g., mutation, loss of plasmid)	Use new QC strain and check all test conditions
	Testing Supplies: Improper storage or shipping conditions, contamination, inadequate volume of broth in tubes or wells, use of damaged (e.g., cracked, leaking) panels, plates, cards, tubes, use of expired materials (i.e., disks)	Use new reagents and supplies and check all test conditions
	Testing Process: Use of wrong incubation temperature or conditions, inoculums suspensions incorrectly prepared or adjusted, inoculum prepared from a plate incubated for the incorrect length of time, inoculums prepared from differential or selective media containing anti-infective agents or other growth-inhibiting compounds, use of wrong	Check all test conditions and the reading and recording of test results

	reagents, ancillary supplies, incorrect reading or interpretation of test results, transcription error	
	Equipment: Not functioning properly or out of calibration (i.e., pipettes)	Check the QC on all equipment and perform maintenance as required; replace if continues to malfunction

[a] Adapted from M100-S23 Table 4G[121].

[b] All results that are out of control will require retesting and withholding of results until QC is back in control (see Tables 17-10 and 17-11).

AST, antimicrobial susceptibility testing; QC, quality control; ATCC, American Type Culture Collection.

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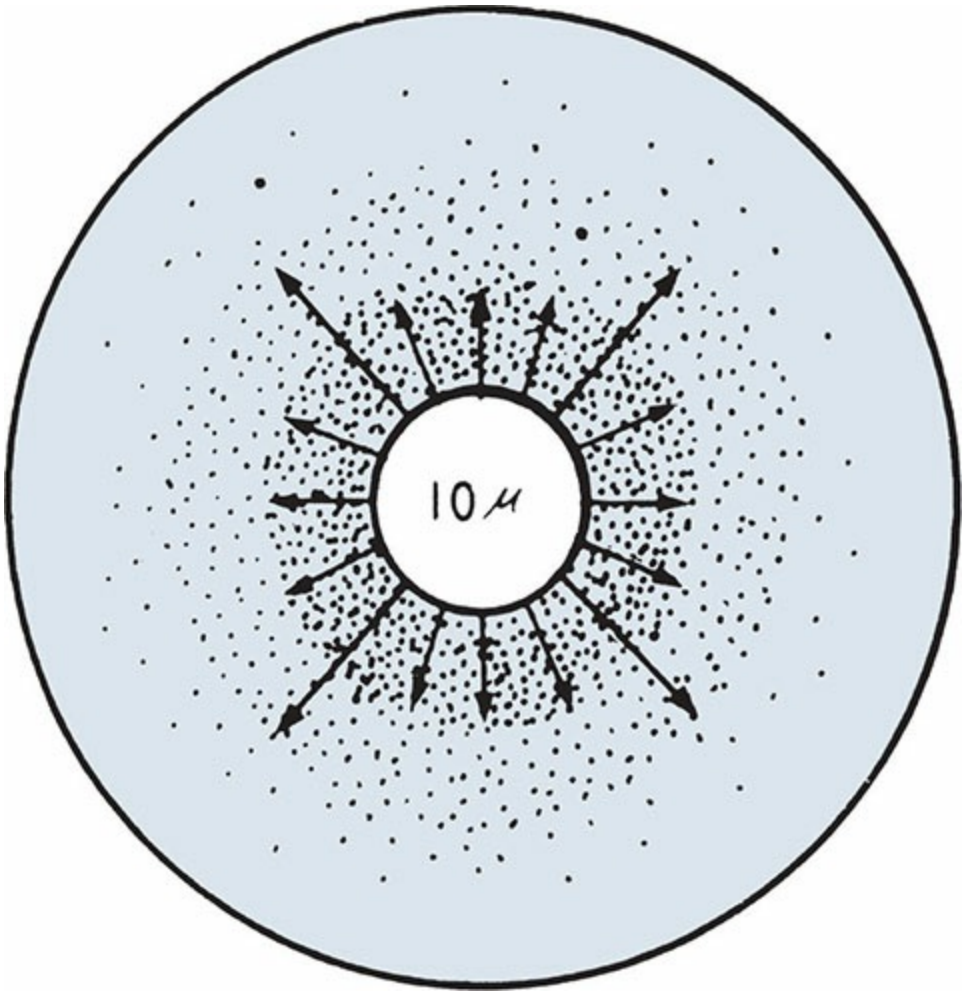
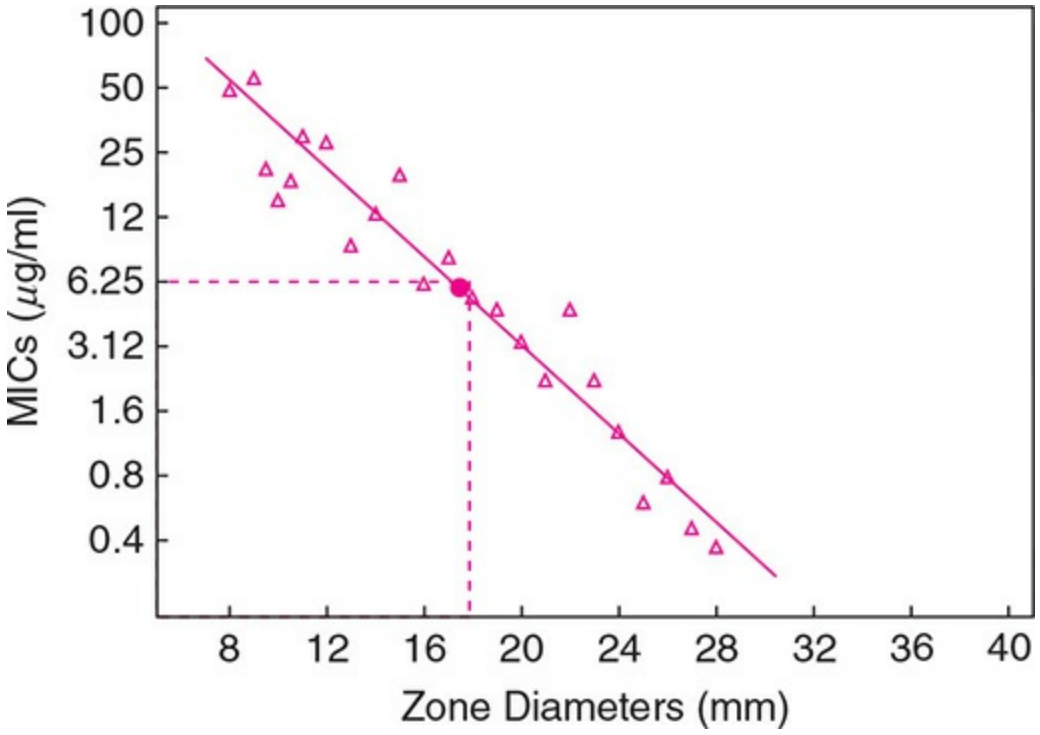


■ **FIGURE 17-12** The principle of antibiotic diffusion in agar. The concentration of antibiotic decreases as the distance from the disk increases.

Interpretation of Results. The zone size observed in a disk diffusion test is compared to the interpretive standards provided by the CLSI.[118,121,652] Zone diameter sizes derived from testing a wide range of strains are correlated with the known MICs of the species tested to assign interpretive breakpoint criteria for susceptible, intermediate and resistance zone sizes. A prototype of a regression curve providing such a correlation is illustrated in Figure 17-13. Multiple strains have been tested against a single antimicrobial agent both by the disk diffusion technique and by a dilution method. Each triangle represents the results of both tests for a single strain. A regression line has been drawn through the many individual points. Once the regression line is established, an approximate MIC result can be inferred from any zone diameter. In this example, a zone size of 18 mm corresponds to an MIC of 6.25 μg/mL; the breakpoint of the broth dilution test illustrated in Figure 17-14.

The interpretive guidelines for the disk diffusion test permit the user to make

approximations of the MIC for each of the antimicrobial agents listed, with zone diameters as determined by the disk diffusion technique. Thus, an antimicrobial agent that produces a zone diameter greater than 18 mm would theoretically have an MIC less than 6.25 µg/mL, and the organism would be considered susceptible. In contrast, a strain producing a zone size less than 18 mm would be considered resistant. In actual practice, regression curves are not that clearly defined, and a 2- to 4-mm zone may be established where it is not possible to determine whether the organism is susceptible or resistant. Isolates that produce an inhibitory zone in this range are characterized as intermediate in susceptibility. In studies conducted late in the 1950s, Bauer, Perry, and Kirby first demonstrated that bacterial strains tested against a given antimicrobial agent tend to fall into either the resistant or susceptible categories; only a small percentage (5% or less) fall into the intermediate range.[31]

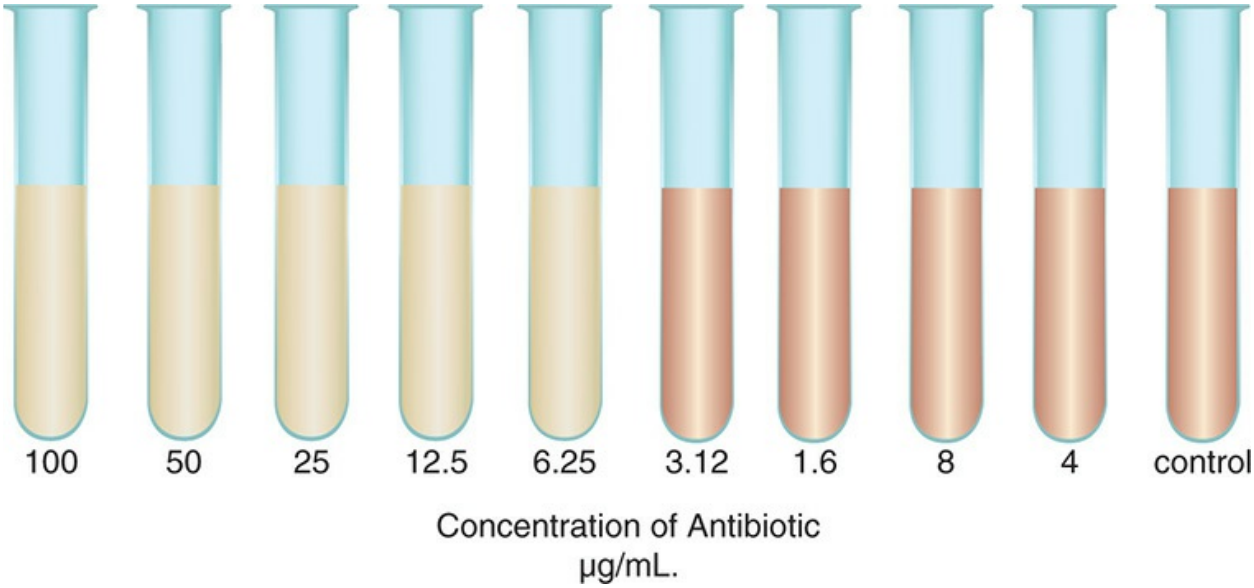


■ **FIGURE 17-13** Prototype regression curve comparing MICs in micrograms per milliliter with zone size in millimeters. Each triangle represents the MIC (*vertical axis*) and inhibitory zone (*horizontal axis*) of a single isolate.

Situations in which the technologist must learn to interpret unclear results correctly are listed in Box 17-4 and illustrated in Figures 17-15 through 17-18.

Quality Control. The objectives of performing QC for disk diffusion tests are to monitor the performance of the medium and disks, and the accuracy of the test

procedure using standard reference bacterial strains. Operator competence in performing the test procedure including the reading of the zone diameter and its interpretation are also being evaluated. Controls should be routinely tested using the methods and frequency of testing set out by the CLSI.[118,121,122] QC should be performed each time a new lot of disks or agar is used. The CLSI has also established limits on zone sizes that are acceptable for the QC strains[118,121,122]; these limits provide a means by which to measure the accuracy of testing. Reference strains recommended by the CLSI for QC of disk diffusion tests for aerobic bacteria include *E. coli* ATCC 25923, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, and *E. coli* ATCC 35218. The latter *E. coli* strain is used as a control only for β-lactamase inhibitor combinations containing clavulanic acid, sulbactam, or tazobactam. Table 17-14 provides an example of a chart for recording the results of weekly disk diffusion QC using one of the standard control test organisms, *E. coli* ATCC 25922.



■ **FIGURE 17-14** Broth dilution susceptibility test. The MIC for the test illustrated here is 6.25 µg/mL.

BOX 17-4

Troubleshooting the Reading of Disk Diffusion Tests

- The diameter is read as the widest part of the outer margin of the circular zone of inhibition:
  - Diameter should not include pinpoint colonies or hazy growth.
  - *Proteus* spp. and other motile organisms may swarm when growing on agar surfaces, resulting in hazy growth within the zone which should be ignored; read the outer margin (Fig. 17-15).
  - Check the purity of the culture and perform separate susceptibility testing on the pinpoint colonies in the zone of inhibition; do not ignore these colonies in determining the MIC as they may represent a more resistant subpopulation (Fig. 17-16).
  - Ignore growth inhibition at the outer margin when reading sulfonamide disk results; the clear zone of <80% inhibition should be read as the zone diameter.
  - Overlapping zones may occur when disks are not correctly placed on the agar; the test must be repeated with more careful placement of the disks so the outer margins of the zone can be read correctly (Fig. 17-17).
  - Tests need to be repeated if the plate is not properly streaked resulting in irregular zone margins (Fig. 17-18).
  - When testing β-hemolytic bacteria, assess the zone of inhibition and ignore the hemolysis.

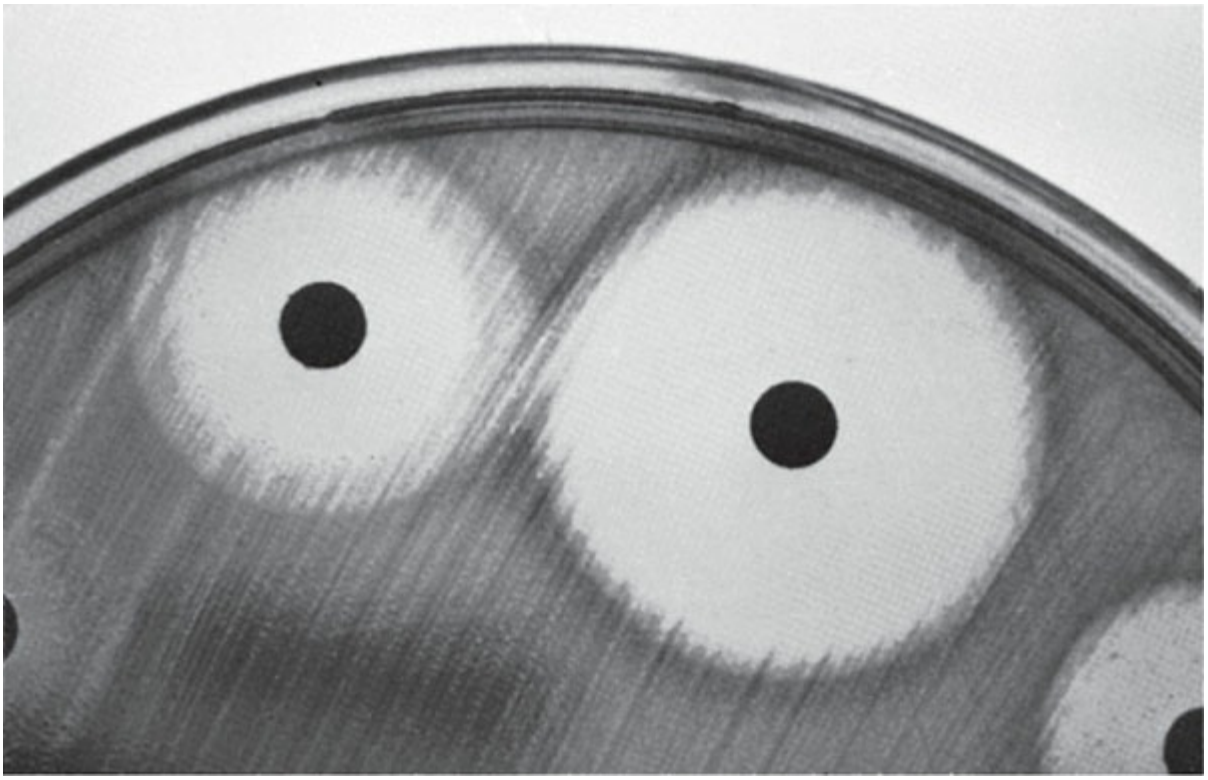
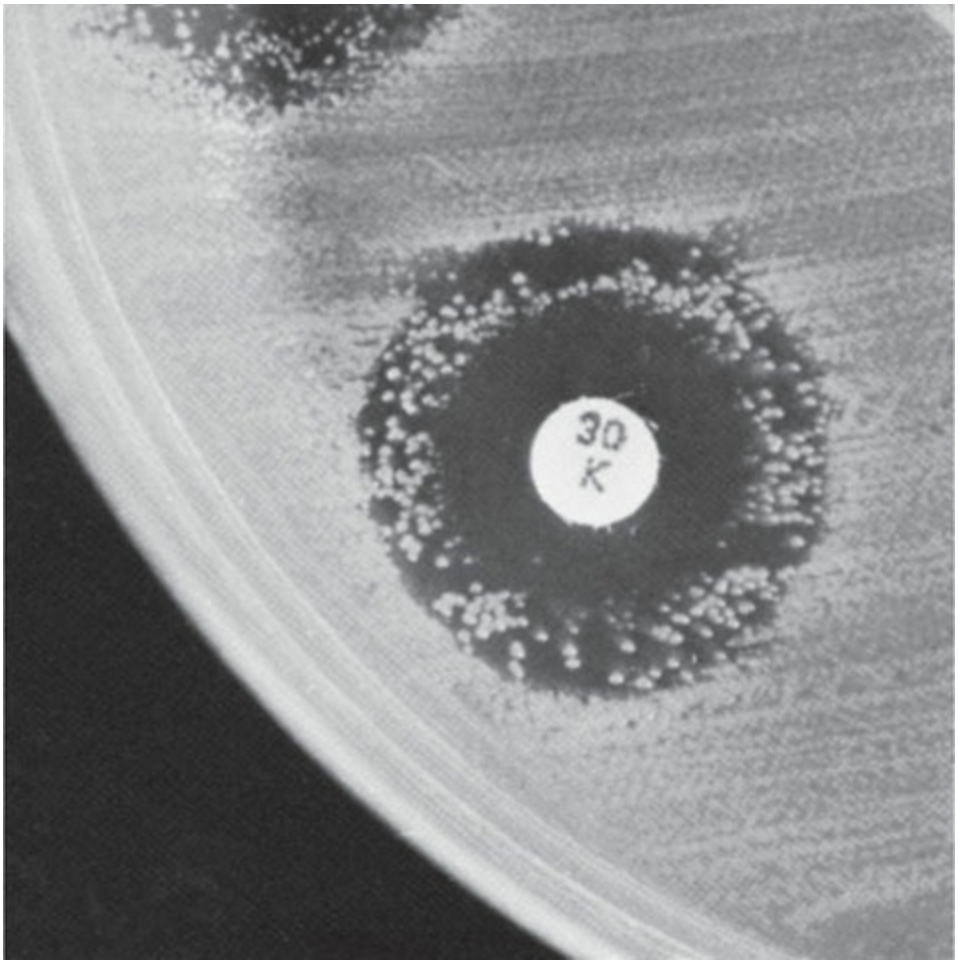
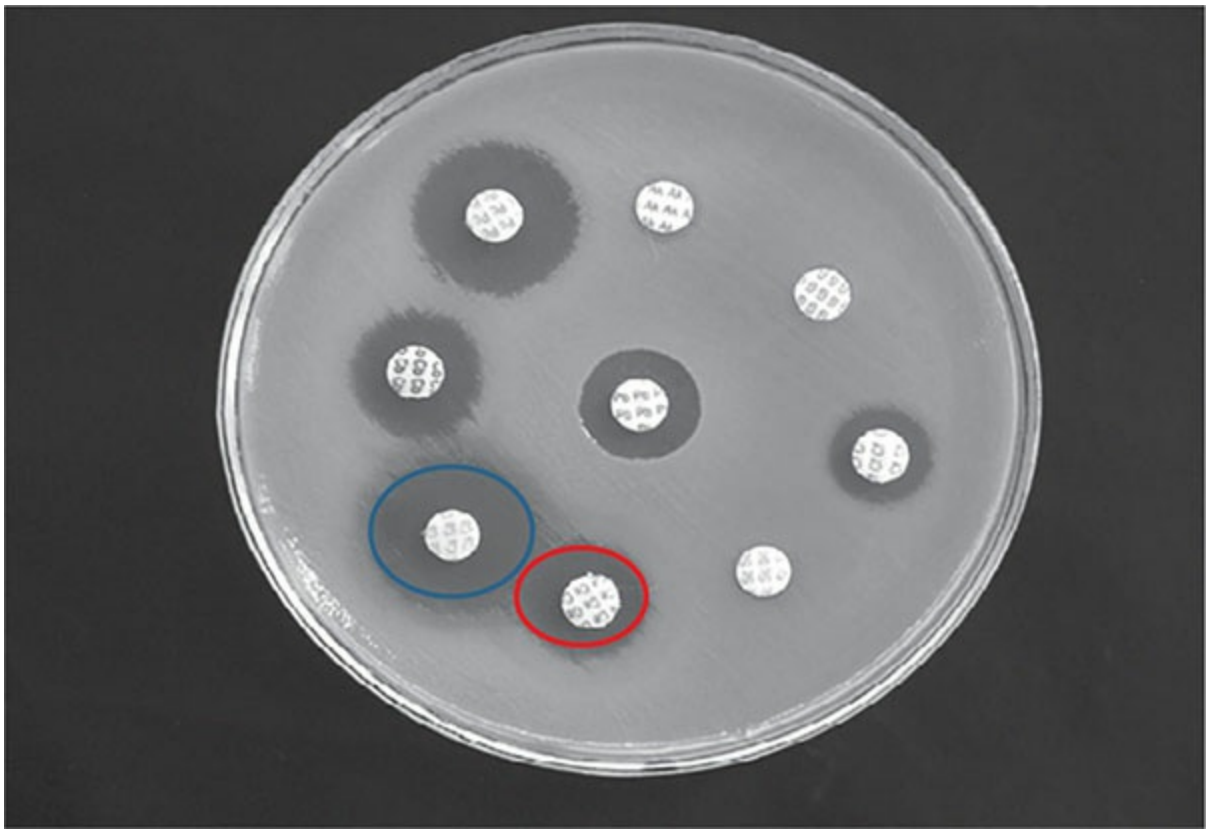


FIGURE 17-15 Photograph of an antibiotic susceptibility plate using a species of

Proteus as the test organism. Note the swarming into the zone of inhibition at the peripheral margins. The second outer zone of growth inhibition should be used when measuring the width of the zone.



■ **FIGURE 17-16** An antibiotic susceptibility plate in which colonies resistant to kanamycin are growing within the zone of inhibition: biochemical tests must be performed to determine if the resistant strain is a mutant of the organisms being tested or represents a second species growing in mixed culture.



■ **FIGURE 17-17** Improper disk placement: demonstrates the difficulty in measuring diameters when there is overlapping adjacent zones of inhibition, or zones that extend beyond the outer margin of the agar. Oval or elliptical zones occur, and it is difficult to decide where to measure the zone diameter. Test should be repeated with correct disk spacing.

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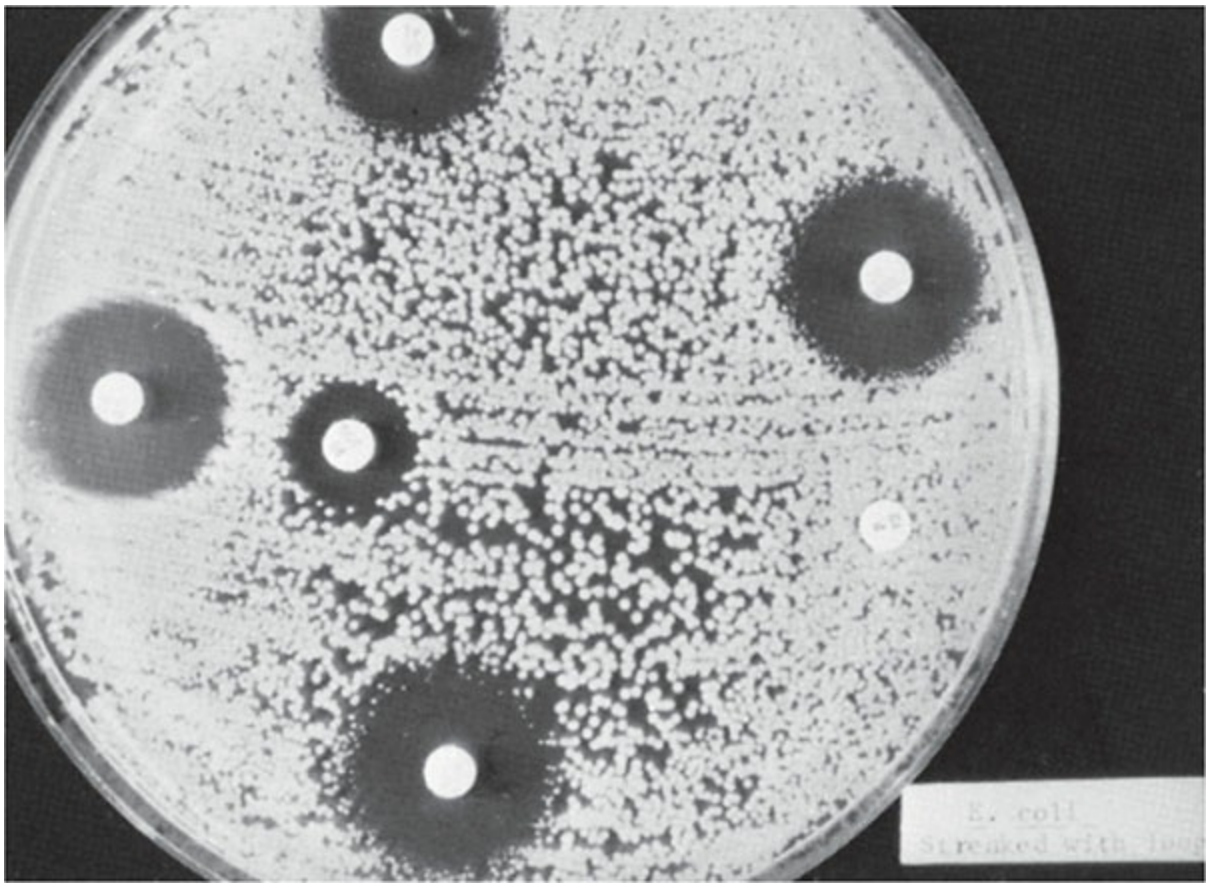

■ **FIGURE 17-18** A poorly streaked antimicrobial susceptibility plate showing uneven growth. The zone margins are indistinct, compromising accurate measurement.

Limitations. The Bauer–Kirby test,[31] as modified by the CLSI, has been accepted in the United States as the standard technique for performing disk diffusion susceptibility tests, and will provide accurate information, provided it is used according to published guidelines.[118,121,122] The test should be applied only to bacterial species that have been thoroughly evaluated. Bacteria that grow slowly, need special nutrients, or require CO_2 or anaerobic conditions for growth should not be tested, unless the validity of the procedure has been documented.[114,115]

Gradient Diffusion Susceptibility Test. Gradient diffusion susceptibility testing or the epsilometer test (Etest) was developed by AB Biodisk in Sweden (Chart 17-3). Although the Etest has now been acquired by bioMérieux, it is still marketed by the same name. The Etest consists of antimicrobial agent-impregnated strips that are placed on the surface of agar, and exploits the same principles as the disk diffusion test, but MIC values are generated by applying a gradient of the antibiotic to be tested along the strip. The antibiotic gradient in the strip is made by applying 15 different

concentrations of antibiotics in repeated arrays on an increasing number of small dots (www.biomerieux-diagnostics.com). Placement of the strip on the inoculation media causes the antibiotic to diffuse into the surrounding medium in a high amount from one end of the strip to a low concentration at the other end. The MIC is read off the linear scale printed on the upper side of the strip where the zone of growth inhibition intersects with the edge of the strip (Fig. 17-19). If the strips are placed on the agar upside down (impregnated side not in contact with the agar), erroneous determinations of the MIC will likely result.[231] The manufacturer indicates, however, that an upside-down strip can be repositioned without problems if the mistake is recognized immediately.

The same factors that influence the disk diffusion susceptibility test also apply to the E test. Diffusion of antimicrobial agents begins immediately after placement of the strip, which cannot, therefore, be moved once the impregnated surface has touched the agar. A plate that is poorly streaked may produce an irregular zone of inhibition at the MIC point. Swarming of *Proteus* spp. and mutant colonies within the zone of inhibition may be challenges with the Etest, as with disk diffusion.

The Etest has been evaluated against a very broad range of bacteria since its advent, and this method produces results similar to those of conventional MIC tests (agar or broth dilution methods).[84,96,509,613] However, despite the simplicity of setting up an Etest compared to other methods, the expense of individual drug strips make this method less attractive for testing multiple antimicrobial agents against organisms that grow well in one of the dilution or disk diffusion procedures. The Etest is invaluable for testing highly selected antimicrobial agents against fastidious organisms that do not grow well in the other tests such as *Streptococcus pneumoniae*[604] or viridans streptococci,[398] for confirming an MIC result for a particular bug/drug combination initially tested by another method[613] and for the detection or confirmation of specific types of antibiotic resistance (i.e., ESBLs, MBLs, VISA/VRSA) as outlined below (see Detection of Special Types of Antibiotic Resistance).[518,573,598]

Laboratories should follow all of the principles and practices of QC for Etest outlined earlier in the chapter for disk diffusion testing. In addition to the general issues common to all susceptibility tests, specific suggestions for troubleshooting the Etest are summarized in Box 17-5. Table 17-15 provides an example of a weekly QC chart for performing gradient diffusion (Etest) testing for *S. pneumoniae* (ATCC 49619).

Dilution Susceptibility Testing

Agar Dilution Susceptibility Test. The agar dilution procedure is the reference method for testing using solid medium. This method has been successfully adapted for routine use in large laboratories by testing only selected concentrations of antimicrobial agent. Although a brief description of the method is outlined, agar dilution testing is

mainly performed by reference laboratories. A standardized suspension of bacteria is inoculated onto a series of agar plates, each containing a different concentration of antimicrobial agent, encompassing the therapeutic range of the drug.[135] For example, if the therapeutic range for a given antimicrobial agent is 2 to 12 µg/mL, a series of agar plates containing 1, 4, 8, 16, and 32 µg/mL of antimicrobial agent might be used to determine the susceptibility of the organism being tested. If the organism grows on the first three plates but not in the plate containing 16 µg/mL of antimicrobial agent, then an MIC value of 16 µg/mL is established.

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TABLE 17-14 Weekly Quality Control Chart for Disk Diffusion Testing of *Escherichia coli* ATCC 25922

PLAIN MUELLER–HINTON AGAR			Year: _____
E. coli ATCC 25922		Date (mm/dd) Initial	
		Zone Size mm	Remember to record Disk & Media Lot # & Expiry date on CLS Form# MI3811
AMOX/CLAV ACID	AMC 30	18–24	
AMIKACIN	AK 30	19–26	
AMPICILLIN	AMP 10	16–22	
AZTREONAM	ATM 30	28–36	
CEFAZOLIN	KZ 30	21–27	
CEFEPIME	FEP 30	31–37	
CEFIXIME	CFM 5	23–27	
CEFOTAXIME	CTX 30	29–35	
CEFOXITIN	FOX	23–29	
CEFTAZIDIME	CAZ 30	25–32	
CEFTRIAXONE	CRO 30	29–35	
CEFUROXIME	CXM 30	20–26	
CEPHALOTHIN	KF 30	15–21	
CIPROFLOXACIN	CIP 5	30–40	
COTRIMOXAZOLE	SXT 25	23–29	
ERTAPENEM	ETP 10	29–36	
FOSFOMYCIN	FOT 200	22–30	
GENTAMICIN	CN10	19–26	
IMPENEM	IPM 10	26–32	
MEROPENEM	MEM 10	28–34	
NITROFURANTOIN	F300	20–25	
NORFLOXACIN	NOR10	28–35	
PIP/TAZOBACTAM	TZP 110	24–30	
TETRACYCLINE	TE30	18–25	
TOBRAMYCIN	TOB 10	18–26	
Weekly Review :			
Monthly Supervisor Review:			Report all out-of-control results to Supervisor immediately

Adapted with permission from Calgary Laboratory Services Manual (QC)

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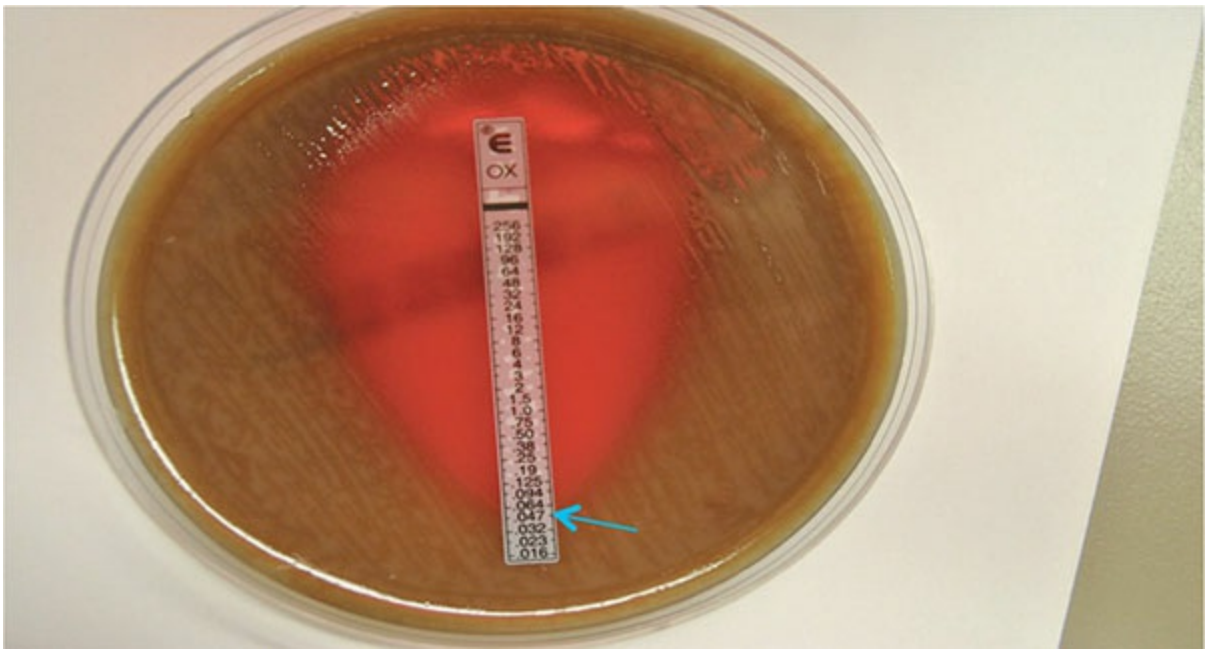

■ **FIGURE 17-19** Oxacillin Etest—*S. pneumonia*. MIC = 0.47 µg/mL (S). (Courtesy of Calgary Laboratory Services.)

To facilitate testing of a large number of isolates, an instrument known as the Steers replicator is used to inoculated large agar dilution plates with 32-26 wells cut into the agar. These wells are tooled in such a manner that when the seed plate is properly aligned within the guide at the base of the replicator, each of the inoculating pins on the movable instrument head fits exactly into the wells. Each well in the seed plate provides a receptacle into which different bacterial suspensions can be placed. Inoculated agar dilution plates are incubated at 35°C for 18 hours. Agar dilution plates ready for interpretation are shown in Figure 17-20. Note that microorganisms that are susceptible to the concentration of antimicrobial agent contained in any given agar plate do not produce a circle of growth at the inoculum site, whereas those that are resistant appear as circular colonies. The agar plates are marked with a grid so that each microorganism can be identified by a number and the results entered on the worksheet.

Macrodilution Broth Susceptibility Test. The macrodilution broth susceptibility test was among the first to be developed by Fleming, and still serves as a reference method.[196] Serial dilutions of antimicrobial agent are made initially in liquid medium, after which a standardized bacterial suspension is added. Figure 17-14 shows a schematic representation of a broth microdilution test with 10 test tubes that contain CAMHB typically in a volume of 1 to 2 mL. Quantities of antimicrobial agent are serially diluted in nine of these tubes from a high concentration of 100 µg/mL down to

0.4 µg/mL. Tube number 10 serves as a growth control and is free of antimicrobial agent. Each of the 10 tubes is inoculated with a calibrated suspension of the microorganism to be tested and incubated at 35°C for 18 hours. At the end of the incubation period, the tubes are visually examined for turbidity; the five tubes to the left are clear and the five to the right appear cloudy (Fig. 17-14). Cloudiness indicates that bacterial growth has not been inhibited by the concentration of antimicrobial agent contained in the medium. The breakpoint of growth inhibition is between tubes 5 and 6, or between 6.25 and 3.12 µg/mL of antimicrobial agent. This breakpoint represents the MIC (i.e., the **MIC** lies somewhere between 6.25 and 3.12 µg/mL); defined as the lowest concentration of antimicrobial agent in micrograms per milliliter that prevents the **in vitro** growth of bacteria. However, by convention, the **MIC** is interpreted as the concentration of the antimicrobial agent contained in the first tube in the series that inhibits visible growth. Therefore, in this example, the **MIC** is 6.25 µg/mL.

BOX 17-5

Troubleshooting the Reading of Etest Assays

- The MIC equals the point of intersection where the ellipse intersects the scale on the strip:
  - Zone of inhibition includes pinpoint colonies and hazy growth within the zone of inhibition.
  - Check the purity of the culture when there are pinpoint colonies in the zone of inhibition; do not ignore these colonies in determining the MIC.
  - Tilt the plate or use a magnifying glass to visualize pinpoint colonies and hazes, especially for enterococci, pneumococci, and *Stenotrophomonas* spp. Include a haze as growth when determining inhibition, but ignore the swarming of *Proteus* spp.
  - When testing β-hemolytic bacteria, assess the zone of inhibition and ignore the hemolysis.
  - If a paradoxical effect (inhibition at low MIC and regrowth at higher MICs), read the MIC as the zone of final, complete inhibition.
  - Bacteriostatic drugs, such as trimethoprim–sulfamethoxazole, may produce growth with a diffuse, fuzzy edge; read the MIC at 80% inhibition (as for the disk diffusion test).
  - When testing combinations of β-lactams and β-lactamase inhibitors, the inhibitor may have intrinsic activity, producing an extended ellipse at the lowest MICs. Extrapolate the upper ellipse of inhibition to determine the MIC.
  - A "dip" effect at the lowest MIC may be seen when inducible macrolide resistance is present, producing an ellipse with an elongated nipple at the end. Extrapolate the curvature of the primary ellipse to determine the MIC.

- Ignore a thin line of growth along the lower portion of the strip, a phenomenon caused by organisms growing in a tunnel of water along the strip.
- If the zone of inhibition intersects the strip between two markings, read the higher value. Similarly, if the zone intersects the strip slightly differently on the two sides, read the higher value.
- If the MIC produced by the strip is between numbers in the classic twofold-dilution scheme (on which all standard recommendations are based), round the "answer" up to the next twofold dilution. For instance, an MIC of 1.5 μg/mL would be reported as 2.0 μg/mL.

Adapted from Etest Technical Guide: Etest for MIC determination, AB Biodisk, Solna, Sweden.

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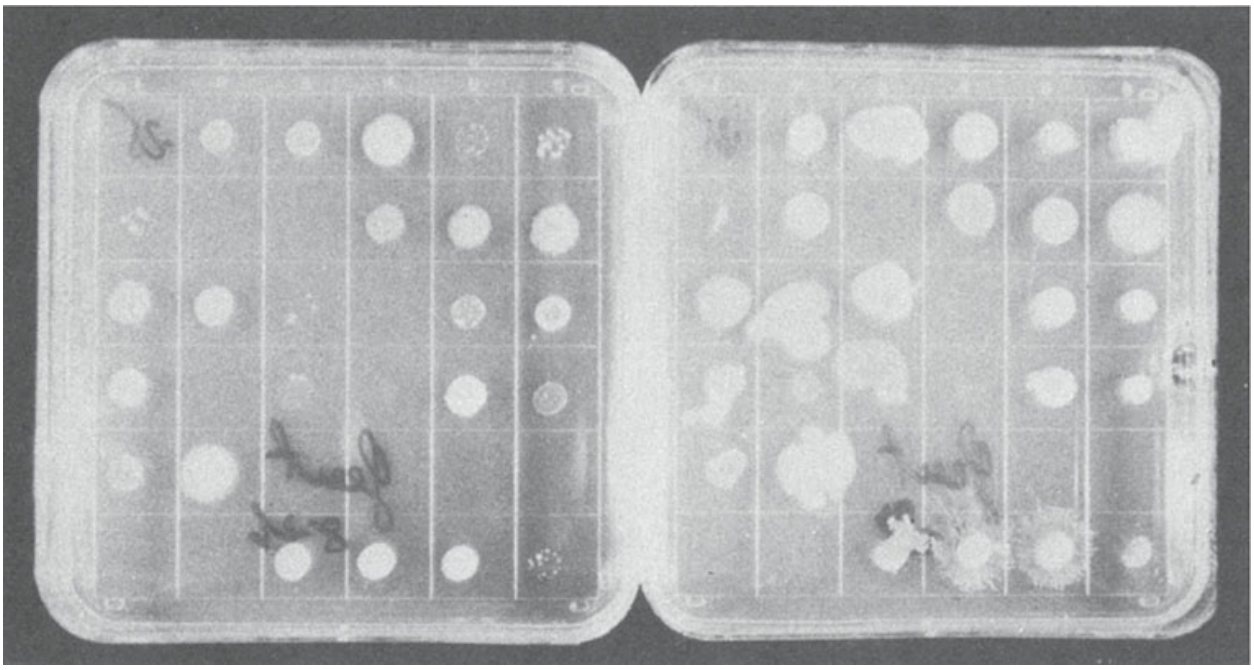
TABLE 17-15 Weekly QC Chart for MIC (Etest) Testing of *Streptococcus pneumoniae* (ATCC 49619)

BMH LOT # EXPIRY _____ DATE: RECEIVED _____ DATE: _____		DATE /TIME SET UP: _____ INITIALS: _____ DATE READ: _____ INITIALS: _____	
STREPTOCOCCUS PNEUMONIAE ATCC 49619			
E TEST LOT # & EXPIRY DATE:		MIC VALUE (μg/mL)	Weekly Review
AMPICILLIN (AM)	0.016–256 μg/mL	>0.03-0.25[a]	
Lot#:	Expiry:		
CEFOTAXIME (CT)	0.016–256 μg/mL	0.03–0.12	
Lot#:	Expiry:		
CEFTRIAXONE (TX)	0.002–32 μg/mL	0.03–0.12	
Lot#:	Expiry:		
PENICILLIN (PG)	0.016–256 μg/mL	0.25–1	
Lot#:	Expiry:		
VANCOMYCIN (VA)	0.016 –256 μg/mL	0.12–0.5	
Lot#:	Expiry:		
ERYTHROMYCIN (EM)	0.016–256 μg/mL	0.03–0.12	
Lot#:	Expiry:		
LEVOFLOXACIN	0.002–32 μg/mL	0.5–2	
Lot#:	Expiry:		
TRIMETHOPRIM/SULFA	0.002–32 μg/mL	0.12–1.0	
Lot#:	Expiry:		
AMOXICILLIN	0.016–256 μg/mL	0.03–0.12	
Lot#:	Expiry:		
Monthly QA/QC Review:			

[a] Use CLSI breakpoints outlined in M100-S24.[122]
Adapted with permission from Calgary Laboratory Services Microbiology Manual (Antibiotic Susceptibility Manual).



■ **FIGURE 17-20** Agar dilution antimicrobial susceptibility plates that have been inoculated with several species of bacteria from a Steers replicator. Sensitive organisms are inhibited by the concentration of antibiotic contained in the plate, and no growth is evident at the points of inoculation; resistant organisms appear as distinct colonies of bacterial growth.

1115

Because of the large increase in the number of antibiotics that have to be simultaneously tested against clinical pathogens, the broth macrodilution procedure is no longer performed in clinical laboratories. Microbroth dilution testing is done instead which is an adaptation of the macrobroth method performed in a much smaller volume of liquid media as described below.

Microbroth Dilution Susceptibility Test. The performance of the microbroth dilution procedure is similar in principle to the macrobroth method, except that the susceptibility of microorganisms to antimicrobial agents is determined in a series of microtube wells that are molded into a plastic plate (Chart 17-2).[210,296] The development of the microdilution method allows a large number of bacteria to be tested simply and inexpensively against a panel of antimicrobial agents.

Method Description. Broth microdilution has been widely used for both clinical and investigational purposes, and is the international reference method used for establishing MICs by EUCAST (www.EUCAST.org/clinical-breakpoints) and CLSI.[117,121,122] Microbroth dilution tests are referred to as the microdilution technique

because the final volume of liquid media is only 50 μL. MHB has been the most widely used and evaluated liquid medium for dilution susceptibility testing and has been designated the international reference method by EUCAST (www.EUCAST.org) and CLSI.[117,121,122] Each plate may contain 80, 96 or more microwells, depending on the number and concentration of antimicrobial agents that are to be included in the susceptibility test panel. Commercial broth microdilution panels (Pasco systems, MicroScan panels, Sensititre, TREK plates) that contain standard horizontal serial dilutions of different antibiotics in the plate microwells, and the automation of plate incubation and reading (MicroScan system, Sensititre system), allow clinical laboratories to readily determine the MICs for a large number of drugs against a pathogen in a timely fashion.

The antimicrobial agent-containing plates must be frozen at −20°C or lower until used. Frozen prepared plates can be obtained from several commercial sources. Storage of large numbers of frozen microplates may be difficult in some laboratories. In this case, plates that contain lyophilized antimicrobial agent solutions may be purchased. Rehydration of the wells adds another step to the procedure, but the extended shelf life of the freeze-dried plates is valuable, especially for laboratories in which small numbers of panel tests are performed. Both the frozen and lyophilized broth dilution systems have performed well in comparison to the reference methods.[226,474,604,685] Few laboratories have production facilities to make their own plates for patient testing, and in-house plates have been shown to be less reliable and reproducible across laboratories than that of the commercial systems.[209] Some of the commercial systems have not performed well, however, in certain special situations, as discussed below.

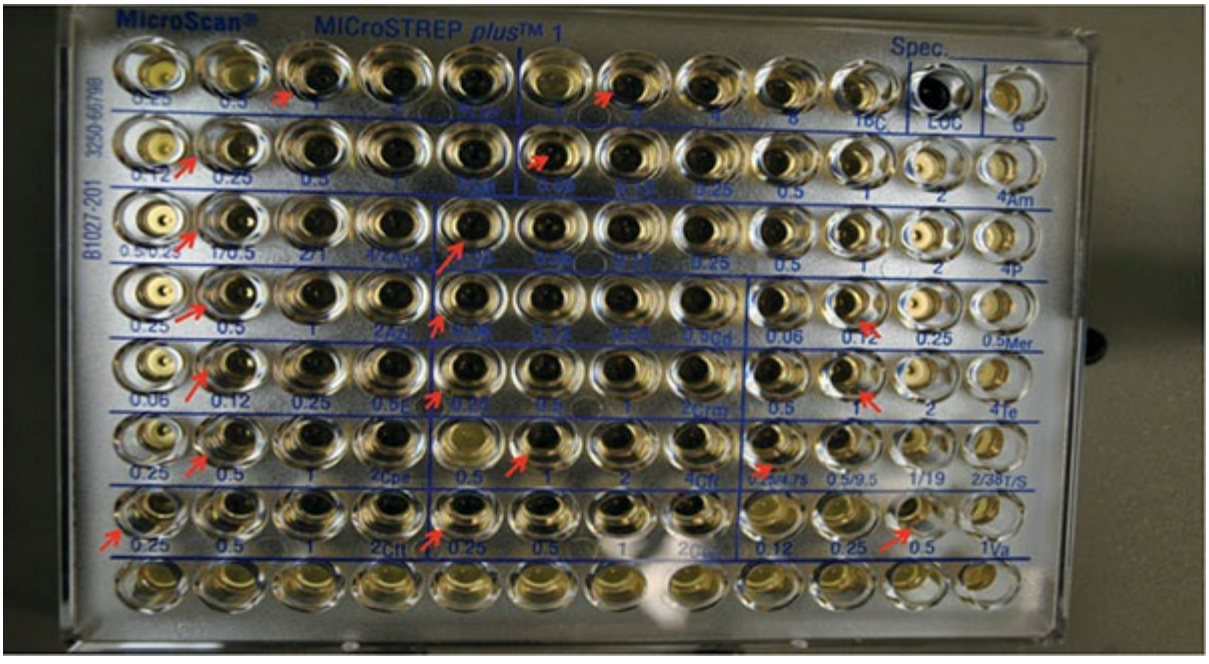
Interpretation of Results. The growth control wells are examined for organism viability and inoculum purity before reading the MICs for the clinical isolate. End points of broth microdilution tests are usually easily defined (Fig. 17-21). Examination of the microplate is facilitated by use of a viewing mirror. Occasionally, skipped wells occur where growth is inhibited in a well that is adjacent to wells with uninhibited growth. If a single well is skipped and the interpretation of the result is not affected, the skipped well may be ignored. The problem should, however, be investigated and the test repeated if multiple wells are skipped, the skipped well occurs at a dilution that is critical for determining susceptibility of the isolate or multiple isolates demonstrate the phenomenon.

The interpretive guidelines outlined by the CLSI should be reported along with or in place of the actual MIC values.[117,121,122] Separate guidelines for interpreting dilution susceptibility test results for fastidious bacteria have been published by the CLSI.[114]

Quality Control. The objectives of performing QC for dilution tests are the same as for disk diffusion tests. International standards have been developed for acceptable performance of susceptibility testing devices including broth microdilution

panels.[121,122] A key principle of the QC of broth dilution tests is the use of reference bacterial strains that are genetically stable and have well-defined MICs that are in the middle range of the expected MICs of each antimicrobial agent to be tested. A dilution series should include at least two concentration increments above and below the previously established MIC for the reference organisms. CLSI has established QC limits for dilution susceptibility tests[117,121,122]; an unacceptable QC result is one that falls outside these published limits. Reference strains recommended by the CLSI for QC of dilution tests for aerobic bacteria include *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213. The β-lactamase-producing strain *E. coli* ATCC 35218 is only recommended for penicillin-β-lactamase inhibitor combinations. Box 17-6 outlines common possible problems that occur with dilution susceptibility testing as well as their explanations.

Quality control tests for dilution susceptibility tests should be performed daily until the QC plan outlined in Table 17-10 (20- to 30-day plan) and Table 17-11 (3- × 5-day plan) is passed.[121] Weekly QC testing may be implemented provided no more than 3/30 consecutive test results for each reference drug–organism combination falls within acceptable limits. However, if a single MIC occurs outside the acceptable range, then daily testing is reinstated unless an obvious source of error is identified and fixed (i.e., incorrect test conditions, wrong inoculum, contamination). In addition, each new batch and lot of commercial plates needs to undergo QC tests before being put into clinical use. Table 17-16 provides an example of a chart for recording the results of daily/weekly QC for a microbroth dilution panel (MicroScan) using one of the standard control test organisms, such as *P. aeruginosa* (ATCC 27853).



■ **FIGURE 17-21** Commercial microdilution panel testing of *S. pneumoniae*. Red arrows indicate the wells where the MIC breakpoint for each drug would be read. (Courtesy of Calgary Laboratory Services.)

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BOX 17-6

Troubleshooting Broth Microdilution Assays

- MIC is read as the lowest dilution where growth is inhibited in the well:
  1. When MICs are lower than expected—the inoculum may be too light. Repeat using McFarland 0.5 turbidity standard or standardizing device. Check steps in inoculum preparation and inoculation procedure.
  2. When MICs are either higher or lower than expected—the composition of the cation-adjusted Müeller–Hinton broth may not be optimal. Check the pH and calcium concentration of in-house prepared media. Use an alternative commercial lot of media, or an alternative lot of commercial panels.
  3. When MICs are higher than expected—the inoculum may be too heavy. Repeat using McFarland 0.5 turbidity standard or standardizing device. Check steps in inoculum preparation and inoculation procedure.
  4. When there are skipped wells—may be caused by several problems:
     a. Check for contamination.
     b. Panel may have been inadequately inoculated or the inoculum may have been inadequately mixed.
     c. Drug concentration in the wells may be inaccurate.
     d. Volume of broth in the wells may be inaccurate.
  5. When several MICs too high or too low—may indicate a possible reading/transcription error. Recheck all of the readings and repeat testing using an alternative lot.

Automated Systems

Commercial automated systems for antimicrobial susceptibility testing have been widely used since the 1990s. Automated systems allow for more rapid test turnaround time, and the ability to produce a large number of results with less labor than manual methods. Automated results are highly reproducible, and the development of expert computer systems allow for the on-board interpretation of results using expert rules built into the system, and the ability to retain and analyze a large amount of

susceptibility data. Although most large laboratories have purchased an automated system, the high capital cost on the instrumentation and required consumables compared to manual methods have curtailed their implementation in some smaller facilities. These systems also do not have the capability to test all of the types of encountered pathogens, and problems have been documented using strains that have unusual resistance phenotypes or types of resistance that are difficult to detect.[296] The major systems have generally performed well in comparisons against reference methods, although laboratories need to be aware of the limitations of their particular automated system, and perform manual and/or molecular confirmation of specific types of resistance flagged by the system (see Detection of Specific Types of Antibiotic Resistance Phenotypes below). This section provides a brief overview of the current automated system approved for use by the FDA in the United States. Readers are referred to Section 5.17 of the Clinical Microbiology Procedures Handbook for a more detailed review of the evaluation of commercial automated susceptibility systems.[407]

Vitek 2. The Vitek 2 system (bioMérieux) has been in use in the United States since 2000 (Fig. 17-22). The standard instrument contains 64 wells for cards that have one to six concentrations for 9 to 20 antimicrobial compounds. The larger Vitek 2XL instrument has a 120-card capacity. The system also runs gram-positive and gram-negative identification panels at the same time that susceptibility testing is being performed. Although the initial bacterial inoculum and dilution of the organisms to be tested must be done manually, all other steps are automated. Vitek 2 takes an average setup time of 1.5 minutes per isolate.[407] Each Smart Carrier Station has a barcode reader, and holds up to 15 cards. A memory chip in the cassette captures and transfers scanned data to the reader-incubator unit. Robotics of the instrument move the loaded cassette through successive stations for barcode reading, test dilution preparation, and card inoculation and sealing before placement of the card on the 6-card carousel for incubation. Each card is read by an optical station every 15 minutes, and the amount of light transmitted is inversely proportional to the amount of growth achieved. Vitek 2 uses computer-assisted analysis of growth in plastic cards to calculate a MIC. In some instances the calculation of the MIC depends on the bacterial identification. The additional accuracy provided by the computer-driven correlation of growth pattern and identification is counter balanced by the uncertainty about the susceptibility result if the identification is not yet known or cannot be provided with adequate certainty by the system. The Vitek 2 Advanced Expert System analyzes MIC patterns and detects phenotypes for most organisms tested. The Vitek 2 Observa information management system allows susceptibility results to be stored so that it can be analyzed and formatted into user-defined reports including the generation of cumulative reports.

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TABLE 17-16 Weekly Quality Control Chart for Microbroth Dilution Panel (MicroScan) Testing of *Pseudomonas aeruginosa* (ATCC 27853)

NOTE: QC WEEKLY & EACH NEW LOT											
METHOD: Microdilution Panel	PANEL TYPE: MIC 38			Gram Negative Panel			ORGANISM: *P. aeruginosa* ATCC# 27853				
Panel Lot # (Expiry Date):				PURITY PLATE Checked:							
Inoculation Fluid Lot #(Expiry Date):											
Set Up By Date/Initials:						Read By Date/Initials:					
Negative Growth	Growth Control	<=2 Am	4 (>128)	8	16 >	<=2 Cfz	4 (>16)	8	16 >	<=8 16 >	CF (>64)
<= 16 Pi	32 (<=8)	64 >	<= 8 P/T	16 (<=8)	32	64 >	<=8 C	16 > (>16)	LOC	<= 8 A/S	16 > (>32)
<=4 Azt	8 (2-8)	16 >	<=4> Crm	8 (>16)	16	<=0.5 Cp	1 (<=0.25-1)	2 >	<=1 Lvx	2 (<=0.5-4)	4 >
<= 1 Caz	2	4	8 (<=1-4)	16 >				<=4 Te	8 > (8-128)	<=32 Fd	64 > (>64)
0.25/4 Caz/CA	2/4	<=8 Cfx	16 > (>16)	<=1 Gm	2 (1–4	4	8 >	<=8 Tim	16 (<=8-32)	32	64 >
<=2 Cft	8 (8-32)	16	32 >	<=4 Ak	8 (<=2-8)	16	32 >	<=4 Cax	8 (8->32)	16	32 >
0.5 Cft/CA	4/4	<=16 Ctn	32 > (>32)	<=1 To	2	4	8 > (<=0.5-1)	<=2 Cpe	4 (<2-4)	8	16 >
<=1 Imp	2 (1–4)	4	8 >	<=1 Mer	2 (<=1)	4	8 >	<=1 Etp	2 (2->4)	4 >	<=2 > T/S (>=8)
Weekly QC Review:						Monthly QA/QC Review:					
Adapted with permission from Calgary Laboratory Services											

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■ **FIGURE 17-22** Vitek 2 automated susceptibility system www.biomerieux-diagnostics/VITEK-2.

Ligozzi and colleagues reported that the time required for Vitek 2 susceptibility results for gram-positive cocci ranged from 6 to 17 hours depending on the species.[343] *S. aureus* results took 8 hours, while results for coagulase-negative staphylococci took longer at 11 hours. Group B *Streptococcus* results took 9 hours as did enterococci, but *S. pneumoniae* took slightly longer at 9 hours. Vitek 2 also generates rapid susceptibility results for *Enterobacteriaceae* in from 3.3 to 17.5 hours; isolates from blood cultures compared well with manual methods.[348]

MicroScan Walkaway SI. The Microscan Walkaway (Dade Behring) has been on the market the longest of the automated instruments, and has been used in the United States since the early 1990s (Fig. 17-23). All of the panels used on the instrument are conventional 96-well microdilution trays including MIC panels that have a broad range of antimicrobials and combination panels that include wells for identification and susceptibility testing. Breakpoint combo panels are also available that provide a limited range of dilutions that determine whether an isolate is susceptible, intermediate, or resistant to a compound, as well as identification wells. Both frozen and lyophilized panel formats are available on the conventional MicroScan and rapid Walkaway systems. The Walkaway instrument is available in two sizes that will accommodate either 40 or 96 panels. The MicroScan uses broth microdilution to determine the MIC. After inoculation and overnight incubation (i.e., 4.5 to 15 hours), the conventional MicroScan system reads panels turbometrically (conventional MicroScan system) while the Walkaway instrument uses a fluorescence detection system that will provide same-day identification and susceptibility testing for some organisms.

■ **FIGURE 17-23** MicroScan walkaway plus automated ID and antibiotic susceptibility system (www.healthcare.siemens.com/microbiology-testing/microscan-systems/walaway-plus-system)

The Walkaway system is primarily a semiautomated plate incubator-reader attached to a computer and printer. The initial inoculum preparation is done manually, and then a manual device (RENOK) is used to rehydrate and inoculate panels. The newer SI version of the Walkaway instrument has also automated the addition of reagents to the identification wells. Robotics within the instrument moves the plates under a central photometer (MicroScan) or fluorometer (Walkaway) for reading. The data management system LabPro interprets the results, generates reports and stores data for analysis and the generation of epidemiologic reports. An expert system LabPro Alert may also be purchased that contains algorithms for expert rules-based interpretation and reporting.

Although the Walkaway system has comparable clinical performance to other automated systems, panel testing using this instrument is more labor intensive than a fully automated system. Due to the number of panels that need to be tested, more biohazardous waste is generated by the MicroScan than either the Vitek 2 or BD

Phoenix instruments.

BD Phoenix. The BD Phoenix system (Becton Dickenson Diagnostics, Baltimore MD) has been in use in the United States since 2004 (Fig. 17-24). The instrument has the largest capacity of any automated system (i.e., 100 panels), other than the Vitek 2XL. The system provides direct rather than calculated MICs and a wide range of antimicrobials can be tested. Each plastic tray has 126 wells divided between the identification side (51 wells) and the susceptibility side

(85 wells) that contain 16 to 22 antimicrobials. A mean time of 3 minutes per isolate has been reported for the BD PHOENIX setup time.[407] A redox indicator (blue) is added to the bacterial inoculum during preparation, and the suspension is poured into the susceptibility side of the panel and sealed. Individual bar codes are used to identify each test panel. The instrument reads panels every 20 minutes. Turbidity is measured as a growth indicator, and a colorimetric change (to pink) is the redox indicator. BD Phoenix has expert software (BCXpert) and data management software (BD EpiCenter) to interpret MIC values according to CLSI guidelines and reporting rules. Data is stored in the instrument and analyzed to generate epidemiologic reports.

■ **FIGURE 17-24** BD phoenix automated ID and antibiotic susceptibility system (www.bd.com/ds/productcenter/is-phoenix.asp)

The BD PHOENIX system had a somewhat faster overall reporting time for most common types of bacteria than Vitek 2 in one study, but a longer time was required to generate results for *Enterobacteriaceae* than Vitek 2.[407]

Limitations. Automated systems have their individual limitations, particularly with the reporting of unusual resistance phenotypes (See Detection of Specific Antibiotic Resistance Phenotypes below). QC of automated susceptibility results has been difficult and largely remains unstandardized. Standard reference strains should be tested on the instrument after routine maintenance is performed. Laboratories should also validate new products with new or improved test capabilities (i.e., the ability to detect specific types of resistance phenotypes), and validation should also be done with each new software upgrade to the system including the addition of new expert reporting rules.

Choice of Test

A detailed summary of the susceptibility methods recommended by the CLSI for selected organisms is outlined in Table 17-17. Although disk diffusion testing is widely used, particularly in smaller laboratories or laboratories in resource limited settings, some antibiotics cannot be reliably tested against some organisms as outlined in CLSI guidelines,[118,121,122] and below in the section on detection of antibiotic resistance in important pathogens. Antibiotic susceptibility testing using dilution tests provides more quantitative information, and may be applied to a wider range of isolates than the diffusion test. For evaluation of susceptibility tests, and comparison between methods, most investigators use the following definitions for determining the levels of error:

- Very major error: characterization of a resistant isolate as susceptible
- Major error: characterization of a susceptible isolate as resistant
- Minor error: characterization of a susceptible or resistant isolate as intermediate, or characterization of an intermediate isolate as susceptible or resistant

Detection of Specific Types of Antibiotic Resistance

β-Lactamase Testing

Several methods have been used to rapidly and reliably to detect the presence of many

β-lactamases. β-Lactamase testing is based on the detection of the end products of β-lactam ring hydrolysis which are visualized using a chromogenic substrate. Although three different reactions methods have been used including the iodometric, acidimetric, and chromogenic cephalosporin methods, the latter has been widely adopted because of its ease of use and sensitivity for the bacterial species for which the test is recommended. A detailed description of each of these methods has been previously published.[39]

The chromogenic cephalosporin test, is performed by smearing a loopful of a colony onto a nitrocefin disk (Cefinase; BD Diagnostic Systems, Sparks, MD), which is then placed in a closed Petri dish to prevent rapid desiccation. Organisms that containβ-lactamase will change the color of the disk from yellow to red (Fig. 17-25). The reaction usually occurs within 30 seconds, but tests are read finally after 15 minutes.

A positive test is correlated with resistance to specific β-lactam drugs for some bacterial species including (1) *H. influenzae* (amoxicillin, ampicillin), (2) *M. catarrhalis* (amoxicillin, ampicillin, and penicillin), (3) *N. gonorrhoeae* (amoxicillin, ampicillin, and penicillin), (4) *Staphylococcus* spp. (amoxicillin, ampicillin, penicillin, mezlocillin or cloxacillin, and ticarcillin or piperacillin), and (5) *Enterococcus* spp. (ampicillin, amoxicillin, penicillin, piperacillin). However, special precautions must be taken to ensure that β-lactamase production in *Staphylococcus* spp. has been induced prior to testing as outlined below. For other organisms, β-lactamase production does not entirely correlate with resistance to β-lactam drugs that may be used for treatment, and the tests described below should not be used for aerobic gram-negative bacteria including *Enterobacteriaceae* or *P. aeruginosa*.

Surveillance in the United States shows that 30% to 40% of clinical isolates of *Haemophilus influenzae* will be resistant to ampicillin/amoxicillin because of β-lactamase production, and the highest rates of this type of resistance occur in nontypeable strains.[280] A more recent study of pediatric clinical isolates found that ~40% of nontypeable *H. influenzae* isolates from serious infections also produce β-lactamases.[236] A β-lactamase test should be done on all clinically significant isolates to rapidly detect resistance to ampicillin or amoxicillin. However, β-lactamase nonproducing, ampicillin/amoxicillin-resistant (BLNAR) strains that have altered PBP3 can only be detected by a diffusion or dilution susceptibility test.[275] *H. influenzae* recovered from sterile sites/sources that are producing clinically significant infections should undergo full susceptibility testing as outlined later in the chapter.

Most clinically significant isolates of *M. catarrhalis* will demonstrate β-lactamase production. CLSI continues to recommend that laboratories perform a β-lactamase test on all isolates, but full susceptibility testing should be done on isolates from invasive infections.[121,122] Recent surveillance of respiratory tract isolates from several geographic locations have shown the *M. catarrhalis* has developed increased

resistance to second-generation cephalosporin (cefaclor, cefprozil, cefuroxime), tetracyclines, macrolides, and trimethoprim–sulfamethoxazole.[462,538,649]

Staphylococci may require induction in order to ensure the accuracy of detecting β-lactamase production. β-Lactamase production by in *Staphylococcus* spp. is responsible for most of the resistance to penicillin G and related compounds, but the cephalosporins or the penicillinase-resistant penicillins, such as methicillin and nafcillin, are not inactivated by these penicillinases unless produced in large amounts. A staphylococcal β-lactamase test should not be reported as negative unless the test has been performed on the isolate after exposure to an inducing agent (i.e., oxacillin or cefoxitin). Microdilution MIC tests using penicillin may also fail to detect staphylococci that are penicillin-resistant due to the presence of a β-lactamase because only a small amount of the enzyme is typically produced by the relatively small inoculum used in this test. However, the presence of a β-lactamase can be inferred if the MIC of a staphylococcal strain to penicillin is greater than 0.25 μg/mL. *Staphylococcus* spp. isolates with penicillin MICs less than 0.03 μg/mL are considered susceptible. Isolates for which the MIC falls between these limits (0.06 or 0.12 μg/mL) should be tested for the presence of β-lactamase before the results are reported. Use of bacterial growth from a well that contains a penicillin-class drug or from the edge of a zone of inhibition around a disk that contains a penicillin compound may induce activity and enhance detection of the enzyme. The results from the testing of penicillin can be extrapolated to ampicillin.

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TABLE 17-17 Summary of Antimicrobial Susceptibility Test Methods for Selected Organisms

Organism	Method	Test Conditions (Medium/Inoculum/Incubation)	Comments
Staphylococcus spp.	Broth microdilution	CAMHB or MHA/DCS/35°C ambient air for 16–20 hours	Use molecular testing for *mecA* gene; confirm VAN I/R strains
	Agar dilution (Reference test)	CAMHB or MHA plus 2% NaCl for OXA, MET or NF/DCS/35°C ambient air for 24 hours	
	OXA–salt agar screen	MHA with 4% NaCl/DCS/35°C ambient air for 24 hours	Chromagar used by many laboratories
	Disk diffusion	MHA/DCS/35°C ambient air X 16–20 hours; 24 hours for OXA, MET or NF	Confirm VAN I/R by an MIC method; low-level resistance not well detected
	Gradient diffusion (Etest)	MHA or MHA with 2% NaCl for OXA, MET, or NF/DCS in broth/35°C ambient air X 16–20 hours; 24 hours for OXA, MET, or NF	Incubate for 48 hours to detect OXA resistant CONS
Enterococcus spp.	Broth microdilution; agar dilution	CAMHB; MHA/Growth method or DCS/35°C ambient air for 16–20 hours; 24 hours for VAN	Confirm VAN R strains; perform β-lactamase test
	Disk diffusion	MHA/Growth method or DCS/35°C ambient air for 16–20 hours; 24 hours for VAN	
	Gradient diffusion (Etest)	MHA/Growth method or DCS/35°C ambient air for 16–20 hours; 24 hours for VAN	
Enterococcus spp. HLAR	Broth microdilution; agar dilution	BHI broth or agar; GM 500 µg or STR 1000 µg/mL (broth) or 2000 µg/mL (agar)/growth method or DCS (standard microdilution inoculum); 10 µL spotted on agar surface/35°C ambient air for 24 hours; reincubate STR an additional 24 hours if susceptible	Perform on invasive isolates only
	Disk diffusion	MHA/120 µg disk GM or 300 µg disk STR/growth method or DCS/35°C ambient air for 16–20 hours	Confirm HLAR results with an MIC method
	Gradient diffusion	MHA; GM and STR high-dose strips/suspension in broth to 0.5–1.0 McFarland standard/35°C ambient air for 48 hours	
Enterococcus spp. VAN resistance	Agar dilution	MHA; VAN 6 µg/mL/growth method or DCS; standard microdilution inoculum; 1–10 µL spotted on agar surface	Chromagar used by many laboratories
Streptococcus pneumoniae	Broth microdilution	CAMHB with 2%–5% (v/v) lysed horse blood/DCS/35°C ambient air for 20–24 hours	OXA disk screen for PEN susceptibility may be used as a preliminary test
	Disk diffusion	MHA with 5% sheep blood/DCS/35°C in 5% CO_2 for 20–24 hours	Disk diffusion not reliable for some antimicrobial agents; MIC test recommended for CNS isolates
	Gradient diffusion	MHA with 5% sheep blood/DCS in broth/35°C in 5% CO_2 for 20–24 hours	Use a 1.0 McFarland inoculum if the bacterial lawn is sparse
Other *Streptococcus* spp.	Broth microdilution	CAMHB with 2%–5% (v/v) lysed horse blood/DCS/35°C ambient air for 20–24 hours	
	Agar dilution	MHA with 5% v/v sheep blood/DCS/35°C ambient air for 20–24 hours/CO_2 if necessary for growth	Use lysed horse blood if testing a sulphonamide
	Gradient diffusion	MHA with 5% sheep blood/DCS in broth/35°C in 5% CO_2 for 20–24 hours	Use a 1.0 MacFarland inoculum for mucoid strains
Listeria spp.	Broth microdilution	CMHB plus 2%–5 % lysed horse blood/DCS/35°C ambient air for 16–20 hours	For AMP and PEN ≤2 µg/mL (susceptible); resistant strains not characterized
Haemophilus spp.	Broth microdilution	HTM broth/DCS/35°C in 5% CO_2 for 20–24 hours	
	Disk diffusion	HTM broth/DCS/35°C in 5% CO_2 for 20–24 hours	
	Gradient diffusion	HTM or MHA with 1% hemoglobin and 1% Isovitalex (BBL) if validated against CLSI/DCS in broth/35°C ambient air for 24 hours	

Neisseria gonorrhoeae	Agar dilution	GC agar base with 1% defined growth supplement (see text)/ DCS/35°C in 5% CO_2 for 20–24 hours	Perform β-lactamase test
	Disk diffusion	GC agar base with 1% defined growth supplement (see text)/ DCS/35°C in 5% CO_2 for 20–24 hours	Cysteine-free medium not required; perform β-lactamase test
	Gradient diffusion	GC agar base with 1% defined growth supplement (see text)/ DCS/35°C in 5% CO_2 for 20–24 hours	
Neisseria meningitidis	Broth microdilution	CAMHB plus 2%–5% (v/v) lysed horse blood/DCS/35°C ambient air for 16–20 hours	
	Agar dilution	MHA plus 5% (v/v) sheep blood/DCS/35°C ambient air for 16–20 hours	Use 2%–5% (v/v) lysed horse blood instead of sheep blood if sulphonamides are tested
	Gradient diffusion	MHA plus 5% sheep blood or MHA plus 1% hemoglobin and 1% Isolvitalex (BBL)/DCS in broth/35°C in 5% CO2 for 18–24 hours	
Vibrio cholerae	Broth microdilution; agar dilution	CAMHB; MHA/growth method or DCS/35°C ambient air for 16–20 hours	
	Disk diffusion	MHA/growth method of DCS/35°C ambient air for 16–18 hours	

CAMHB, Cation-adjusted Müeller–Hinton broth; MHA, Müeller–Hinton agar; MIC, Minimum inhibitory concentration; DCS, direct colony suspension; VAN, vancomycin; S, susceptible; I, intermediate; R, resistant; OXA, oxacillin; MET, methicillin; NF, nafcillin; BHI, brain–heart infusion; GM, gentamicin; STR, streptomycin; HLAR, high-level aminoglycoside resistance; CNS, central nervous system; AMP, ampicillin, PEN, penicillin; HTM, Haemophilus Test Medium; GC, *Neisseria gonorrhoeae*.
Adapted from Etest application sheets, bioMérieux; and from references 114,117, 118, 121, and 122.

■ **FIGURE 17-25** Positive β-lactamase nitrocefin test for *S. aureus*. (Courtesy of Calgary Laboratory Services.)

First-generation cephalosporins and penicillinase-resistant penicillins (methicillin, nafcillin, oxacillin, dicloxacillin, etc.) have a modified β-lactam ring that resists enzymatic digestion by staphylococcal β-lactamases. These antibiotics can, however,

be inactivated in vitro by a strain that produces a large quantity of penicillinase. These *S. aureus* strains usually exhibit borderline resistance to oxacillin (MIC, 4 μg/mL) (called BORSAs), but retain susceptibility to other commonly used antimicrobial agents.[291] In contrast, MRSA strains that are resistant to oxacillin by means of altered penicillin-binding proteins usually have an oxacillin MIC ≥ 4 μg/mL, and are typically multi-resistant to other classes of antimicrobial agents, as discussed below. The clinical significance of BORSA strains has been restricted to superficial skin and soft tissue infections. Kernodle and colleagues described a group of such strains of *Staphylococcus aureus* of a certain phage type that produced clinically significant wound infections.[313]

High-level resistance to penicillin in enterococci can also be detected by the nitrocefin test. A positive nitrocefin test indicates resistance to all penicillinase-labile penicillins (i.e., ampicillin, amoxicillin, piperacillin). The β-lactamase, which resembles that of staphylococci, is carried on a plasmid,[409] but the enterococcal and staphylococcal plasmids themselves

are not homologous.[651] Strains of *Enterococcus faecalis* that produce β-lactamase that also exhibit high-level resistance to aminoglycosides have become epidemic in some institutions.[17,527] If the nitrocefin disk test is not performed on isolates, resistance to β-lactam antibiotics may not be recognized. In a survey of New Jersey laboratories by Tenover and colleagues, a β-lactamase-producing strain of *E. faecalis* was recognized as resistant to penicillin by only 66% of 76 participants and to ampicillin by only a few (8%) laboratories. Only 3 of the 76 laboratories recognized that the strain produced β-lactamase, emphasizing the importance of performing a β-lactamase test on enterococci, especially if they have been isolated from sterile sites.[611] A combination of piperacillin and tazobactam is effective against β-lactamase-producing enterococci,[438] and this agent may provide synergy with gentamicin provided the enterococcal strain does not demonstrate high-level resistance to aminoglycosides as discussed below.

Detection of Resistance in Gram-Positive Bacteria

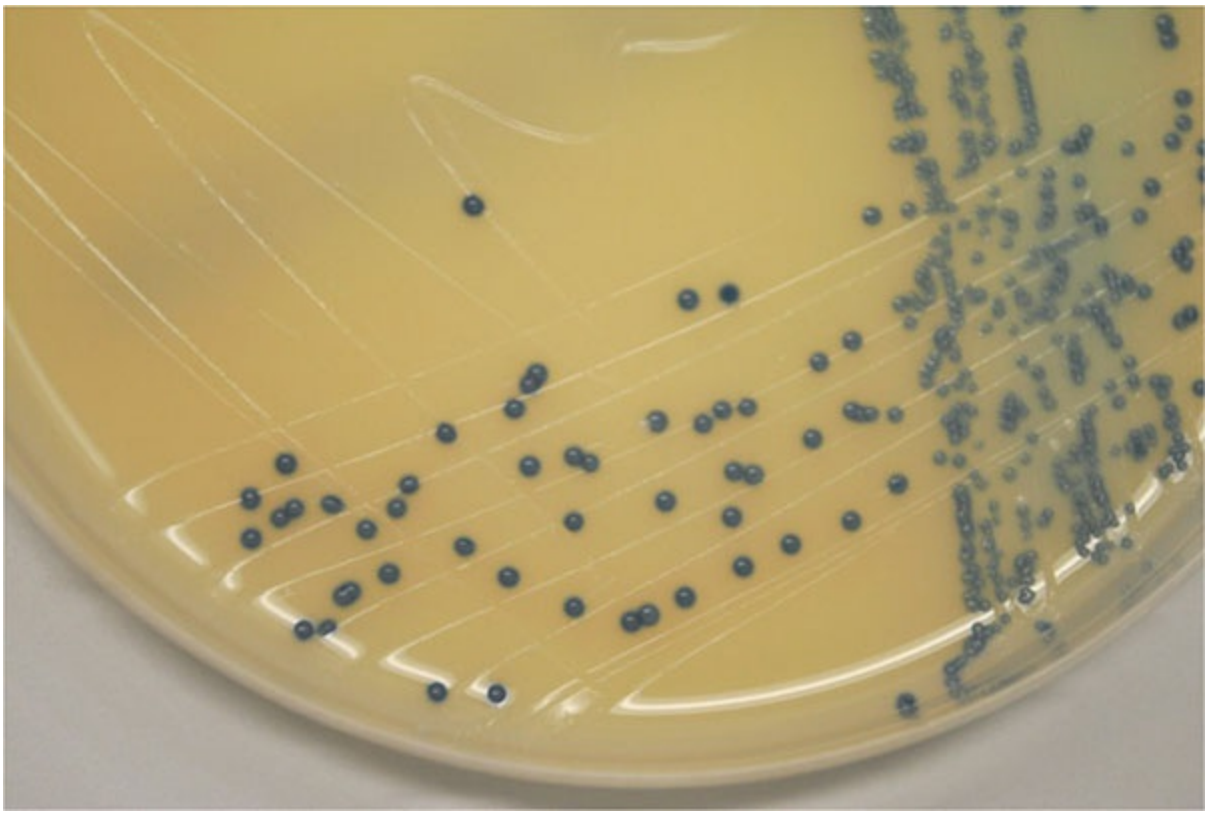
Staphylococci

Methicillin (Oxacillin)-Resistant Staphylococci

Phenotypic Detection. *Staphylococcus* infections are the most common cause of

nosocomial infections worldwide. *Staphylococcus* spp. resistant to semisynthetic penicillins (i.e., methicillin, nafcillin, oxacillin, and cloxacillin) have become a serious threat in both nosocomial and ambulatory healthcare settings.[317,374,688] Worldwide, the rates of MRSA in clinical isolates range from a low of <1% to 3% in the Netherlands and Denmark, to the mid-level of ~20% to 30% for countries such as Germany and Canada, to a high of 50% to 60% in Japan and the United States.[557,579,581] Because most MRSA surveillance data is accrued for nosocomial isolates, the true incidence of ambulatory community-acquired MRSA infections in most jurisdictions is not known.

Because of the global problems being encountered within acute-care facilities due to both hospital-acquired and community-acquired MRSA, many countries have implemented targeted or universal screening of patients on admission. Although MRSA isolates readily grow on routine nonselective media, chromogenic agar has been widely adopted in clinical laboratories to rapidly screen for the presence of MRSA in surveillance and clinical specimens. Several chromagar formulations are available that contain chromogenic substrates and various inhibitors that combine primary growth and selective detection of MRSA, including MRSA ID (bioMérieux), MRSA Select (BioRad), CHROMagar MRSA (BD Diagnostics), and Denim Blue (Oxoid) (Fig. 17-26).[195,414] However, some chromogenic agars will not grow all strains of MRSA, but a cefoxitin disk may be placed onto chromogenic or nonselective media as an alternate method for enhancing detection of MRSA by growth around the disk.[247] Few studies have, however, compared the performance of chromagar with either secondary susceptibility testing of the isolates or detection of the *mec*A gene. Flayhart and colleagues found that the CHROMagar MRSA recovered 95.2% of MRSA isolates and have a specificity of 99.7% compared to a sensitivity and specificity of 94.4% and 96.7% for oxacillin broth microdilution MIC; 94.3% and 96.7% for oxacillin screen agar; 93.7% and 98.5% for PBP2′ latex agglutination; 95% and 98.1% for cefoxitin disk diffusion; and 95.1% and 98.1% for *mec*A polymerase chain reaction (PCR).[195] Healthcare jurisdictions with high rates of MRSA have also implemented rapid commercial real-time PCR assays in place of culture-based methods because of their greater sensitivity and much more rapid turnaround time (see Detection of the *mec*A Gene below).



■ **FIGURE 17-26** MRSA colonies growing on denim blue agar (Oxoid). (Courtesy of Calgary Laboratory Services.)

CLSI defines oxacillin resistance in *S. aureus* as an MIC ≥ 4 μg/mL,[121,122] and all strains meeting this definition are characterized as "MRSA" even though methicillin is no longer used either to test strains in the laboratory or as a therapeutic agent. Although several other resistance mechanisms may result in MRSA (i.e., high level of β-lactamase production (BORSA) or production of modified PBPs (MOD-PBP) with decreased drug affinity), the vast majority of clinical strains that exhibit this type of resistance have acquired the *mec*A gene that encodes for PBP2a which has decreased affinity for binding the compound (See Mechanisms of Resistance).[146,239,628] MRSA strains with a *mec*A gene may either be homogenous or heterogenous in their expression of this resistance phenotype. Accurate detection of heterogenous strains is particularly challenging because some cells will test susceptible while others will be resistant (i.e., typically only 1×10^4 to 1×10^8 cells in a population will express the *mec*A gene).[238] Staphylococci carrying a *mec*A gene with heterogenous expression, may therefore either be missed using the established MIC breakpoint alone because they can have borderline MICs between 2 to 4 μg/mL, or be confused with strains that have borderline resistance due to another mechanism (i.e., BORSA if the β-lactamase test is also positive). Table 17-18 outlines several scenarios that may be encountered in

the clinical laboratory when screening *S. aureus* for oxacillin resistance. MRSA strains carrying the *mec*A gene are also usually multidrug-resistant to one or more classes of other antimicrobial agents. The overall antibiogram profile of a clinical isolate should, therefore, also be used as another important clue that points to this type of resistance mechanism. In most of these cases, however, molecular detection of the *mec*A gene is the most expedient way to determine whether one is truly dealing with a *mec*A-positive strain, because errors in the identification and reporting could have potentially serious clinical and epidemiologic outcomes.

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TABLE 17-18 Possible *S. aureus* Oxacillin Resistance Phenotypes

Scenario	Vitek Oxacillin Results	Cefoxitin (30 µg) Disk Screen Results	Resistance Profile to Other Drugs
1	Resistant	Resistant	Multidrug resistant[a]
2	Sensitive	Sensitive	Multidrug resistant[a]
3	Resistant	Resistant	R to β-lactams[b] but S to all other drugs
4	Sensitive Resistant	Resistant Sensitive	Discrepant results between oxacillin and cefoxitin screen

[a] Resistant to two of gentamicin, tetracycline, clindamycin, or erythromycin.
[b] Resistant to penicillin, oxacillin, and cefazolin.

Antibiotic susceptibility testing for reliable detection of MRSA should be done using an MIC method. Broth microdilution is the reference method for detection of oxacillin resistance in *S. aureus*, and in most strains reliably demonstrate the breakpoints recommended by CLSI.[121,122] As of 2013,[121] CLSI has eliminated the use of oxacillin disk testing for *Staphylococcus* spp. Cefoxitin has been shown to be a more sensitive surrogate than oxacillin for detection of MRSA because of its more reliable induction of the *mec*A gene.[121,122,525,559,592] A large multilaboratory study involving the Centers for Disease Control and Prevention (CDC) also investigated whether a cefoxitin broth microdilution MIC breakpoint would correlate with the presence of the *mec*A gene in staphylococci.[70] Using breakpoints of ≤4 µg/mL for *mec*A-negative and ≥6 or 8 µg/mL for *mec*A-positive isolates, sensitivity and specificity compared to the presence of the *mec*A gene for *S. aureus* at 18 hours of incubation were 99.7% to 100% for three CAMHBs. For coagulase-negative staphylococci isolates at 24 hours of incubation, breakpoints of ≤2 µg/mL for *mec*A-negative and ≥4 µg/mL for *mec*A-positive strains gave sensitivities and specificities of 94% to 99% and 69% to 80%, respectively. Routine disk diffusion procedures may be used to perform the cefoxitin disk (30 µg) test on *S. aureus* isolates, and a zone diameter of ≥20 mm is interpreted as susceptible

to oxacillin, while one that is ≤19 mm is interpreted as resistant. A broth microdilution screen using the CLSI cefoxitin breakpoints for *S. aureus* would therefore provide an alternate method that is highly reliable for detection of MRSA.[121,122] This method, however, appears to be less reliable for some species of coagulase-negative staphylococci.

MHA supplemented with 2% sodium chloride and 6 µg of oxacillin, methicillin, or nafcillin per mL is the agar screen method recommended by CLSI.[121,122] Colonies are grown overnight on nonselective agar and transferred to tryptic soy broth to make a 0.5 McFarland suspension. The plate is then inoculated in one quadrant or an area 10 to 15 mm in diameter using either a cotton swab or 1-µL loop. Resistance to oxacillin is indicated if there is growth of more than one colony of the plate after incubation a full 24 hours at 35°C. QC of oxacillin screen plates is done using *S. aureus* ATCC 29213 (susceptible strain) and *S. aureus* ATCC 43300 (resistant strain).

Standardizing testing for coagulase-negative staphylococci has proven to be more of a challenge because of the variability between the many species contained under this umbrella term. Broth microdilution breakpoints for diagnosis of oxacillin resistance in coagulase-negative staphylococci were lowered by CLSI in 1999 due to concerns that *mec*A-positive strains were not being reliable detected (Table 17-19). Although these revised breakpoint criteria improved detection in certain species (i.e., *S. epidermidis*, *S. haemolyticus,* and *S. hominis*), they had less specificity for other species (i.e., *S. saprophyticus* and *S. lugdenensis*) so that disk and MIC methods indicated resistance but the strains did not carry an *mecA* gene.[191,224] In 2005, CLSI recommended that the disk and MIC oxacillin breakpoints for *S. aureus* be used to interpret resistance in *S. lugdenensis*.[119] Similar to *S. aureus*, the cefoxitin-30-µg disk test has replaced the oxacillin-1-µg disk test for accurate detection of *S. lugdenensis* and other coagulase-negative staphylococci that are *mec*A-positive.[121,122] The recommended breakpoints for the cefoxitin-30-µg disk test for *S. lugdenensis* are the same as *S. aureus*, but the zone diameters are higher for all other species of coagulase-negative staphylococci with ≤24 mm being susceptible and ≥25 mm being resistant (Table 17-20).

TABLE 17-19 CLSI Breakpoints (MIC µg/mL) for *Staphylococcus* spp.

Antibiotic	Species	S	I	R
Oxacillin	*S. aureus*	≤2	—	≥4[a]
Oxacillin	*S. lugdenensis*	≤2	—	≥4
Oxacillin	Other CONS	≤0.25	—	≥0.5
Vancomycin	*S. aureus*	≤2	4–8[b]	≥16
Vancomycin	*S. lugdenensis*	≤4	8–16	≥32
Vancomycin	Other CONS	≤4	8–16	≥32

[a] Report as MRSA—send isolates with MIC >4 µg/mL to a reference laboratory.
[b] hVISA/VRSA—send isolates with MIC >8 µg/mL to a reference laboratory.
S, susceptible; I, intermediate; R, resistant.
Adapted from reference 122.

Detection of the *mecA* Gene. The gold standard for confirmation of phenotypic oxacillin resistance in clinical strains of staphylococci is detection of the *mec*A gene using either a commercial latex agglutination test and/or molecular detection methods. Table 17-18 outlines four scenarios that will be routinely encountered clinically when performing oxacillin resistance testing of *S. aureus* that warrant such confirmation.

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TABLE 17-20 CLSI Zone Diameter Breakpoints for Cefoxitin 30 µg Screen

Species	S	I	R
S. aureus	≥22 mm	—	≤21 mm[b]
S. lugdenensis	≥22 mm	—	≤21 mm
Other CONS	≥25 mm	—	≤24 mm

[a] Adapted from reference 122.
[b] Report as MRSA.
S, susceptible; I, intermediate; R, resistant, CONS, coagulase-negative staphylococci.

A latex agglutination test that rapidly detected PBP 2′ as an indicator of MRSA was originally developed in Japan.[417] Several commercial latex agglutination methods that can be rapidly performed on a high number of strains are available, but only the MRSA-Screen test (Deika-Seiken Co. Ltd., Tokyo, Japan) and the PBP 2′ test (Oxoid Ltd, Basingstoke, United Kingdom) are approved for use by the FDA in the United States. Both of these tests use latex agglutination to detect the presence of altered PBP 2a (PBP 2) as evidence for the presence of the *mec*A gene. A slide latex agglutination test may also differentiate MRSA from *S. aureus* isolates with borderline oxacillin resistance.[361] The MRSA-Screen test should only be used to test *S. aureus* for which it has been extensively evaluated and shown to have a high sensitivity and specificity for accurate detection of the *mec*A gene encoded resistance.[593] The MRSA-Screen test has, however, not performed as well in directly testing coagulase-negative isolates, and the procedure has required an increased inoculum or incubation time or, in some cases, induction with either oxacillin or cefoxitin for accurate detection to occur.[677] Coagulase-negative staphylococci including *S. lugdenensis* are tested using organism growth around either a oxacillin-1-μg or cefoxitin-30-μg disk. The Oxoid PBP 2′ test can be used for all staphylococcal isolates, but again, induction with cefoxitin or oxacillin should be performed when testing coagulase-negative staphylococci. Few comparative clinical evaluations of the Oxoid PBP 2′ test have so far been done.

Many larger clinical microbiology laboratories have been using in-house PCR tests to confirm the presence of the *mec*A gene. In-house PCR tests for *mec*A detection have also included the *nuc* and *coa* genes as a specific marker that allows simultaneous identification of *S. aureus*.[75] A quadriplex assay has been developed that will simultaneously distinguish *S. aureus* from coagulase-negative staphylococci using

the 16S rRNA and *nuc* gene markers, that also detect both methicillin (*mec*A) and mupirocin (*mup*A) resistance.[687] Other PCR MRSA detection assays have also used other staphylococcal gene markers (i.e., *femA*, *femB*, *sa*442, and *orfX*), and of these, the open reading frame (*orfX*) where the staphylococcal cassette chromosome *mec* (SCCmec) integrates with the *S. aureus* chromosome has been commercially exploited to develop several rapid detection assays as outlined below.[95] Figure 17-27 shows real-time PCR detection of the *nuc* and *mec*A genes in a *S. aureus* blood culture isolate that demonstrated a Rule 1 phenotypic resistance profile according to Table 17-18.[178] This isolate was confirmed to be *S. aureus* due to the presence of the *nuc* gene, and MRSA due to the presence of the *mec*A gene.

Recent commercial development of rapid test platforms has, however, made real-time PCR detection of the *mec*A gene available to most facilities. Readers are referred to several excellent recent reviews for a detailed discussion of all of the rapid molecular tests currently available for detection of MRSA, but only a brief discussion is provided herein of the two commercial assays currently in use in the United States.[27,95,442,579] The BD GeneOhm MRSA assay (formerly IDI-MRSA assay) (BD GeneOhm, San Diego, CA) was the first commercial real-time PCR assay to be approved by the US FDA for detection of MRSA from nasal surveillance swabs. This multiplex assay uses molecular beacons directed toward multiple targets within SCC*mec* and *orfX* genes to simultaneously identify and detect resistance and differentiate

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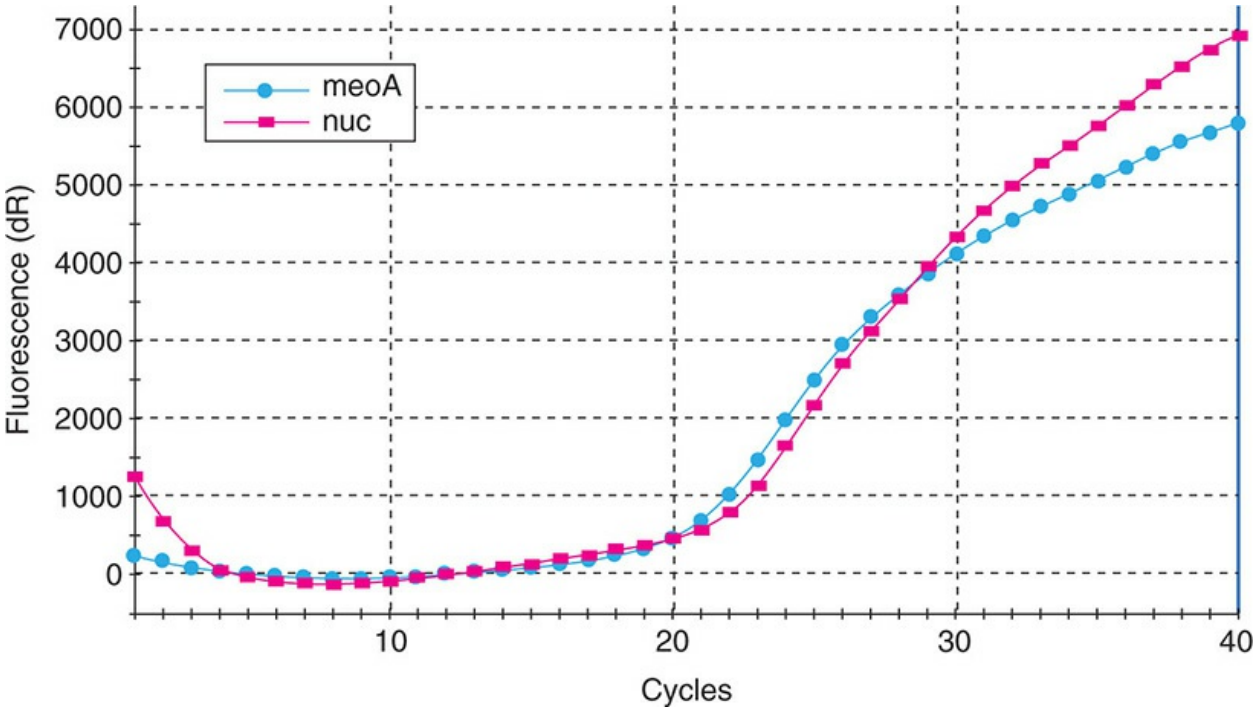
methicillin-sensitive *S. aureus* from MRSA isolate and *mec*A carrying strains of coagulase-negative staphylococci.[95] The BD GeneOhm assay was initially evaluated by Huletsky and colleagues at IDI,[267] and many subsequent clinical evaluations have shown similar results. The overall ranges for reported sensitivity, specificity, PPV, and NPV were 84.3% to 100%, 93.5% to 99%, 61.1% to 94%, and 97% to 99.7%, respectively.[95,144,186,363,449,568] The high false-positivity rate for this test (i.e., up to 5%) occurs because some *mec*A-negative *S. aureus* continues to carry a fragment of the SCC*mec* element near the insertion site.[95,554] However, this assay has shown reliable detection of a variety of SCC*mec* genotypes in a large number of well-characterized strains from the United States and Taiwan.[59]



■ **FIGURE 17-27** Real-time PCR to detect *mec*A gene in *S. aureus*. (Courtesy of Calgary Laboratory Services.)

The second test to receive US FDA approval was the GeneXpert MRSA assay (Cepheid Diagnostics, Inc., Sunnyvale, CA).[27] This assay also targets SCC*mec* insertion site. The overall ranges for assay performance published by the company for sensitivity, specificity, PPV, and NPV were 86.3% to 96.5%, 90.4% to 94.9%, 80.5% to 90.4%, and 96.6% to 99.7%, respectively.[95] Although some recent clinical evaluations of the GeneXpert assay report even better performance,[13,259,337] studies found a high false-negative rate (7.7% to 12.9%), again due to *S. aureus* isolates that harbor a SCC*mec* element that is *mec*A-negative.[12,554] The GeneXpert MRSA assay is easy to perform on a rapid, fully automated random-access platform that requires minimal specimen processing and provides results in 1.5 to 2 hours. Brenwald and colleagues found that the GeneXpert MRSA assay had a similar performance when performed as a point of care test on the ward compared to the laboratory, but results were available>10 hours quicker at the bedside.[67]

Because of the lack of homology between the nucleotide sequence of the *mec*C gene compared to that of *mec*A, *S. aureus* strains carrying a *mec*C determinant may be difficult for diagnostic laboratories to detect using *mec*A-based in-house or commercial PCR assays. Latex agglutination assay that detects PBP2a will also not detect *S. aureus* strains that have a *mec*C gene because of their altered PBP structure. Skov and colleagues, however, demonstrated that cefoxitin used with EUCAST methodology

reliably detected *mec*C-positive *S. aureus* isolates.[559] Newer version of the commercial real-time PCR MRSA detection assays will need to use primers designed to detect both the *mec*A and *mec*C genes as this type of resistance may be more widespread than currently documented. One such assay has been released on the BD Max system (BD, Sparks, MD).

Reporting. There is clinical evidence that isolates of *S. aureus* that are resistant to oxacillin do not respond in vivo to cephalosporin therapy; the same approach has recently been suggested for *S. epidermidis*. As of 2013,[121] CLSI has eliminated breakpoints for all β-lactam drugs for *Staphylococcus* spp. except for penicillin, oxacillin/cefoxitin, and has added ceftaroline, a cephem with anti-MRSA activity that should be selectively tested and reported for MRSA isolates. This decision was based on the fact that breakpoints had never been extensively evaluated for many β-lactam drugs for *Staphylococcus* spp., and the results of penicillin and oxacillin can be used to predict susceptibility to penicillinase-labile penicillin drugs and penicillinase-stable penicillins respectively. Table 17-21 outlines the current CLSI recommendations for reporting β-lactam results for *Staphylococcus* spp. based on testing penicillin and oxacillin/cefoxitin alone. Laboratories should also have protocols in place for performing extended susceptibility testing on other antibiotics for multidrug-resistant isolates. This protocol should include criteria for referral of multidrug-resistant isolates to a reference laboratory for confirmatory susceptibility testing and molecular genotyping. Infection Prevention and Control and/or public health agencies should be immediately notified of patients who are suspected or confirmed to have MRSA detected either from surveillance or clinical samples.

TABLE 17-21 Prediction of Susceptibility of *S. aureus* to β-Lactams Based on Penicillin/Oxacillin Test Results

Penicillin	Oxacillin (Cefoxitin)	Predicts
S	S	*Susceptible to:* • All penicillins • β-Lactam/β-lactamase inhibitor combinations • Cephems • Carbapenems
R	S	*Resistant to:* • Penicillinase-labile penicillins *Susceptible to:* • Penicillinase-stable penicillins • β-Lactam/β-lactamase inhibitor combinations

		• Cephems • Carbapenems
R	R	*Resistant to:* • All β-lactams except cephems with anti-MRSA activity (e.g., ceftaroline)

Adapted from reference 122.

Decreased Vancomycin Susceptibility in Staphylococci. Increased use of vancomycin has most likely resulted in the rapidly evolving problem of decreased susceptibility of staphylococci to this agent. Currently, the incidence of decreased vancomycin susceptibility in *S. aureus* remains low in some countries such as the United States (<1%),[556] and nonexistent in others.[393] Howden and colleagues have recently published an excellent review on this topic.[264]Although this problem has been found in *Staphylococcus* spp. other than *S. aureus*, the emergence of VISA, and more recently VRSA are of utmost concern because of the potential for nosocomial transmission of these strains, and increased mortality would undoubtedly result.[97,556,665] Invasive infections due to *S. aureus* are common, and few drugs are currently available to effectively treat infection due to VISA/VRSA/VRSA.[325,556,660] Glycopeptide-intermediate (GISA) or –resistance (GRSA) are also terms used for these types of *S. aureus* because these strains also exhibit resistance to other glycopeptide antibiotics such as teicoplanin.[605]

MRSA with decreased vancomycin susceptibility was first reported by Hiramatsu and colleagues in 1997.[254] The resistance mechanism heterogeneously expressed on initial

testing by broth microdilution gave an MIC that was susceptible, but a subgroup of cells tested intermediate according to CLSI criteria with an MIC of 4 to 8 mg/L. Similar to oxacillin resistance in staphylococci, decreased susceptibility to vancomycin may be expressed homogeneously or strains may be heteroresistant (i.e., designated as hVISA/VRSA).[264] VRSA strains in contrast that have acquired a *van*A gene from enterococci continuously express high-level resistance (i.e., MICs ≥ 32 μg/mL).[612]

Phenotypic Detection. Detection of hVISA/VRSA strains has been especially problematic, and an ideal method that is highly sensitive and easy to perform has yet to be developed. Table 17-22 summarizes the performance of methods that have been

used to detect hVISA/VRSA. The reference method for hVISA/VRSA detection is the population analysis profile—area under the curve calculation (PAP-AUC), which involves population analysis to determine the percentage of bacteria is resistant at each of a series of MIC levels.[264] A standard inoculum of an overnight broth culture is plated onto brain heart infusion (BHI) agar plates containing increasing concentrations of vancomycin from 0.5 to 4 μg/mL. Plate counts are graphed to calculate the proportion of cells that are vancomycin-resistant at each dilution. Wootton and colleagues showed that a PAP–AUC concentration ratio between 0.9 and 1.3 is indicative of hVISA/VRSA while a ratio <0.9 is susceptible.[669] The PAP–AUC method even in its more efficient forms is too labor intensive to be routinely performed in a clinical laboratory on a large number of isolates.

Broth microdilution remains the recommended clinical method for detection of hVISA/VRSA. CLSI lowered its breakpoint in 2007 for the vancomycin-intermediate range to 4 to 8 μg/mL because of vancomycin failure to treat serious hVISA/VRSA infections caused by strains with an MIC = 4 μg/mL.[121,122] *S. aur*eus that have an MIC ≤ 4 μg/mL are considered susceptible while those with an MIC ≥ 16 μg/mL are resistant. EUCAST does not define an intermediate range for *S. aureus* but the vancomycin resistance breakpoint was decreased in 2009 to an MIC > 2 μg/mL which is at the upper range for the MIC distribution for wild-type strains (www.eucast.org/clinical-breakpoints). *S. aureus* isolates that have an elevated vancomycin MIC (i.e., ≥8 μg/mL) should be sent to a reference laboratory for confirmatory testing. Laboratories that do not routinely perform broth microdilution as their main susceptibility method may elect to use this method to confirm hVISA/VRSA in clinical isolates with an elevated vancomycin MIC result using another method as outlined below. Alternatively, some laboratories perform broth microdilution on all invasive *S. aureus* but use another susceptibility method to test noninvasive isolates (i.e., superficial wounds).

TABLE 17-22 Ability of Different Methods to Detect Vancomycin Susceptibility in *S. aureus*

Vancomycin MIC (mg/L)	MIC Method	Disk Diffusion Method[a]	Vancomycin Agar Screen
≤2 (S)	Yes	No	Yes
4 (I)	Yes	No	Variable
8 (I)	Yes	No	Yes
16 (R)	Yes	No	Yes
≥32 (R)	Yes	Yes	Yes

[a] Isolates that have a zone diameter of ≥7 mm should be retested using an MIC method.
Adapted from reference 122.

Although coagulase-negative staphylococci have been reported with decreased susceptibility to vancomycin, so far no *van*A gene positive strains have been found. Broth microdilution vancomycin breakpoints for coagulase-negative staphylococci remain higher than *S. aureus*; susceptible is defined as an MIC ≤ 4 μg/mL, intermediate is defined as an MIC of 8 to 16 μg/mL, and resistant is defined as an MIC ≥ 32 μg/mL.[121,122] Coagulase-negative staphylococcal isolates with an elevated vancomycin MIC ≥ 32 μg/mL should be sent to a reference laboratory for further confirmatory testing.

Other screening methods that have been used to detect hVISA/VRSA include a BHI agar screening plate containing either 5 or 6 μg/mL of vancomycin similar to the original method used by Hiramatsu,[254,273] and also currently used to screen for vancomycin resistance in enterococci as discussed below. A 10-μL drop on a 0.5 MacFarland isolate suspension is used to spot a 10 to 15 mm diameter of a BHI plate. More than one colony or light growth on the plate after 24 hours of incubation is considered positive and presumptive evidence for the presence of VISA/VRSA or VRSA. However, confirmatory testing should be done using an MIC method, either broth microdilution or an Etest.

Vancomycin Etest strips have been used alone or in combination with a teicoplanin strip (i.e., macro Etest or MET).[393,517] However, Etest glycopeptides resistance detection (GRD) strips have recently been developed to detect hVISA/VRSA. Etest GRD strips have had limited clinical evaluation to date, but a recent comparison of

Etest GRD and the curve (PAP–AUC) analysis showed less than optimal performance.[517] Etest methods are known to produce higher vancomycin MIC results than broth microdilution and so "false resistance" may be reported using these methods if confirmatory tests are not done. *S. aureus* isolates with an elevated Etest MIC ≥ 8 μg/mL should be referred to a reference laboratory for confirmatory testing (i.e., CDC in the United States).

Disk diffusion tests are unreliable and will not distinguish VISA/VRSA from wild-type strains and should not be used. For this reason, CLSI has recently eliminated vancomycin disk screening criteria for VISA/VRSA.[122] Laboratories using automated susceptibility systems will also miss detecting hVISA/VRSA unless additional confirmatory testing is done on *S. aureus* strains with elevated vancomycin MIC ≥ 2 mg/L.[587] Similarly, *S. aureus* strains with vancomycin MICs between 1.5 and 2 μg/mL according to the MET Etest method should have a broth microdilution test performed.

Reporting. Isolation of hVISA/VRSA is considered to be an urgent clinical, infection control and public health issue. Because hVISA/VRSA isolates are often multidrug-resistant, extended susceptibility testing for alternate therapeutic drugs should be routinely performed and reported on invasive strains including linezolid, daptomycin, fluoroquinolones, rifampin, tigecycline, and ceftaroline. Laboratories

should have protocols in place for performing extended susceptibility testing on other antibiotics for multidrug-resistant isolates, or immediately refer the isolate to a reference laboratory where such testing can be done. Criteria for referral of multidrug-resistant isolates to a reference laboratory for confirmatory susceptibility testing and molecular genotyping should be included in the laboratory protocol. Infection Prevention and Control and/or the public health agencies should be immediately notified of patients who are suspected or confirmed to have hVISA/VRSA detected either from surveillance or clinical samples.

Inducible Clindamycin Resistance. Resistance to macrolides among staphylococci is mediated either by active efflux of antibiotics from the bacterial cells (***msrA***) or by modification of ribosomal targets (usually *ermA* or *ermC*).[344]Ribosomal modification may be expressed constitutively or it may be inducible.[135] Resistance to erythromycin is more easily detected than resistance to clindamycin. Strains that are susceptible to erythromycin are reliably susceptible to clindamycin, but the opposite is not necessarily true.

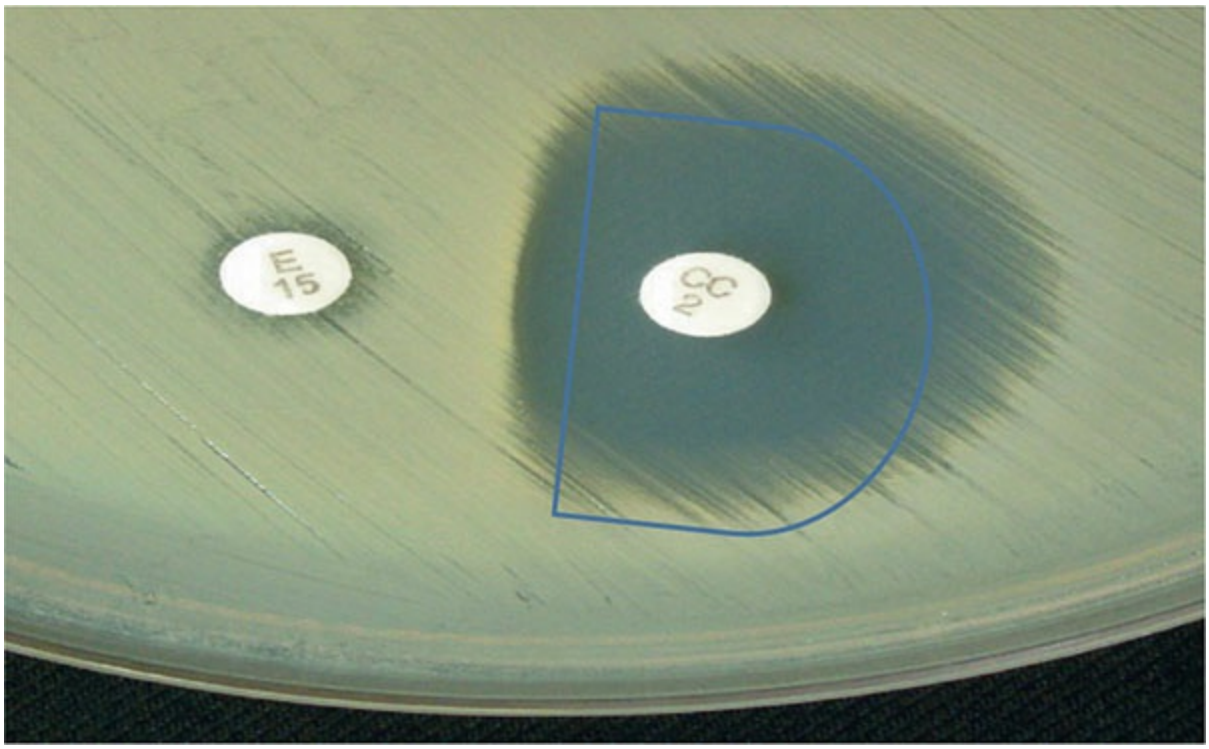
Strains of staphylococci that express constitutive resistance to erythromycin and clindamycin present little difficulty, because they will be detected as resistant in vitro. When resistance is inducible, however, there may be a dissociation occurring between

the erythromycin and clindamycin susceptibility results (Table 17-23). Resistance to 14-membered and 15-membered macrolides, such as erythromycin, is more readily induced than is resistance to 16-membered macrolides and lincosamides, such as clindamycin. There is evidence that inducible resistance to clindamycin is clinically significant in not only staphylococci, but also β-hemolytic streptococci and *S. pneumoniae*.[300,341,390] CLSI recommends that an induction test be performed before reporting clindamycin as susceptible in *S. aureus* isolates that demonstrate discrepant susceptibility to erythromycin and clindamycin (i.e., erythromycin is resistant but clindamycin is susceptible).[121,122] For other staphylococci, β-hemolytic streptococci and *S. pneumoniae*, a clindamycin induction test should be done if requested.[121,122] The D-zone double-disk test for inducible resistance to clindamycin is based on the principle that erythromycin is a potent inducer of the *erm* gene (i.e., MLS_B type).[192,295] If disks containing erythromycin (15 µg) and clindamycin (2 µg) are placed in proximity (i.e., 15 to 26 mm) and resistance is induced by erythromycin, the zone of inhibition around the clindamycin disk will be distorted. The resultant flattening of the zone of inhibition produces a D-shape and is indicative of inducible resistance to clindamycin (Fig. 17-28). Alternatively, a broth microdilution test where 1 µg/mL of erythromycin and 0.5 µg/mL of clindamycin are placed in the same well can be used to detect inducible clindamycin resistance in β-hemolytic streptococci and *S. pneumoniae*.[55,121,122] This pattern indicates inducible resistance, and clindamycin should then be reported as resistant.

TABLE 17-23 Phenotypic Macrolide/Lincosamide Resistance Profiles

Mechanism	Resistance Gene	Erythromycin	Clindamycin
Efflux	*msrA*	R	S D-ZONE Test (–)
Ribosome alteration	*ermA* or *ermC*	R	S→R (inducible) D-ZONE Test (+)
Ribosome alteration	*ermA* or *ermC*	R	R (constitutive) D-ZONE Test (–)

*msr*A, macrolide streptogramin (type B) resistance; *erm*, erythromycin ribosome methylase (MLS_B) resistance.



■ **FIGURE 17-28** D-zone test for Clindamycin inducibility. (Courtesy of Calgary Laboratory Services.)

Reporting. Isolates that have a positive D-zone test, or those that show induction in the broth microdilution test should be reported as resistant to clindamycin. A comment should be added to the report stating "This isolate is presumed to be clindamycin resistant based on detection of inducible clindamycin resistance.[121,122] In the absence of inducible resistance (i.e., negative D-zone test), the isolate may be reported as clindamycin susceptible based on the in vitro susceptibility result. Laboratories should also have protocols in place for performing extended susceptibility testing on other antibiotics for multidrug-resistant isolates. This protocol should include criteria for referral of multidrug-resistant isolates to a reference laboratory for confirmatory susceptibility testing and molecular genotyping. Infection Prevention and Control and/or public health agencies should be immediately notified of patients who are suspected or confirmed to have MRSA detected either from surveillance or clinical samples.

Enterococci

The mainstay of antimicrobial therapy of serious invasive enterococcal infections for decades has been combined therapy with a cell wall agent, either a β-lactam or

vancomycin and an aminoglycoside agent.[17,520] Although enterococci are often tolerant to the actions of β-lactams and are inherently resistant to aminoglycosides (i.e., MICs range of 8 to 256 μg/mL), reliable synergistic bactericidal activity results from combination therapy because the cell wall agent allows the aminoglycoside to be taken up into the cell where it acts on its ribosomal target. However, this approach has been

seriously undermined in the last 50 years, initially by the development of resistance to penicillin but more recently due to the increasing incidence of VRE,[98] and the development of high-level aminoglycoside resistance so that synergy to kill enterococci can no longer be readily achieved.[17] The subject has been recently reviewed by several authors.[216,258,657] More recently, resistance has also been detected in newer agents including linezolid and daptomycin dubbed the "antibiotics of last resort," especially in strains of *E. faecium*.[15,18,197]

Several other factors have coalesced to bring about the current situation of having to routinely treat serious multidrug-resistant enterococcal infections in hospitals worldwide.[17,15,250,658] Enterococci were mostly *E. faecalis* in US hospitals in the late 1970s.*E. faecium* has since become the predominant *Enterococcus* spp. isolated in hospitalized patients, and is now second only to *S. aureus* as a cause of nosocomial infection.[17] *E. faecium* is much more commonly resistant to ampicillin and vancomycin than *E. faecalis*.[250] This "enterococcal species displacement" has primarily occurred because of the increased use of vancomycin and other broad-spectrum antibiotics such as the third-generation cephalosporins, coupled with suboptimal infection control measures.[17,198] There is also evidence for transmission of multidrug-resistant *Enterococcus* strains from humans, animals, and the environment.[142,166,657]

β-Lactam Resistance

Many strains of enterococci, particularly *Enterococcus faecium*, are intrinsically resistant to β-lactam antibiotics because they possess binding proteins with low affinity for these drugs. In general, ampicillin is more effective than penicillin in vitro and is the preferred agent for susceptible enterococci. The cephalosporins are uniformly ineffective against enterococci and should not be tested. The detection of β-lactamase production in *Enterococcus* spp. is outlined above under β-Lactamase Testing.

High-Level Aminoglycoside Resistance

Acquired high-level aminoglycoside resistance (HLR) results in MICs in enterococci that are well above the normal concentration tested in the clinical laboratory (e.g., ≥2,000 μg/mL for streptomycin and ≥500 μg/mL for gentamicin). Detection of HLR of enterococci to aminoglycosides is important because these strains are not inhibited synergistically by combination therapy with β-lactam antibiotics.[404] Although labor-intensive time-kill studies were used to predict synergy between an aminoglycoside and a cell wall agent, several screening methods are now used in the clinical laboratory. *E. faecium,* however, is inherently resistant to synergy between tobramycin, netilmicin, kanamycin, or amikacin and a cell wall agent, and in vitro testing need not be done. Synergy testing is only done to detect HLR for either gentamicin and/or streptomycin in *E. faecium* or *E. faecalis*.[408] Commonly used methods for detection of HLR in enterococci are briefly described below.

Many clinical laboratories use an automated broth microdilution system to screen for HLR. Concentrations of 1,000 mg/L of streptomycin and 500 mg/L of gentamicin are used as recommended breakpoints.[121,122,590] Alternatively, a broth microdilution panel (Pasco, MicroScan) using BHI media supplemented with similar antibiotic concentrations in two aminoglycoside synergy wells may be used.[595] The same standard inoculum is used as a routine gram-positive MIC panel (i.e., 5×10^5 CFU/mL). After a minimum of 24 hours' incubation, any growth is considered resistant. Plates may have to be reincubated for an additional 24 hours if there is no growth for streptomycin after the initial incubation period.

Agar dilution plates are also made using BHI media supplemented with the same amount of gentamicin (500 μg) but twice the amount of streptomycin (2,000 μg) as used for HLR microdilution tests.[117,121,122] Agar dilution plates for HLR testing can be purchased from a commercial supplier. A 0.5 MacFarland isolate suspension is prepared and 10 μl is spotted to provide a final inoculum of 10^6 CFU per spot. Plates should be incubated a full 24 hours in ambient air before reading, and streptomycin plates should be reincubated for another 24 hours if there is no growth after the initial incubation. Growth of a single colony on agar dilution plates indicates resistance. Standard disk diffusion testing is performed using antibiotic disks with high concentrations of gentamicin (120 μg) or streptomycin (300 μg).[408] Resistance is defined by a zone diameter ≤6 mm (no zone), and susceptibility by a zone of <10 mm. Strains with zone diameters of 7 to 9 mm should be tested by agar dilution or microdilution methods because a few HLR enterococcal strains have only moderately elevated MICs.[408] Several comparative method evaluation of HLR testing in *Enterococcus* spp. found virtually complete agreement among high-potency disks, commercially available microdilution panels, and a commercial agar dilution "synergy" plate.[408,590,595]

Decreased Susceptibility to Vancomycin

Dilution susceptibility methods may be used to reliably detect VRE (*van*A or *van*B phenotypes) or intrinsic low-level vancomycin resistance (MICs 2 to 32 µg/mL) associated with *E. gallinarum* or *E. casseliflavus* (*van*C phenotype). *E. faecium* or *E. faecalis* that carry a *van*A gene typically have a high vancomycin MIC ≥ 64 µg/mL and teicoplanin MIC ≥ 16 µg/mL, while strains that carry a *van*B gene have a lower vancomycin MICs between 16 to 512 µg/mL, and remain susceptible to teicoplanin (Table 17-24). Historically, many laboratories used the vancomycin agar dilution screen

test to detect VRE.[588] Agar plates contain BHI media supplemented with 6 µg of vancomycin per mL. A 0.5 MacFarland isolate suspension is used to spot a 10 to 15 mm diameter area on the plate providing a final inoculum of 10^5 to 10^6 per spot. The presence of more than one colony or a haze of growth indicates resistance. BHI vancomycin screen plates may also grow *Enterococcus* spp. that carries a *van*C gene but these can be easily identified by phenotypic methods. *E. casseliflavus* typically grow as yellow pigmented colonies, both *E. casseliflavus* and *E. gallinarum* ferment 1% methyl-α-D-glucopyranoside (MGP), and most strains are motile. Commercial vancomycin agar dilution screening plates that contain 6 µg/mL of drug may be purchased from several suppliers.

TABLE 17-24 CLSI Breakpoints (MIC µg/mL) for *Enterococcus* spp.

Antibiotic	Susceptible	Intermediate	Resistant
Penicillin or ampicillin	≤8	—	≥16
Vancomycin	≤4	8–16[a]	≥32[b]
Teicoplanin	≤8	16	≥32[b]

[a] VRE with decreased susceptibility—VanB genotype if teicoplanin (S).
[b] VRE with high-level resistance—VanA genotype if teicoplanin (R).
S, susceptible; I, intermediate; R, resistant.
Adapted from reference 122.

Automated systems may also be used to detect glycopeptides resistance in enterococci, but laboratories should be aware of reported detection problems.[277,636] Recent versions of all of the automated microdilution systems (i.e., Vitek 2, BD Phoenix, and MicroScan) have improved their ability to detect VRE, including low-level intrinsic resistance, but that was not always the case.[176,572] The Vitek Legacy and MicroScan systems initially had problems detecting moderate (*van*B) and low-level (*van*C) resistance in enterococci. Tenover and colleagues evaluated 10 commercial systems for detection of VRE using a broth microdilution method as the standard for testing 50 reference strains.[610] Although an agar dilution screening test accurately detected VRE, increased numbers of minor errors occurred with the disk diffusion test, very major errors were produced by the Vitek Legacy system (10.3%) and the MicroScan Rapid system (20.7%), and the later system also produced some major errors (13.3%). Recent studies also show that automated systems may have problems detecting specific VRE strains. Abele-Horn and colleagues recently performed a clinical validation of Vitek 2 version 4.01 software and found that the system was able to identify 114(94.2%) of enterococcal strains correctly to the species level and classified 119(98.3%) correctly to the glycopeptides resistance genotype level (i.e., VanA, VanB, or VanC genotypes).[3] Six *E. faecium* strains carrying a *van*A gene, and one *E. casseliflavus* strain (i.e., *van*C gene) were identified with low discrimination and required additional testing. One VanA strain was misclassified as the VanB type because the system did not detect resistance to teicoplanin, and one glycopeptide-susceptible *E. faecium* was misclassified as the VanA type (i.e., resistant to both vancomycin and teicoplanin). Raponi and colleagues also recently compared the ability of Vitek 2, Phoenix, Etest, broth microdilution, and disk diffusion tests to detect glycopeptides and linezolid resistance in 30 clinical isolates of *E. faecium*.[505] All of the strains had undergone molecular testing to detect the presence of the *van*A and *van*B resistance genes and the 23S RNA G2576T mutation conferring resistance to linezolid. Resistance rates to teicoplanin ranged from 3% for Vitek 2 to 57.6% for the Phoenix test, and those to vancomycin ranged from 56.7% for Vitek 2 to 86.7% for the Phoenix test. Only 2/25 strains known to carry the *van*A gene were definitively recognized as the VanA phenotype. Only one strain had the G2576T mutation and did not carry the *van*A gene; disk diffusion, broth microdilution and the Vitek 2 system showed resistance (MIC > 8 μg/mL) but both Phoenix and Etest gave susceptible results (MIC ≤ 4 μg/mL). These studies highlight that more evaluations need to be done of automated dilution susceptibility system against a broader range of molecularly characterized VRE strains.

Disk diffusion tests are not as reliable as dilution assays, and should be performed strictly according to standard methods to minimize errors.[118] Disk diffusion tests should be incubated for a full 24 hours and read under transmitted light. VRE strains carrying a *van*B gene may be missed unless the inhibition zone is inspected carefully

for a diffuse edge. Performance of Etest strips has been shown by some studies to be equivalent to agar dilution and broth microdilution testing.[610] However, in a recent study, Etest failed to detect four *E. faecium* isolates that had been confirmed to carry the *van*B gene.[506] Grabsch and colleagues also found that a vancomycin Etest performed on either standard MHA or BHI agar repeatedly failed to detect a *van*B2-containing *E. faecium* (MIC ≤ 4 μg/mL) during a nosocomial outbreak.[223] Etest results improved when either MHA or BHI agar was supplemented with 1 g/L oxgall and plates were incubated a full 48 hours before reading. More studies are needed to evaluate this media to improve early detection of VRE strains with a low-level resistance phenotype in laboratories where *van*B detection by PCR is not possible.

Reporting. The results of penicillin and/or ampicillin, vancomycin and the screen for HLR to gentamicin and streptomycin should be reported for all *E. faecium* and *E. faecalis* isolates recovered from serious invasive infections. Laboratories should also have protocols in place for performing extended susceptibility testing on other antibiotics (i.e., daptomycin, linezolid) for invasive multidrug-resistant isolates. This protocol should include criteria for referral of multidrug-resistant isolates to a reference laboratory for confirmatory susceptibility testing and molecular genotyping. Infection Prevention and Control and/or public health agencies should be immediately notified of patients who are suspected or confirmed to have VRE detected either from surveillance or clinical samples.

Molecular Detection of Vancomycin Resistance Genes. Molecular testing of enterococci is required to detect a vancomycin gene, and to distinguish the type of gene(s) that are present. Aside from the more common vancomycin resistance genotypes discussed above (i.e., *van*A, *van*B and *van*C), several other vancomycin gene clusters have been reported (i.e., *van*D, *van*E, *van*G, *van*L, and *van*M).[2,58,154,672] The latter genes have been rarely described in clinical isolates, and discovered when routine phenotyping identified the strain as vancomycin-resistant, but molecular testing showed that neither *van*A nor *van*B gene clusters were present. Newer vancomycin gene clusters will likely continue to be found as other studies have documented a high prevalence of glycopeptides resistance genes (i.e., *van*B, *van*D, and *van*G) in human fecal flora that are not associated with enterococci.[164] In a subsequent study, these investigators identified *van*D and *van*G-like gene clusters in a *Ruminococcus* **spp**.[165] Clinical isolates of *Clostridium clostridioforme* and *Atopobium minutum* carrying a *van*B cluster have also been recently found.[376] Other gram-positive bacteria in the human bowel flora, particularly anaerobes, may represent a large pool of vancomycin resistance genes.

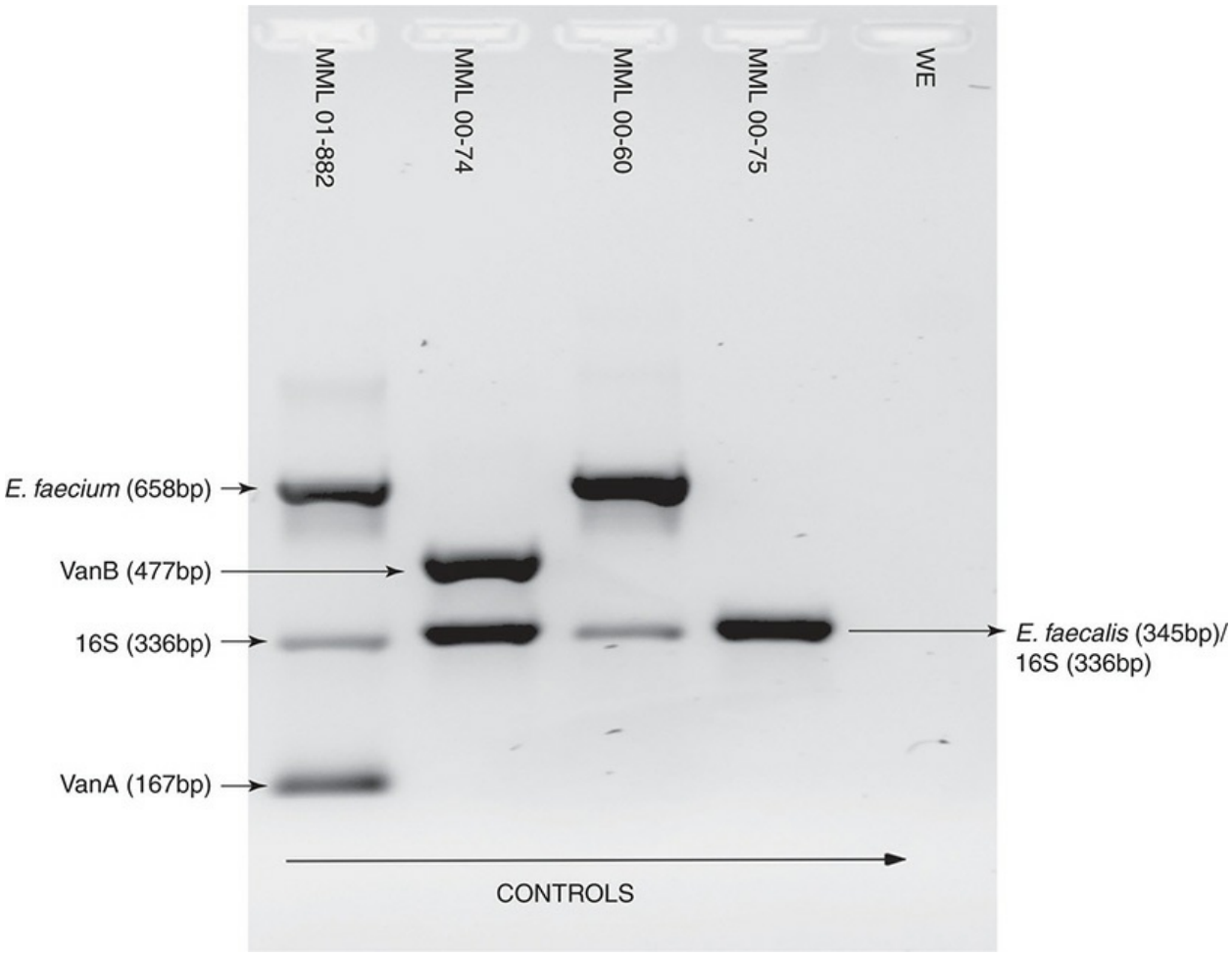
Several laboratory developed multiplex methods using PCR and real-time PCR have been developed to primarily detect the *van*A and *van*B gene clusters that are of utmost clinical importance (Fig. 17-29).[179,443,461] Molecular detection of the *van*C1,*van*C2, and *van*C3 genes can also definitively identify *E. gallinarum* as well as to distinguish *E. casseliflavus* from *E. flavescens*.[111] Specific molecular assays can also be done to detect other vancomycin genes.

Recent commercial development of rapid test platforms has, however, made real-time PCR detection of the *van*A/B genes directly from clinical specimens (i.e., rectal swabs and stool specimens) available to most facilities. Initial clinical evaluations of the two assays that have received FDA approval in the United States are briefly described herein. The BD GeneOhm VanR assay (BD GeneOhm, San Diego, CA) is a real-time PCR assay that amplifies specific targets of the *van*A and/or *van*B genes. The VanR assay is performed using the SmartCycler (Cepheid, Sunnyvale, CA). Stamper and colleagues compared the performance of the VanR assay with selective culture using bile esculin azide agar with 6 μg/mL vancomycin (BEAV), and BEAV broth with 8 μg/mL of vancomycin for detection of VRE from rectal swabs and stool specimens.[569] Overall, the VanR assay had a sensitivity of 96.6% and specificity of 87%, and several false-positive tests occurred with the *van*B portion of the assay. Although the sensitivity of the assay for rectal swabs (98.3%) was higher than stool specimens (95.4%), the specificity was similar for both specimen types. When used only to detect *van*A resistance, the VanR assay had sensitivity, specificity, PPV, and NPV of 94.4%, 96.4%, 91.3%, and 97.7%, respectively. Similar performance for the VanR assay was also reported recently in a study that tested 1,786 samples including a set of 50 reference strains known to be of the *van*A-G genotypes.[659] Although the overall sensitivity was 93.1%, the lower specificity (87%) was mainly due to false-positive *van*B results.

The GeneXpert *van*A/*van*B assay (Cepheid Diagnostics, Inc., Sunnyvale, CA) is also a real-time PCR assay that detects both the *van*A and *van*B genes. An evaluation of this test on 804 rectal swab specimens compared to enriched culture[54] demonstrated that although the molecular assay had a high sensitivity and NPV (100%), it had a lower specificity (85.4%) and PPV (8.7%) because of false-positive tests, especially for the *van*B gene. Another more recent evaluation compared direct and broth-enriched culture methods for detection of VRE to the *van*A/*van*B assay, and reported sensitivity, specificity, PPV, and NPV of 96.4%, 93%, 92%, and 96.9%, respectively.[375] The GeneXpert assay for VRE is easy to perform on a rapid, fully automated random-access platform that requires minimal specimen processing and provides results in 1.5 to 2 hours.

Laboratories that do not have the ability to perform molecular testing may consider implementing a chromogenic agar-based culture method to detect VRE directly from

rectal swabs or stool specimens (Fig. 17-30). Two chromogenic media (Chromagar VRE and chromID VRE) were compared for the direct detection of VRE from stool specimens after an overnight enrichment step and 48 hours of incubation of the plates.[456] Both of these media had a high sensitivity (98.2%) and comparable specificity of 96.5% and 97.5%, respectively. BBL CHROMagar VanRE (CVRE) (BD Diagnostic Systems, Baltimore, MD) was also recently compared with BEAV plates to screen for VRE colonization, and had sensitivity, specificity, PPV, and NPC of 98.6%, 99.1%, 95.9%, and 99.7%, respectively.[306] CVRE agar also showed improved performance for detecting VRE in a study of 517 surveillance swabs when compared to BEAV plates, and had sensitivity, specificity, PPV, and NPV of 99.1%, 94.8%, 84.2%, and 99.7%, respectively.[570] Another chromagar formulation (Spectra VRE, Remel, Lenexa, KS) was also much more sensitive (98.2%) and specific (99.3%) compared to BEAV in testing rectal swabs.[460] Currently, only one study has compared PCR-based screening for VRE against a chromogenic agar-based culture method which tested a total of 8,815 rectal swab specimens.[548] VRE were isolated from 8.4% of the samples while PCR detected VRE from 8.9% of the samples. Overall, the PCR has sensitivity, specificity, PPV, and NPV of 98.2%, 99.6%, 95.7%, and 99.8%, respectively, but no *van*B-positive specimens were included in this study.



■ **FIGURE 17-29** Fast multiplex PCR for distinguishing *E. faecium*/*E. faecalis* and the presence of vancomycin-resistance genes (*van*A and *van*B). (Courtesy of Calgary Laboratory Services.)

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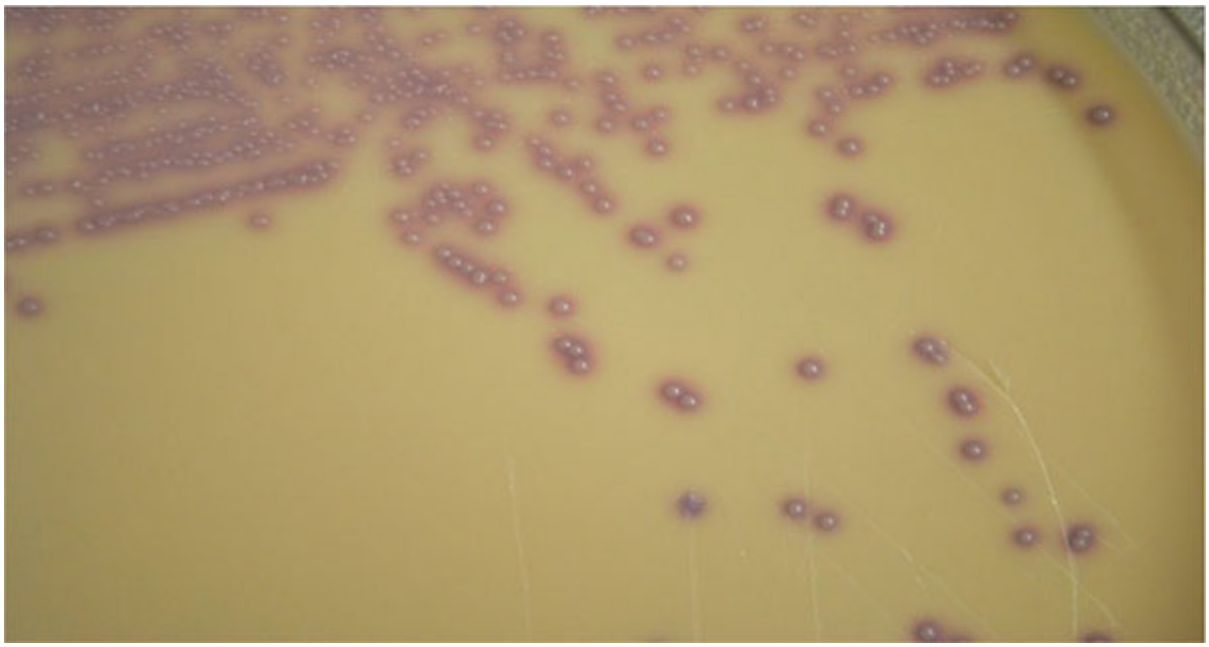

■ **FIGURE 17-30** *E. faecium*—*van*A gene positive growing on VRESelect (Bio-Rad). (Courtesy of Calgary Laboratory Services.)

Enterococcal Resistance to Newer Agents

Linezolid and daptomycin are newer agents that have increasingly been used to treat serious invasive infections due to MRSA and VRE. Laboratories should have protocol for extended testing of these antibiotics for isolates from serious invasive infections, or upon clinical request. If susceptibility of these drugs cannot be done in-house, the isolate should be immediately sent to a reference laboratory for confirmation and additional susceptibility testing. Although resistance to linezolid remains rare in both MRSA and VRE, laboratories should be prepared to recognize these strains because not surprisingly, an increased risk of acquiring a strain resistant to linezolid was associated with the use of the antibiotic and with the duration of therapy.[542] Linezolid breakpoints have been published for both disk diffusion (30 µg) and microdilution methods. However, when using a linezolid disk (30 µg) method, disk diffusion zones should be examined using transmitted light. If a clear zone is not found or the isolate is resistant, an MIC method should be used to perform repeat testing. Linezolid Etest strips also are in agreement with broth microdilution results for MRSA and VRE isolates, but the MIC results may be slightly higher.

A similar situation exists for daptomycin where few strains of either *S. aureus* or VRE have yet to be reported with resistance, but when they do arise, the patient has often been exposed to this agent, sometimes for a prolonged treatment period. Daptomycin is increasingly being used to treat serious invasive infections such as bacteremia and endocarditis due to MRSA or VRE provided the patient does not have

pneumonia because surfactant inactivates this compound. As recently reviewed, in vitro susceptibility testing of daptomycin requires the presence of standardized levels of total calcium (50 µg/mL, using calcium chloride salts).[320] Disk diffusion testing is unreliable for this reason and should not be used for daptomycin susceptibility testing for either *Staphylococcus* spp. or *Enterococcus* spp. Daptomycin Etest strips may be used along with calcium-supplemented MHA where the calcium concentration has been adjusted to the proper level. The in vitro activity of daptomycin in the Etest strip is dependent on the fixed amount of calcium (40 µg/mL) that is present in the strip, as well as the calcium in the agar. One of the largest evaluations of 1,800 *S. aureus* isolates including MRSA showed that the essential agreement of the Daptomycin Etest and microdilution testing was 82.4% but Etest MICS were 0.5 to 1 $\log_2$ dilution higher.[537] Daptomycin Etest MICs agreement rates have been even more variable (66.7% to 100%).[292,320]

Streptococci

Streptococcus pneumoniae

Decreased Susceptibility to Penicillin. Penicillin has been the standard treatment for pneumococcal infections for decades, and susceptibility testing was not indicated because strains remained susceptible. However, highly penicillin-resistant strains of *S. pneumoniae* have recently emerged, and increasingly, serious invasive infections are being reported due to multidrug-resistant clones that have been transmitted worldwide. Recent surveillance studies in the United States show that *S. pneumoniae* strains with multidrug resistance (i.e., resistant to two or more antimicrobial classes including β-lactams, macrolides, tetracycline, and trimethoprim/sulfamethoxazole, but rarely fluoroquinolones) are widespread; overall 20% of the isolates tested were multidrug-resistant, while the prevalence of isolates with intermediate-penicillin resistance (MIC 0.1 to 1 µg/mL) was 17.9% versus penicillin resistance (MIC ≥ 2 µg/mL) was 14.6%.[516]

Serotyping date for pneumococci causing invasive and noninvasive disease in 2008 to 2009 and 2010 to 2011 for more than 43 centers in the United States compared with data from preconjugate vaccine (PCV7) (1999 to 2000) and postconjugate vaccine (2004 to 2005) periods, show that the prevalence of PCV and pneumococcal serotypes decreased from 64% of invasive and 50% of noninvasive isolates in 1999 to 2000 to 3.8% and 4.2%, respectively, in 2004 to 2005.[516] Although the use of 7-valent pneumococcal conjugate vaccine (PVP7) dramatically decreased the incidence of invasive pneumococcal disease (IPD) in both children and adults, IPD continues to occur in individuals at the extremes of age (<2 years or ≥65 years), and those with

comorbidities and/or immunodeficiency because the vaccine only provides limited

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protection against the more than 90 *S. pneumoniae* serotypes known to cause human disease.[326,599] In addition, specific *S. pneumoniae* serotypes (i.e., 19A, 3, 6C, and 7F) have emerged in the United States and worldwide that may have heightened virulence and increased antimicrobial resistance not encompassed in the pneumococcal conjugate vaccine (PVP7) administered to children prior to 2010.[227,290,345,655] From 1998 to 2005, the incidence of pediatric IPD caused by 19A in the United States increased from 0.8 cases/100,000 population to 2.5 cases/100,000 population, which directly coincided with an increase of IPD caused by penicillin-resistant 19A strains (i.e., from 6.7% to 35%) belonging to a rapidly emerging international clone (clonal complex 320).[599] Since 2010, when 13-valent pneumococcal vaccine (PCV13) was introduced in the United States, the increase in serotype 19A ceased, and the prevalence of other predominant serotypes included in PCV13 (3, 6C, 7F) have also remained constant between 2008 to 2009 and 2010 to 2011.[599] Other factors that have been shown to enhance the selection of resistant pneumococcal strains include the spread of a few international clones, and the frequency with which oral antibiotics are used in the community.[349]

The mechanism of pneumococcal resistance to penicillin by the alteration of PBPs is described in the section entitled Mechanisms of Antimicrobial Resistance. *Streptococcus pneumoniae* does not produce β-lactamases. Penicillin resistance occurs with a variety of serogroups and among diverse strains that can be differentiated by molecular typing,[515] but relatively few clones are the dominant source of multiply resistant strains.[671] Pneumococci that are resistant to penicillin also have decreased susceptibility to other β-lactam antibiotics.[74] Some β-lactams (i.e., carbapenems, cefotaxime, ceftriaxone, and cefpirome) are more active in vitro than penicillin against penicillin-resistant pneumococci, while other drugs (i.e., ampicillin, cefdinir, cefuroxime, cefoperazone, azlocillin, mezlocillin, piperacillin, cephalothin, and cefamandole) demonstrated slightly less activity. Oxacillin, cefixime, ceftizoxime, cefetamet, cefaclor, ceftazidime, cefoxitin, cefonicid, and latamoxef show very poor activity against penicillin-resistant *S. pneumoniae* strains. The mechanism for resistance to these other β-lactam antibiotics is also an alteration in penicillin-binding proteins.[406] The results of penicillin susceptibility testing, however, cannot be used to predict the susceptibility of other β-lactam antibiotics, because strains with mosaic PBP genes can have low penicillin MICs (<1 μg/mL) and high cefotaxime MICs (2 to 32 μg/mL), or low penicillin MICs (1 to 2 μg/mL) but higher amoxicillin MICs (4 to 8 μg/mL).[163,385]

Resistance to penicillin and other classes of antimicrobial agents in *S. pneumoniae* has serious clinical consequences for adequately treating serious infections, including community-acquired pneumonia, empyema, meningitis, and other invasive infections.[290,599] Vancomycin must be prescribed for suspected or confirmed cases of IPD with meningitis due to high-level penicillin-resistant strains, and multidrug resistance in *S. pneumoniae* has also resulted in a dramatic increase in the use of levofloxacin for community-acquired pneumonia.

Phenotypic Detection of Penicillin Resistance. Disk diffusion remains the primary screening method used by most laboratories for detecting penicillin resistance in *S. pneumoniae*.[591] This method is highly sensitive for *S. pneumoniae* but it does not differentiate strains that have intermediate resistance from strains that are resistant based on determination of the MIC according to the source of the isolate and the formulation of penicillin being used (Table 17-25). The disk diffusion procedure recommended by the CLSI for detection of resistance to penicillin incorporates a 1-µg disk of oxacillin.[118,121,122] Swenson and colleagues found that oxacillin was a better predictor of penicillin susceptibility than was methicillin.[591] Strains that have a zone diameter ≤19 mm should be not be reported as nonsusceptible to penicillin before confirmation by an MIC method.[121,122] Dilution or gradient diffusion MIC testing must be routinely performed on invasive *S. pneumoniae* isolates from sterile fluids, tissues, and blood sources. Although CLSI has published an agar dilution method using MHA with 5% blood, this method produced lower MICs compared to broth microdilution in a recent evaluation.[686] Broth microdilution testing is considered to be the reference MIC method for testing *S. pneumoniae*. Several commercial systems produce microdilution panels for MIC testing of *S. pneumoniae* (e.g., PASCO Strep Plus, Trek Sensititre). Trek Sensititre panels have performed well with only minor errors for penicillin, ceftriaxone, and meropenem in recent evaluations against a bank of *S. pneumoniae* strains that consisted of a selection of strains that span the CLSI criteria.[686] Earlier evaluations also have shown that other manufacturers' microdilution panels give reliable results, except when testing trimethoprim/sulfamethoxazole.[226,398] The major advantage of this approach is that the MICs of an isolate against many drugs can be established.

Due to the fastidious nature of *S. pneumoniae*, isolates should be grown on MHA supplemented with 5% sheep blood to ensure adequate growth when performing Etest. Plates may also be incubated in CO_2 if necessary for growth of the strain. QC of these tests should include known resistant pneumococcal strains in addition to standard control organisms. Figure 17-19 shows the Etest results for penicillin for a highly resistant strain from CSF. Etest has been shown to be a reliable method overall for testing *S. pneumoniae* for penicillin and extended-spectrum cephalosporin resistance but low minor error rates with penicillin (9.5%) and cefotaxime (5.4%) may occur.[297]

Hashemi and colleagues have noted that the Etest produces higher MIC results than does the microdilution test,[242] but reports of false resistance have not subsequently appeared.

TABLE 17-25 CLSI Breakpoints (MIC μg/mL) for *S. pneumoniae*

Antibiotic	Source	S	I	R
Penicillin V	Meningitis	≤0.06	—	≥0.12
Penicillin V	Nonmeningitis	≤2	4	≥8
Penicillin (oral penicillin V)	—	≤0.06	0.12–1	≥2
Third-generation cephalosporin(s)	Meningitis	≤0.5	1	≥2
Third-generation cephalosporin(s)	Nonmeningitis	≤1	2	≥4

S, susceptible; I, intermediate; R, resistant.
Adapted from reference 122.

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CLSI changed the breakpoints in 2008 for interpretation and reporting of MICs of parenteral penicillin and other commonly used β-lactams for *S. pneumoniae* depending on whether the isolate is recovered from cerebrospinal fluid (i.e., meningitis) or other sources (i.e., nonmeningitis) (Table 17-25). Clinically, the MIC breakpoints for oral versus parenteral penicillin drug formulations also remain different. The rationale for this change was to not over-estimate resistance in *S. pneumoniae* when treating invasive localized infections (i.e., pneumonia, bacteremia) that had not disseminated to cause meningitis.[656] Use of a single set of breakpoints for all invasive *S. pneumoniae* isolates regardless of source (i.e., nonmeningitis and meningitis) was discouraging the effective use of penicillin in cases where the strain demonstrated intermediate but not high-level resistance. Although patients with relatively resistant strains of *S. pneumoniae* causing pneumonia have responded to penicillin treatment, meningitis may not be treated successfully because concentrations of antimicrobial agent are

lower in cerebrospinal fluid.[591] Strains that have an MIC > 4.0 µg/mL are unlikely to respond to penicillin therapy in any clinical situation. The predictable epidemiologic effects of establishing differential MIC breakpoints for parenteral penicillin has been to lower the incidence of penicillin resistance in nonmeningitis isolates of *S. pneumoniae*, but increase the incidence of penicillin resistance in meningitis isolates.[103,274]

Current CLSI guidelines also include new breakpoints based on MIC and PK/PD data for doxycycline, and revised breakpoints for tetracycline.[121,122] This drug is an option for treating community-acquired pneumonia, but some *S. pneumoniae* isolates may be resistant to tetracycline but susceptible to doxycycline.[371]

Decreased Susceptibility to Macrolides and Lincosamides. Resistance of *S. pneumoniae* to macrolides and clindamycin has been increasing for many years, primarily due to selection pressure from oral antimicrobial agent use.[287,290] In *S. pneumoniae* and other viridans streptococci macrolide resistance occurs due efflux out of the cell due to the *mefA* gene,[584] which is constitutively controlled giving a typical phenotype of erythromycin resistant but clindamycin susceptible. *S. pneumoniae* strains are also increasingly encountered that have inducible resistance to clindamycin (i.e., MLSB due to the presence of an *erm* gene).[192] CLSI now recommends that an inducible clindamycin resistance test (i.e., either a D-zone disk test or a broth microdilution test) be done on request for *S. pneumoniae* isolates that have an antibiogram profile of resistance to erythromycin but susceptibility to clindamycin.[121,122]

Reporting. For a nonmeningitis *S. pneumoniae* isolate, if the penicillin MIC is ≤0.06 µg/mL and the β-lactam drug of interest is listed in CLSI M100-S23 and M100-S24 Table 2G, then the laboratory can extrapolate the susceptible penicillin result to other β-lactam agents.[121,122] As for staphylococci, pneumococcal strains that exhibit inducible resistance to clindamycin should be reported as resistant; strains that lack both intrinsic and inducible resistance to clindamycin may be reported as susceptible. Resistance to other macrolides can be inferred from the erythromycin result.[121,122] Therapeutic failure has been reported when a macrolide was used to treat systemic *S. pneumoniae* infection due to a strain that demonstrated in vitro resistance.[279]Doxycycline may be routinely reported on lower respiratory *S. pneumoniae* isolates if the drug is on the test panel, and stakeholders agree it should be reported, or this drug may be selectively tested and reported at the request of the physician.

Other Viridans Group Streptococci

In addition to *S. pneumoniae,* many other viridans group streptococci (VGS) can

cause serious invasive infections, particularly bacteremia and endocarditis. Increased penicillin resistance in VGS was likely transferred from pneumococcal strains.[171]Reduced susceptibility to penicillin among clinical isolates of VGS range from 20% to around 40%.[232,288,679] Similar to pneumococci, VGS with decreased susceptibility to penicillin are usually more susceptible to third- and fourth-generation cephalosporins.[463] Penicillin resistance occurs due to changes in penicillin-binding proteins (i.e., PBP2b and PBP2x).[185] Alterations in PBPs have produced resistance to other β-lactam antibiotics among several of the species, including *S. mitis*, *S. sanguis,* and *S. oralis*.[185] Several recent studies of VGS bacteremia demonstrated the high levels of multidrug resistance (i.e., decreased susceptibility to clindamycin, tetracycline, erythromycin, and ceftriaxone) that are typical of strains with decreased susceptibility to penicillin.[182,232,288,679] In particular, *S. mitis* blood isolates from cancer patients that had a high degree of nonsusceptibility to penicillin (28%) were also resistant to fluoroquinolones and other classes of antibiotics.[171] Prior antibiotic exposure was a significant risk factor for the isolation of nonsusceptible VGS from bloodstream infections.[232,679] In a study of VGS recovered from the normal oropharynx, resistance to erythromycin, tetracycline, and quinupristin-dalfopristin was documented frequently; strains expressed resistance to levofloxacin and moxifloxacin much less commonly.[549] Vancomycin resistance has so far not been found in penicillin-resistant VGS, and remains the drug of choice for invasive infections due to penicillin-resistant strains.

Disk diffusion is unreliable for detection of resistance to penicillin or carbapenems and should not be used to test VGS. Disk diffusion can be used to test susceptibility to other β-lactams and antimicrobial classes.[118] Microdilution panels or Etest should be used to determine the penicillin MIC of invasive isolates of VGS recovered from sterile fluids, tissues and blood.[121,122] In vitro susceptibility results obtained with the Etest have correlated well with agar dilution for penicillin and the cephalosporins, but somewhat less well with some VGS species for vancomycin.[399] Laboratories that only perform disk diffusion testing should have policies and procedures in place for immediate referral of invasive VGS strains with either decreased susceptibility to penicillin or vancomycin to a reference laboratory for confirmatory MIC testing.

β-Hemolytic Streptococci

This section pertains to large colony-forming pyogenic strains of streptococci with group A (*S. pyogenes*) (GAS),

C, or G antigens and strain with group B (*S. agalactiae*) (GBS) antigen. Penicillin and ampicillin remain the drugs of choice for treatment ofβ-hemolytic streptococcal infections. Routine susceptibility testing of penicillin and other β-lactams approved for treatment of pyogenic streptococcal infections need not be performed because nonsusceptible isolates (i.e., penicillin MICs > 0.12 μg/mL and ampicillin MICs > 0.25 μg/mL) have not been reported for GAS and are remain extremely rare for the other β-hemolytic species.[121,122] Because of the life-threatening nature of invasive infections due to β-hemolytic streptococci, however, laboratories should routinely perform antibiotic susceptibility testing on invasive isolates recovered from sterile fluids, tissues, and blood. β-hemolytic streptococci isolates found to have nonsusceptible to β-lactams should be reidentified, retested, and if confirmed, immediately referred for confirmation to a public health laboratory.[99,122]

Resistance to macrolides and clindamycin in β-hemolytic streptococci has increased, particularly for GAS and GBS. Strains that are resistant to erythromycin and susceptible to clindamycin may carry an inducible MLS_B mechanism and have an inducible clindamycin resistance test done according to CLSI methods (i.e., either the double-disk test (D test) or broth microdilution with 1 μg/mL erythromycin and 0.5 μg/mL clindamycin in the same well).[121,122] If neither intrinsic nor inducible resistance is documented, the strain may be reported as susceptible to clindamycin. GAS and GBS isolates with high-level resistance to fluoroquinolones have also been described.[315,513] For patients who are allergic to penicillin, the oral therapeutic choices may become increasingly limited. Vancomycin is the drug of choice for invasive β-hemolytic streptococci infections in patients with penicillin allergy, and no resistance has been reported.

Macrolide and clindamycin resistance in GAS is primarily mediated by an efflux mechanism, but ribosomal methylation (i.e., MLS_B mechanism that is either constitutively expressed or inducible) due to the presence of an *erm* gene is becoming more widespread, and reports of inducible clindamycin resistance in GAS have recently increased.[390,504] During 2002 to 2003, a total of 1,885 GAS strains were obtained from 45 US medical centers (85% were pharyngeal isolates), and the macrolide-resistance phenotype distribution was as follows: MLS_B 56% of isolates (inducible, 47%; constitutive, 9%; and macrolide alone, 44%).[514] The most common mechanism of resistance in GBS is by a ribosomal methylase (*erm*A or *erm*B), but a minority of strains may express resistance through an active efflux mechanism, either alone or in combination with the methylase.[150,493]

Laboratories should perform susceptibility tests on group A streptococci isolated from throat swabs, and group B Streptococci isolated from vaginal-rectal swabs for surveillance screening in near-term pregnant women if there is an indication that the patient is penicillin allergic, and/or when there has been an indication of treatment

failure with a β-lactam. Recent case reports document failure of peri-partum chemoprophylaxis with clindamycin in preventing transmission of GBS from mother to infant.[47] Therapeutic failures for bacterial pharyngitis may also result from inadequate penetration of antibiotic into the crypts of the tonsils and adenoidal tissue, or failure of therapy due to infection with a macrolide-resistant GAS strain.[360] In these clinical situations, antibiotic susceptibility testing for alternative drugs, such as erythromycin, clindamycin, and levofloxacin, is appropriate.

If testing is performed by the disk diffusion procedure, penicillin (10U) or ampicillin (10 μg) disks may be used to predict susceptibility; use of oxacillin disks, as for pneumococci, is not recommended.[121,122] β-hemolytic streptococci that demonstrate a dissociated susceptibility pattern for macrolides and clindamycin (i.e., erythromycin resistant but clindamycin susceptible) should have an inducible clindamycin resistance test performed according to CLSI methods (i.e., D-zone test or broth microdilution with 1 μg/mL erythromycin and 0.5 μg/mL clindamycin in the same well).[121,122]

Reporting. Alternative antibiotics including erythromycin and clindamycin should be reported on GAS isolates from the pharynx if patients have a penicillin allergy. Although all GBS isolated from vaginal-rectal swabs should be tested for susceptibility to erythromycin, according to the new CDC GBS guidelines this drug should no longer be used for peri-partum chemoprophylaxis,[642] and laboratories should not report the results. However, the erythromycin result assists laboratories with the identification of GBS isolates that may have inducible clindamycin resistance, so in vitro susceptibility testing for this drug should be continued. GBS vaginal–rectal swab cultures should have a comment appended to indicate that susceptibility to penicillin and other β-lactam antibiotics can be assumed, but that the laboratory should be consulted for testing of alternate agents (i.e., clindamycin and vancomycin) if the patient has an allergy to penicillin. Clindamycin should be reported as resistant if the inducible resistance test is positive, and a comment may be added stating that the isolate is presumed to be clindamycin resistant based on the detection of inducible clindamycin resistance.[121,122]

Fastidious Gram-Positive Bacteria

Abiotrophia spp. and *Granulicatella* spp., formerly known as nutritionally deficient streptococci, may cause invasive infection, particularly bacteremia and endocarditis. These organisms are very fastidious and may be hard to grow and identify using biochemical methods. Definitive identification can be achieved using partial 16S rRNA gene sequencing. Due to the fastidious nature of these isolates, susceptibility testing may also be difficult unless adequate growth is achieved. CLSI recommends microdilution testing using CAMHB supplemented with 2% to 5% lysed horse blood and 0.0001% (1 µg/mL) pyridoxal hydrochloride with incubation at 35°C in ambient air with incubation at 35°C in CO_2 for a full 24 to 48 hours.[114] Some strains may also grow better on chocolate agar with cysteine supplementation incubated in CO_2 for a full 24 to 48 hours. Laboratories that cannot get adequate growth to perform susceptibility testing, or those that do not perform microdilution tests should refer these organisms to a reference laboratory.

Corynebacterium spp. have variable susceptibility profiles,[190] and are increasingly being recognized as important emerging pathogens. These are an important cause of

invasive prosthetic joints and heart valve infections, as well as, a cause of infections of

central lines and pacemaker leads. *Corynebacterium* spp. are fastidious bacteria that grow best in the presence of blood. Some isolates such as *C. striatum* or *C. jeikeium* are multidrug-resistant so that susceptibility testing should be done on isolates recovered in pure culture from sterile sites, tissues, prosthetic devices, central lines and blood. CLSI recommends testing by microdilution using cation-adjusted MHB supplemented with 2% to 5% lysed horse blood with incubation at 35°C in ambient air for a full 24 to 48 hours.[528]

Listeria monocytogenes is a major food-borne pathogen that can cause diarrhea, bacteremia, and meningitis, particularly in immunodeficient patients. *Listeria* species are inherently resistant to the cephalosporins. CLSI recommends testing invasive *Listeria* isolates for susceptibility to ampicillin and trimethoprim-sulfamethoxazole.[114] Recent clinical antibiotic surveillance, and surveys from the food industry show that *Listeria* remains susceptible to these agents, with the exception of rare isolates that have decreased susceptibility to trimethoprim-sulfamethoxazole.[364,507] Microdilution should be used to test invasive *L. monocytogenes* isolates using CAMHB supplemented with 2% to 5% lysed horse blood with incubation at 35°C in ambient air for a full 24 to 48 hours.[114]

Vancomycin resistance is commonly found in strains of *Leuconostoc*, *Pediococcus*, and *Lactobacillus*.[589] These genera are infrequently pathogens, but on occasion cause bacteremia and septicemia. Resistance to vancomycin may be a useful clue to the correct identification, because streptococci, with which these genera may be confused, are uniformly susceptible to vancomycin. CLSI recommends testing these genera by microdilution using CAMHB supplemented with 2% to 5% lysed horse blood with incubation at 35°C in ambient air (*Leuconostoc*, *Pediococcus*) or CO_2 (*Lactobacillus* spp.) for a full 24 to 48 hours.[114]

Detection of Resistance in Gram-Negative Bacteria

Haemophilus influenzae *and* Haemophilus parainfluenzae

Tristram and colleagues recently reviewed antibiotic resistance in *H. influenzae*.[622] Susceptibility testing should be performed on *Haemophilus influenzae* and other *Haemophilus parainfluenzae* recovered from sterile fluids, tissues and blood. A β-lactamase test should be done as previously described in the chapter. CLSI recommends testing invasive isolates against ampicillin, a third-generation cephalosporin, meropenem, trimethoprim–sulfamethoxazole, and chloramphenicol. Although chloramphenicol is now rarely used, resistance to this antibiotic was infrequent among isolates of *H. influenzae* (0.5%) in a national collaborative study

when it was commonly prescribed for serious invasive infection.[159] Chloramphenicol remains available as an alternate agent that may have to be used to treat serious invasive infections in patients with severe antibiotic allergies, or due to the presence of a multidrug-resistant *H. influenzae* strain. Although *H. influenzae* strains that are BLNAR remain rare in North America, Japan and Europe have experienced emergence of a *H. influenzae* clone that has decreased susceptibility to ampicillin often at a low level (i.e., ampicillin MICs 0.5 to 4 μg/L) because of alteration in PBP3 due to mutations in the *ftsl* gene.[25,379,622]

Additional agents including amoxicillin clavulanate, a second-generation cephalosporin, and a fluoroquinolone may be tested for isolates causing pneumonia. First-generation cephalosporins have poor activity against *Haemophilus influenzae* and need not be tested. Second-generation cephalosporins, such as cefamandole and cefuroxime, are active against *Haemophilus* species in vitro, but decreased susceptibility may occur due to β-lactamase production or alteration of PBP3 as a result of mutations in the *ftsl* gene.[577] Third-generation cephalosporin drugs, such as ceftriaxone, remain uniformly effective against *H. influenzae*.

Disk diffusion should be done using HTM plates incubated at 35°C for 20 to 24 hours. Proper preparation of the inoculum is very important for testing *Haemophilus* species. The alternative direct method of standardizing the bacterial suspension from an overnight culture should be used as outlined in CLSI M02-11.[118] The 0.5 MacFarland suspension contains approximately 1 to 4 × 10^8 CFU/mL. A higher inoculum concentration may lead to false-resistant results with some β-lactam drugs, particularly when β-lactamase-producing strains of *H. influenzae* are being tested.[121,122] Zone diameters should be read as the area showing no obvious, visible growth using the unaided eye. Ignore faint growth at the edge of the zone that can only be seen with magnification. Agonists in the media may cause growth of *Haemophilus* spp. when testing trimethoprim-sulfamethoxazole using disk diffusion. CLSI recommends that slight growth (20% or less of the lawn of growth) be ignored when reading the zone diameter.[121,122]

Microdilution is recommended to determine the MICs for *Haemophilus* spp. Jorgensen and colleagues, however, found a good correlation of the Etest and standardized microdilution tests for *Haemophilus influenzae*.[299] Another study of five different susceptibility testing methods for *H. influenzae* also documented a generally acceptable performance among these commonly used methods.[214] Disk diffusion or Etest, however, may not detect some BLNAR strains of *H. influenzae*, and confirmatory testing should be done using broth microdilution.[40,206,434]

Reporting. **BLNAR** strains of *H. influenzae* should be considered resistant to other β-lactam agents (amoxicillin clavulanate, ampicillin sulbactam, cefaclor, cefamandole,

cefetanet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin–tazobactam) despite in vitro susceptibility of some strains.[121,122] Although chloramphenicol and/or a fluoroquinolone may be tested, the results should only be selectively reported.

Fastidious Gram-Negative Bacteria

HACEK group organisms including *Aggregatibacter* spp. (formerly *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* and *Actinobacillus actinomycetemcomitans*), *Cardiobacterium* spp., *Eikenella* spp., and *Kingella*

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spp. may cause invasive infections including bacteremia and endocarditis. HACEK organisms are common pathogens in periodontitis, particularly *Aggregatibacter* spp.[14] *Eikenella corrodens* is also a common pathogen in human and canine bite wound infections. HACEK group species commonly produce β-lactamase, and these strains should be reported as ampicillin resistant.[14] HACEK species are very fastidious and growth is slow on routine media including blood and chocolate agar incubated in CO_2. Due to the fastidious nature of these isolates, susceptibility testing may also be difficult unless adequate growth is achieved. CLSI recommends microdilution testing using CAMHB supplemented with 2% to 5% lysed horse blood with incubation at 35°C in $CO_2$for a full 24 to 48 hours.[114] CLSI breakpoints for the carbapenem drugs are higher for *Aggregatibacter* spp. (MIC ≤ 4 μg/mL is susceptible, 8 μg/mL is intermediate, and ≥16 μg/mL is resistant) than other HACEK species (MIC ≤ 0.5 μg/mL is susceptible, 1μg/mL is intermediate, and ≥2 μg/mL is resistant).[114]

Pasteurella multocida is the most prevalent pathogen in cat-bite infections, and may spread to cause osteomyelitis or bacteremia. *Pasteurella* are fastidious and require blood supplemented media for adequate growth. Penicillin or ceftriaxone are the drugs of choice but alternate agents such as a fluoroquinolone, a tetracycline, a macrolide or trimethoprim-sulfamethoxazole may need to be used in patients with severe penicillin allergy. Disk diffusion testing is done using MHA supplemented with 5% sheep blood incubated at 35°C in ambient air for 24 hours.[114] Microdilution MIC tests are performed using CAMHA with 2.5% to 5% lysed horse blood incubated under similar conditions as the disk diffusion method. Gradient diffusion appears to not be as reliable as other MIC methods.[410]

Moraxella. *Moraxella catarrhalis* is an important cause of respiratory infections, and dissemination may result in bacteremia and seeding of other sites. This organism grows well on blood agar in ambient air. Broth microdilution testing should be done on

invasive isolates. CAMHB is used for microdilution testing, and plates are incubated in ambient air at 35°C for 20 to 24 hours. CLSI only recommends using disk diffusion to test susceptibility to amoxicillin clavulanate, macrolides, tetracycline, and trimethoprim–sulfamethoxazole.[121,122] A microdilution method should be used to test cephalosporin susceptibility.

Neisseria

Neisseria gonorrhoeae. The gonococcus (GC) has a propensity to rapidly develop antibiotic resistance. Treatment options for *N. gonorrhoeae* infections are becoming extremely limited worldwide because of the successive spread of gonococcal strains, which are resistant to sulfonamides, tetracyclines, penicillins, fluoroquinolones, and more recently cephalosporins.[256] Penicillinase-producing gonococci (PPNG) strains were detected initially in Asia in the mid-1970s,[421,502] but they rapidly became widespread.[104] This β-lactamase resembles the TEM enzyme found in enteric bacilli and Haemophilus influenzae. All GC isolates should therefore be tested for β-lactamase production as described earlier in the chapter. GC strains appeared shortly after which were β-lactamase-negative but penicillin-resistant due to altered PBPs, and these strains have since produced epidemic as well as sporadic infection.[169,188] A decade later, GC strains with high-level plasmid-mediated tetracycline resistance were reported in the United States.[318] In the mid-1980s fluoroquinolones became the drugs of choice for treating GC infections. But as of April 2007, the CDC in the United States removed fluoroquinolones as recommended agents for GC infections because of the high incidence of surveillance strains (13.3%) found to be resistant.[105] Azithromycin-resistant strains of GC have been increasingly reported in certain parts of South East Asia,[329] and the first isolate was also recently reported in the United States.[308]

Sexually transmitted disease treatment guidelines published by the CDC in 2010 recommended the use of oral cefixime or IM ceftriaxone for treatment of uncomplicated genital or rectal GC infections because even multidrug-resistant strains remained universally susceptible.[670] However, cephalosporin resistance has been rapidly increasing worldwide, including in the United States.[102] GC isolates with increased cefixime MICs (≥0.25 mg/L) dramatically increased from 0.1% in 2006 to 17% in 2011, mainly in the western states (from 0.2% to 3.6%) and among men who have sex with men (0.2% to 4.7%).[102] The proportion of GC strains having increased ceftriaxone MIC (≥0.125 mg/L) also increased (0.05% to 0.50%) in the same time period with the same geographic and epidemiologic pattern. The genetic basis of reduced cephalosporin susceptibility in these strains is due to a combination of several chromosomal gene mutations in *pen*A that encodes an altered mosaic PBP2, *pen*B

which results in decreased entry of the drug through the PorB1b OM channel, and *mtr*R, a repressor of the Mtr CDE-encoded pump.[346,630] Clinical cure rates are lower (85% to 95%) for infections due to GC strains with decreased susceptibility to cephalosporins compared to susceptible strains (i.e., >95%). Because of increasing reports of cefixime treatment failures associated with infection due to GC strains with elevated cefixime MICs,[6] the CDC no longer recommends using oral cephalosporins for treatment except for situation where an IM injection cannot be given.[106] Combination therapy with ceftriaxone 250 mg IM and either azithromycin 1 g orally as a single dose or doxycycline 100 mg orally twice daily for 7 days should currently be used to treat uncomplicated GC. This regimen not only treats GC but also *Chlamydia trachomatis*. Penicillin allergic patients should receive an increased dose (2-gm) of azithromycin or spectinomycin, if the GC strain is confirmed to be susceptible. A test of cure should also be done for treatment failure where symptoms persist, and/or a positive GC culture is obtained ≥72 hours, and/or a positive NAAT is obtained ≥7 days after completing therapy.[106]

Culture and susceptibility test of *N. gonorrhoeae* should be done in cases of treatment failure. Antibiotic susceptibility testing of GC using either disk diffusion or agar dilution methods has been reviewed by Doern and Jones.[158] Disk diffusion tests are performed on GC agar base supplemented with 1% of the defined growth supplement. Cysteine-free medium is not required for disk testing; enriched chocolate agar is not recommended.[118,121,122] Susceptibility plates for testing *N. gonorrhoeae* must be incubated at 36°C±1°C (not to exceed 37°C) in 5% CO_2 for 20 to 24 hours. A zone diameter of ≤26 mm for a penicillin (10 U) disk indicates resistance, while a zone size ≤ 19 mm is indicative of β-lactamase

production. But β-lactamase detection using the nitrocefin method is preferred for assessing this mechanism of resistance. CLSI has also developed interpretive criteria for disk diffusion susceptibility testing of GC isolates against tetracycline, fluoroquinolones, and spectinomycin.[118,121,122] Plasmid-mediated tetracycline resistance is indicated by a zone diameter of ≤19 mm around a 30-mg tetracycline disk; such strains should be evaluated by a dilution test. CLSI has defined susceptible breakpoints for parenteral third-generation cephalosporins (i.e., ceftriaxone, cefotaxime, ceftazidime) and oral cephems (e.g., cefixime). However, GC strains that demonstrated decreased susceptibility to cephalosporins (i.e., smaller zone size than susceptible) should have repeat testing using agar dilution.

Table 17-26 outlines the CLSI MIC interpretive criteria for testing *N. gonorrhoeae*. Agar dilution tests are performed using GC agar supplemented after autoclaving with a

1% defined growth supplement (1.1 g L-cysteine, 0.03 g guanine HCl, 3 mg thiamine HCl, 13 mg para-aminobenzoic acid [PABA], 0.01 g vitamin B_{12}, 0.1 g cocarboxylase, 0.25 g NAD, 1.0 g adenine, 10 g L-glutamine, 100 g glucose, 0.02 g ferric nitrate in 1 L H_2O). Cysteine must be omitted if carbapenems or clavulanate are to be tested.[114] The Etest has shown good correlation with agar dilution techniques for non–β-lactamase-producing strains,[637] but resistance may not be detected in strains that produce β-lactamase,[682] emphasizing the necessity for performing a test for β-lactamase production in all strains.

Reporting. Patients with GC infection are empirically treated in the United States based on management guidelines for sexually transmitted diseases published by the CDC.[670] Updates to the CDC treatment guidelines are based on surveillance data from antibiotic susceptibility testing of locally recovered GC isolates. Clinical laboratories should refer GC isolates, particularly those that demonstrate decreased susceptibility to one or more major classes of antimicrobial agents to a public health laboratory for confirmatory testing. Any GC isolate from treatment failures should also be referred to a reference laboratory.

Neisseria meningitidis. *N. meningitidis* causes invasive meningococcal disease, and is an important cause of bacteremia and meningitis. Although effective vaccines are available for serotypes A, C, Y, and W-135, vaccination is given only to select groups, and not all serotypes are included as immunogens (i.e., serotype B). Prompt, effective management is paramount for invasive meningococcal infections, which may have a high mortality despite appropriate antibiotic treatment. Because of its universal susceptibility, penicillin has been the drug of choice for invasive *N. meningitidis* infections, but relative resistance is increasingly being reported worldwide although its clinical relevance remains controversial.[38,73,222] However, *N. meningitidis* strains that demonstrate decreased susceptibility to penicillin remain susceptible to third-generation cephalosporins which are the drugs of choice for treatment of bacterial meningitis. The incidence of decreased susceptibility to penicillin (MIC ≥ 0.125μg/mL) in these recent surveillance studies from Brazil, Europe, and Canada ranged from 13% to 21.5%. Brown and colleagues found that patients with higher mortality from invasive meningococcal disease had infection with serotype B or C strains, while serotype Y and W-135 strains accounted for most of the decreased susceptibility to penicillin.[73] Alterations in the structure of PBP2 encoded by the *pen*A gene account for most of the decreased penicillin susceptibility in *N. meningitidis*. Penicillin nonsusceptible strains of *N. meningitidis* have been shown to have mosaic *pen*A genes, and the variable structure has come about through transfer from commensal *Neisseria* species.[616] Modification of PBP2 caused changes in the peptidoglycan structure of the bacterial cell wall which results in decreased binding affinity for penicillin. Several investigators have shown that these changes result in less sensitivity to penicillin

overtime with a gradual increase in the MIC, rather than a rapid conversion to resistance.[38,616] Plasmid-mediated transfer of β-lactamase to *Neisseria meningitidis* has also been documented, but these strains remain rare.[157] Laboratories need to be able to accurately assess the MICs for penicillin, ampicillin and third-generation cephalosporins such as ceftriaxone and cefotaxime on invasive meningococcal isolates.[121,122,639] In addition, antibiotic testing of chloramphenicol may be indicated for treatment on invasive meningococcal disease in patients with a severe penicillin allergy, or if high-level penicillin resistance emerges in *N. meningitidis* that precludes our ability to use β-lactam drugs for therapy.

TABLE 17-26 CLSI Breakpoints (MIC μg/mL) for *N. gonorrhoeae*

Antimicrobial	S	I	R
Penicillin	≤0.06	0.12–1	≥2
Tetracycline	≤0.25	0.5–1	≥2
Cefixime	≤0.25	—	—
Ceftriaxone	≤0.25	—	—
Ciprofloxacin	≤0.06	0.12–0.5	≥1
Spectinomycin	≤32	64	≥128

S, susceptible; I, intermediate; R, resistant.
Adapted from reference 122.

Perform all antibiotic susceptibility testing of *N. meningitidis* in a biologic safety level-2 or preferably, -3 cabinet (BSC-3) using level-3 practices including person protective equipment. Generated aerosols and exposure of laboratory staff outside the BSC-3 has an increased risk of laboratory staff-acquired *N. meningitidis* infection that is associated with a case fatality rate of 50%.[121,122] Disk diffusion is unreliable for testing penicillin or ampicillin susceptibility in *N. meningitidis*; MIC tests should be used for this organism.[294] Disk diffusion zone diameters to establish susceptibility versus nonsusceptibility have been published by CLSI for third-generation cephalosporin drugs and meropenem.[121,122] The CLSI reference method for

susceptibility testing of *N. meningitidis* is microdilution using CAMHB supplemented with lysed horse blood (2.5% to 5%). However, most clinical laboratories rely on either agar dilution using MHA supplemented with 5% sheep blood, or performing antibiotic Etest using the same media.[639] One study evaluated the use of agar dilution and Etest using several media formulations (MHA; MHA supplemented with 5% sheep blood; and

MHA supplemented with chocolated blood) using standardized methodology among national and regional reference laboratories in 14 countries.[640] Overall agreement in each antibiotic/method/medium combination defined as the percentage of laboratories with a result within one dilution of the modal result was 90.6% for agar dilution and Etest. However, agreement among this reference laboratory pool ranged from 98.2% to 69.7%, with only six laboratories demonstrating agreement higher than 90%, while it was more than 80% for the other 11 laboratories. Most concerning was the ability of these reference laboratories to detect decreased penicillin resistance in *N. meningitidis* ranged from 100% to 18.2%, indicating that even with standardized methodology, laboratories may encounter difficulty performing accurate susceptibility testing on this critical organism.

Laboratories also need to be able to accurately test antimicrobial agents used for prophylaxis of contacts of patients with invasive meningococcal disease, including azithromycin, a fluoroquinolone, rifampin, trimethoprim–sulfamethoxazole, and minocycline. CLSI has published MIC breakpoints and corresponding zone diameters for this purpose, but specific guidelines for susceptibility testing for clinical treatment purposes have not been defined. Jorgensen and colleagues recently performed susceptibility testing on a collection of 442 meningococcal clinical isolates from 15 countries to 16 antimicrobial agents as an essential first step in defining susceptibility testing breakpoints specific for *N. meningitidis*.[293] Elevated penicillin and ampicillin MICs among this isolate collection were 14.3% and 8.6%, respectively; none of the isolates produced β-lactamase. Among drugs used for prophylaxis, resistance to trimethoprim–sulfamethoxazole (21%) was the highest, but resistance to all of the other compounds was rare or not found. Burgess and colleagues then used PK-PD modeling and the MICs for 15 antimicrobial agents derived from their prior testing of the above large international culture collection (i.e., 442 clinical isolates) to develop antimicrobial breakpoints for *N. meningitidis*.[82] The PK-PD breakpoint was defined as the MIC at which the calculated target attainment was ≥95% for both serum and CSF, and the reported values were the first human studies to specifically define parameters for this organism.

Reporting. Invasive meningococcal isolates recovered from sterile fluids such as CSF,

tissues, or blood should routinely be tested against penicillin and/or ampicillin, third-generation cephalosporins (ceftriaxone or cefotaxime), rifampin, and ciprofloxacin (or other fluoroquinolone).[121,122] In addition, meropenem and chloramphenicol may be tested and reported upon request by the Infectious Disease service or other clinicians in specific clinical situations. Isolation of *N. meningitidis* should be immediately reported to both Infection Control and Prevention and public health so that contact tracing and antibiotic prophylaxis can be administered to prevent secondary cases.

Enterobacteriaceae

β-Lactamases. This section describes the currently used methods for detecting β-lactamases in *E. coli*, *Klebsiella* spp., and other genera of the family *Enterobacteriaceae*. The currently recommended CLSI MIC breakpoints and zone diameters (as of M100-S20, 2010) were established to predict susceptible and resistant interpretations for detection of extended-spectrum β-lactamases, AmpC enzymes, or carbapenemase-type enzymes, and identification of a specific β-lactamase-resistance mechanism (e.g., CTX-M-type ESBL, *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase [NDM], etc.) as required for clinical care. However, tracking of such strains within a care facility of specific geographic region or globally requires further work to identify the specific mechanism(s) at work in *Enterobacteriaceae* confirmed to have multidrug resistance to β-lactams and other classes of antimicrobials. These enzymes are carried on plasmids that often mediate resistance to other antimicrobial agents, such as aminoglycosides, tetracycline, and sulfonamides, so the appearance of unusual resistance patterns can also serve as a clue that the extended-spectrum enzymes are present.[450] Molecular methods are briefly described for detection of specific types of β-lactamases commonly encountered in *Enterobacteriaceae*.

Extended-Spectrum β-Lactamases. Extended-spectrum β-lactamases (ESBLs) have primarily been detected in either *Klebsiella pneumoniae* or *Escherichia coli*. Although ESBLs occur in other *Enterobacteriaceae*, such as *Enterobacter* spp. and *Citrobacter* spp., detection is more difficult because other types of β-lactamases, especially AmpC enzymes, are commonly present in these genera.[281,450] The CLSI has recently revised the MIC breakpoints and corresponding zone diameters for cephalosporins including cefazolin, cefotaxime, ceftazidime, ceftizoxime, and ceftriaxone and aztreonam in 2010. EUCAST also revised their criteria in 2010, but the breakpoints and corresponding zone diameters are somewhat different. These new breakpoints were established after review of the pharmacokinetic/pharmacodynamic (PK-PD) properties of these drugs, limited clinical data, and the current MIC distribution of clinically relevant *Enterobacteriaceae* species, including ESBL producers. If laboratories have implemented the new MIC breakpoints shown in Table

17-27 or the revised zone diameters for disk testing of these drugs, then confirmatory testing is no longer required before reporting susceptibility results for *Enterobacteriaceae*. The revised breakpoints for these compounds have been based on specific drug dosage regimens outlined by CLSI in Table 2A of the M100-S20-22 document.[120] These drug dosages are required to achieve plasma drug levels in adults with normal renal and hepatic function, and laboratories using these criteria should provide this information to important stakeholders (i.e., infectious diseases physicians, pharmacists, infection control practitioners, and their respective committees).

TABLE 17-27 CLSI Previous (M100-S19) and Revised (M100-S20) Breakpoints (MIC µg/mL) *Enterobacteriaceae* for Cephalosporins and Aztreonam

	Previous (M100–S19)			*Revised (M100–S20)*		
Antimicrobial	**S**	**I**	**R**	**S**	**I**	**R**
Cefazolin	≤8	16	≥32	≤2	4	≥8
Cefotaxime	≤8	16–32	≥64	≤1	2	≥4
Ceftriaxone	≤8	16–32	≥64	≤1	2	≥4
Ceftazidime	≤8	16	≥32	≤4	8	≥16
Ceftizoxime	≤8	16–32	≥64	≤1	2	≥4
Aztreonam	≤8	16	≥32	≤4	8	≥16

S, susceptible; I, intermediate; R, resistant.
Adapted from CLSI M100-S19 (2009) and M100-S20 (2010).

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A recent study compared the revised CLSI breakpoints with those of EUCAST for ESBL detection in 236 well-characterized clinical isolates including 118 ESBL producers using modified double-disk testing (DDT) with cefpodoxime, ceftriaxone, cefepime, cefotaxime EUCAST (5 µg/disk), ceftazidime EUCAST (10 µg/disk), cefotaxime CLSI (20 µg disk), and ceftazidime CLSI (30 µg disk).[483] Antibiotic synergy was also recorded from the modified DDTs between clavulanic acid disk, and

both the EUCAST and CLSI cephalosporin disks. Overall, the sensitivity (99.2%) for both the EUCAST and CLSI cefotaxime breakpoints were similar. The EUCAST nonsusceptible breakpoint for ceftazidime did not detect 27 ESBL producers, but the CLSI also missed 41 of them. The EUCAST cefpodoxime resistant breakpoint was more sensitive than that of CLSI (100% vs. 98.3%). A modified DDT using clavulanate and cefipime had the highest overall sensitivity (96.6%) of all of the combinations tested, and this disk combination also had excellent sensitivity (100%) and specificity (97.4%) in isolates that also produced an AmpC enzyme.

Reporting. Use of the revised CLSI MIC breakpoints and zone diameters for cephalosporins and aztreonam allows laboratories to report the result for each β-lactam agent; it is no longer necessary to change susceptible results for penicillins, cephalosporins, or aztreonam to resistant results as occurs using the "old" breakpoints and to perform the secondary ESBL-confirmation testing described below. Laboratory stakeholders need to know that the revised MIC breakpoints for *Enterobacteriaceae* will enhance detection of resistance regardless of the mechanism of resistance. Because some ESBLs hydrolyze some cephalosporins but not others, laboratories will likely be reporting discrepant susceptibility test results for isolates that produce this type of enzyme (i.e., an **ESBL**-producing *E. coli* may have a resistant **MIC** for cefotaxime but may be susceptible to other third-generation drugs). When the laboratory implements the revised breakpoints, stakeholders may initially request testing of specific isolates to determine the mechanism of β-lactam resistance.

A rate-limiting step for many laboratories in readily adopting these breakpoint changes has been the delay in automated susceptibility test system accommodating these lowered cutoffs for specific cephalosporins and aztreonam. Despite the CLSI revising existing breakpoints, the FDA in the United States must review the data to determine the clinical affects these changes may have on the indication for use of these agents, and patient safety. Until the FDA approves the breakpoint changes for these drugs, the commercial manufacturers of automated systems cannot proceed to change their systems. Currently the FDA has not approved these changes, the automated susceptibility systems have not been changed and many laboratories continue to screen for ESBL-producing *E. coli* and *Klebsiella* spp. using the "previous" CLSI breakpoints (Table 17-27), and subsequently perform ESBL-confirmation testing as outlined below.

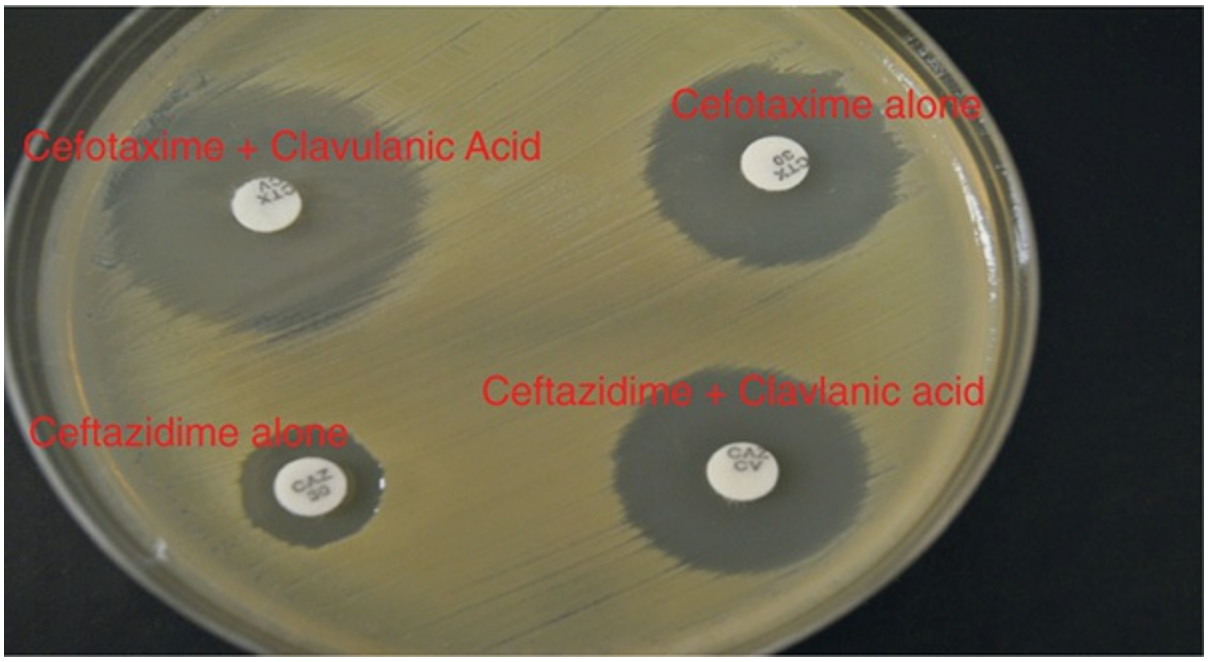
Laboratories that have not yet implemented the revised breakpoints changes for select cephalosporins or aztreonam must continue to perform confirmatory testing on isolates suspected of being ESBL producers according to the CLSI recommended screening test. Routine disk diffusion and MIC tests using the "previous" breakpoints outlined in Table 17-27 may not always identify isolates that produce ESBLs. The previous CLSI recommendations for ESBLs screening for *Klebsiella* spp., *Escherichia coli* and *Proteus mirabilis* are summarized in Table 17-28. ESBL-producing isolates typically demonstrate high-level resistance to one or more of the

screening drugs (e.g., aztreonam, cefotaxime, cefpodoxime, ceftriaxone, ceftazidime), but retain susceptibility to cephamycins (i.e., cefoxitin or cefotetan). However, no single screening antibiotic detects all types of ESBLs, so the use of multiple screening compounds increases the sensitivity of screening. Cefpodoxime is most likely to detect ESBL production, but even with revised breakpoints this indicator may have a lower specificity for *E. coli*.[609]

Phenotypic confirmation of the presence of an ESBL relies on the fact that these enzymes are inhibited by the inhibitor compound clavulanic acid. All of the currently available phenotypic tests summarized in Table 17-28 are based on enhanced activity of the β-lactam (cefotaxime, ceftazidime) when tested with clavulanic acid compared to testing of the β-lactam alone. Before commercial detection tests became available, laboratories relied on combination testing of β-lactams/clavulanic acid either using the DDT or broth microdilution methods recommended by CLSI (Figs. 17-31 and 17-32). These methods may be used to detect ESBLs in *Klebsiella oxytoca*, *K. pneumoniae*, *E. coli,* and *P. mirabilis,* but have not been validated for other *Enterobacteriaceae* species. Another modified DDT procedure has been described that reliably detect ESBLs in other genera of *Enterobacteriaceae*, but routine use of this method in the clinical laboratory can be exacting, particularly the challenging interpretation.

TABLE 17-28 Confirmatory Tests for ESBL-Producing *Enterobacteriaceae*

Method	Comments	Reading and Interpretation	Illustration
Double-disk test (DDT)	Placement of the disks is critical; separation by 20 mm and 30 mm may be necessary	Increase of inhibition zone facing disk that contains β-lactamase inhibitor (clavulanic acid) by ≥5 mm	Figure 17-31
Microdilution	Commercial panel with wells that contain cefotaxime and ceftazidime with and without clavulanic acid	Decrease in the MIC of ≥8-fold (three doubling dilutions)	Figure 17-32
Etest ESBL	Strip contains β-lactam on one end and β-lactam + clavulanic acid on the other	Reduction in the MIC by ≥8 fold in the presence of β-lactamase inhibitor	Figure 17-33
Vitek 2, BD Phoenix, MicroScan ESBL	Automated detection of ESBL	Decrease in the calculated MIC in the presence of β-lactamase inhibitor	Figures 17-22, 17-23, 17-24
Multiplex PCR	Differentiates the type of ESBL present (i.e., TEM, SHV, CTX-M, etc.)	Positive for one or more enzymes	Figure 17-34



■ **FIGURE 17-31** Confirmatory double-disk test for *K. Pneumoniae* producing an ESBL (CTX-M 15 type). (Courtesy of Calgary Laboratory Services.)

The DDT is performed using disks with and without 10 μg of clavulanic acid (Fig. 17-31). The DDT is considered positive for an ESBL production if the zone diameter of either β-lactam increases by ≥5 mm in the presence of clavulanic acid. The types of drug substrates used, the drug concentrations in the disks, and the placement of the disks on the media may all affect the performance of the DDT. All DDT should include the recommended QC strain ESBL-producing *K. pneumoniae* ATCC 700623; the accepted zone diameter ranges for this isolate are provided by CLSI. For broth microdilution testing, the same two third-generation cephalosporins are tested alone and with 4 μg clavulanic acid. A ≥ eightfold decrease (three doubling dilutions) in the MIC with combination testing of the β-lactam/clavulanic acid per well compared to β-lactam testing alone is indicative of an ESBL.

A modified DDT has been developed that will simultaneously detect all types of β-lactamase (i.e., ESBLs, AmpC enzymes, and cephalosporinase overproduction) in *Enterobacteriaceae*, and may improve detection of an ESBL in strains where multiple enzymes are operative.[173,473] The sensitivity of the MDDT has been enhanced by decreasing the distance between the disks of cephalosporins and clavulanate. The addition of a cefepime (fourth-generation cephalosporin) disk that is more slowly inactivated by cephalosporinases than by ESBL improves the detection of synergy with clavulanate when there is also overproduction of a cephalosporinase.[309,580] Resistance to cefoxitin (cephamycin) indicates the presence of an AmpC enzyme.[281]

Commercial disc kits are also available that simultaneously detect the presence of

ESBLs and AmpC enzymes in *Enterobacteriaceae*. Donaldson and colleagues compared

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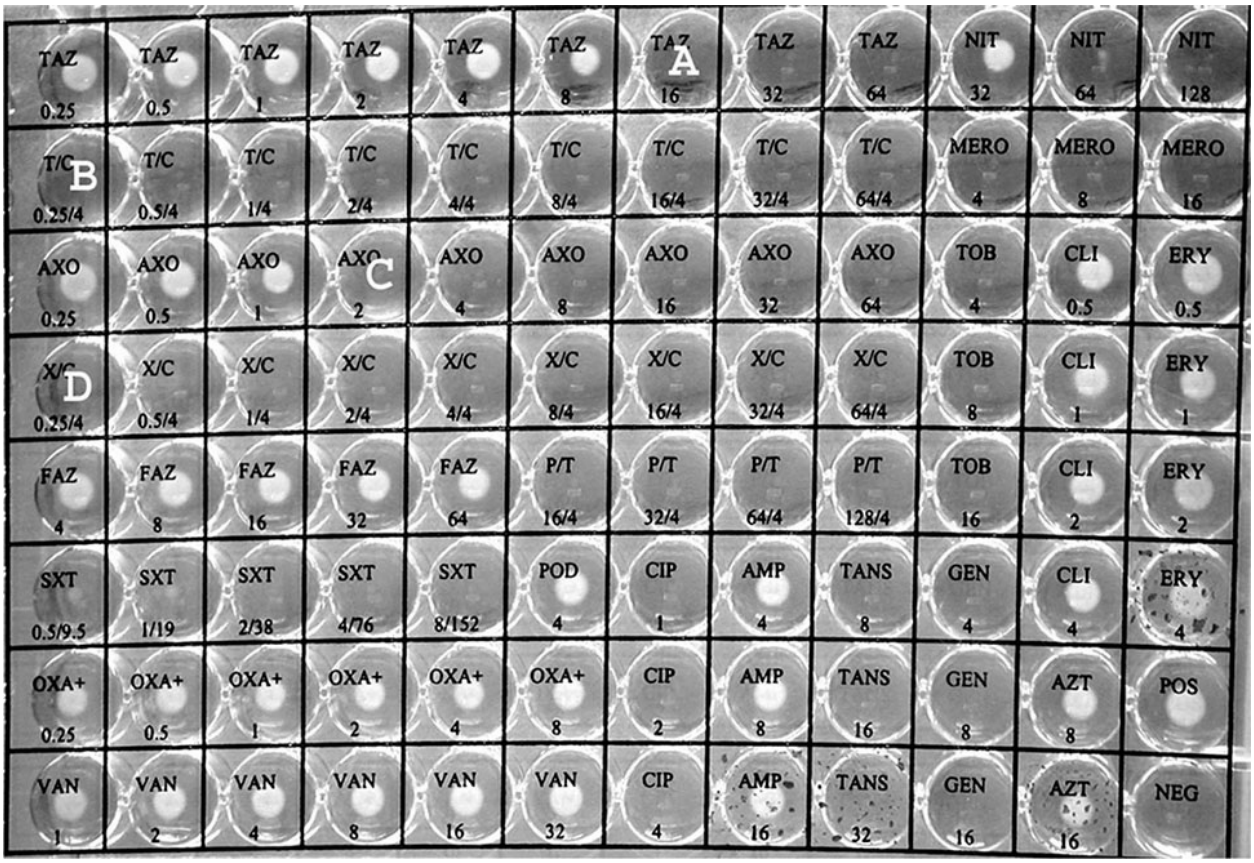
the Vitek 2 AST N-054 card with the MASTdiscs ID ESBL (MAST Group Inc., Bootle, UK) detection disc diffusion test for detecting ESBL production in 137 well-characterized clinical *E. coli* isolates.[167] The MAST disc test correctly detected ESBL in 135 *E. coli* isolates (98.5%; 95% CI, 94.5 to 99.9)] whereas the Vitek card only detected ESBL in 93 (67.9%; 95%CI, 59.7 to 75.1). The MASTdiscs ID AmpC and ESBL 4 disc detection method has recently been compared with a double-disk synergy test for detection of ESBL production in 241 rectal swabs.[339] Of the 41 rectal swabs confirmed positive for ESBL production, the MASTdisc test detected 20(49%) versus 12(29%) by the double-disk synergy test (p = 0.013) after only 24 hours of incubation. The MASTdisc test also confirmed significantly more rectal swabs as negative for ESBL production (183[88%] vs. 158[76%] [$p < 0.001$]), and had a much lower rate of inconclusive results (46[18%] vs. 79[32%]) after 24 hours of incubation. A combination four disc test for simultaneous detection of ESBL and AmpC enzyme production in *Enterobacteriaceae* can also be purchased from Rosco Diagnostica, Tasstrup, DK (www.rosco.dk). Although in-house or commercial modified double-disk synergy tests are not routinely performed in the clinical laboratory, they can assist with differentiation of the enzymatic resistance mechanisms operative when molecular testing is not available.

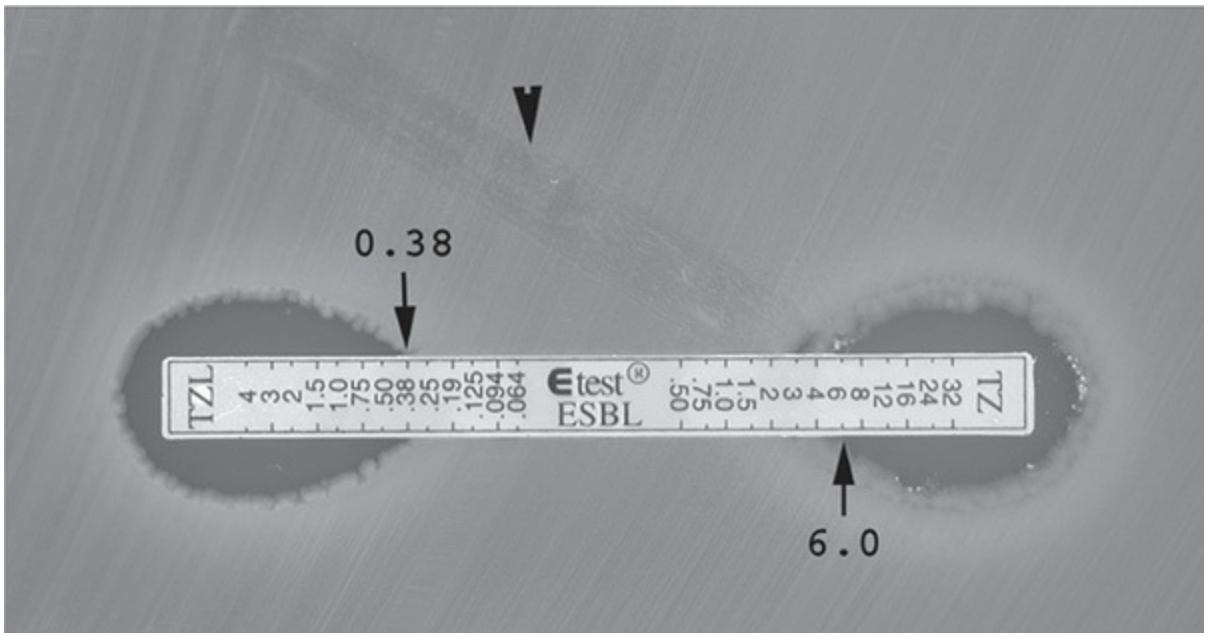


■ **FIGURE 17-32** MIC test for ESBL production in an isolate of *Escherichia coli*. The MIC of this isolate for ceftazidime is 16 μg/mL **(A)**; when combined with clavulanic acid, the MIC is ≤0.25 μg/mL **(B)**. The MIC of the isolate for ceftriaxone is 2 μg/mL **(C)**; in the presence of clavulanic acid, the MIC is ≤0.25 μg/mL **(D)**. Thus, in each case, there is a >fourfold reduction of the MIC in the presence of clavulanic acid, demonstrating the presence of an ESBL.

Nordmann and colleagues have also developed a rapid biochemical test, the ESBL NDP, for detection of ESBLs in *Enterobacteriaceae*.[431] This biochemical test is based on the in vitro detection of a cephalosporin (cefotaxime) hydrolysis that is inhibited by the addition of the β-lactamase inhibitor tazobactam. ESBL activity is evidenced by a color change (red to yellow) of a pH indicator (red phenol) due to carboxyl-acid formation resulting from cefotaxime hydrolysis that is blocked by addition of tazobactam (positive test). Evaluation of the ESBL NDP test for detection of ESBL production in cultured strains (215 ESBL producers and 40 ESBL nonproducers) showed that the test had a sensitivity and specificity of 92.6% and 100%, respectively. A few ESBL producers (n = 16) that remained susceptible to cefotaxime were not detected. Its sensitivity for detection of CTX-M producers was 100%. The ESBL NDP test can be performed in less than 1 hour, and is cost-effective compared to

conventional detection methods for ESBL production which remain time-consuming and take 24 to 48 hours before a result can be reported. The ESBL NDP test is being commercialized and will be available from bioMérieux (www.bioMérieux.com).

Although many laboratories rely on the testing and reporting of the presence of an ESBL from their automated system (e.g., Vitek 2, BD PHOENIX, etc.) or by using an Etest ESBL strip gradient diffusion test (Fig. 17-33), commercial detection has not been widely validated against other methods. Although earlier assessments of commercial methods indicated they were reliable for ESBL detection,[350,565] more recent data are not as favorable. A multicenter evaluation of all of the currently recommended ESBL-confirmation methods was recently reported from the Netherlands.[475] Twenty laboratories submitted 443 *Enterobacteriaceae* with a positive ESBL screen test (i.e., cefotaxime and/or ceftazidime MIC > 1 μg/mL) as well as the results of their confirmatory test (74% *E. coli*, 12% *E. cloacae*, 8% *K. pneumoniae*, 3% *P. mirabilis*, 2% *K. oxytoca*). All isolates used in this study had ESBL genes detected as the reference method. Overall, accurate detection of ESBLs by local laboratories using a phenotypic method was 88% with a PPV of 70%. However, the PPV varied depending on the method used (Vitek 2 69%, BD Phoenix 68%, DDT 92%) and species (95% *K. pneumoniae* vs. 27% *K. oxytoca*). Overall, combination DDT testing for ESBL confirmation was also more specific than Etest ESBL strips (91% vs. 61%), but the PPV for the DDT was poor for certain species (*P. mirabilis* 33% and *K. oxytoca* 38%, although the NPV for these isolates was 100%). ESBL confirmatory tests were also only read and interpreted correctly 93% of the time. These data suggest that ESBL confirmatory testing, regardless of the method used can be an exacting procedure that requires enhanced education of technologists to improve their interpretation. Overall, the DDT had the best performance for the most common species (*E. coli* and *K. pneumoniae*), but the low PPV of automated systems and Etest ESBL strips indicates that the only definitive way to detect all types of ESBLs in *Enterobacteriaceae* is molecular detection of the gene encoding the enzyme(s) present in a given isolate.

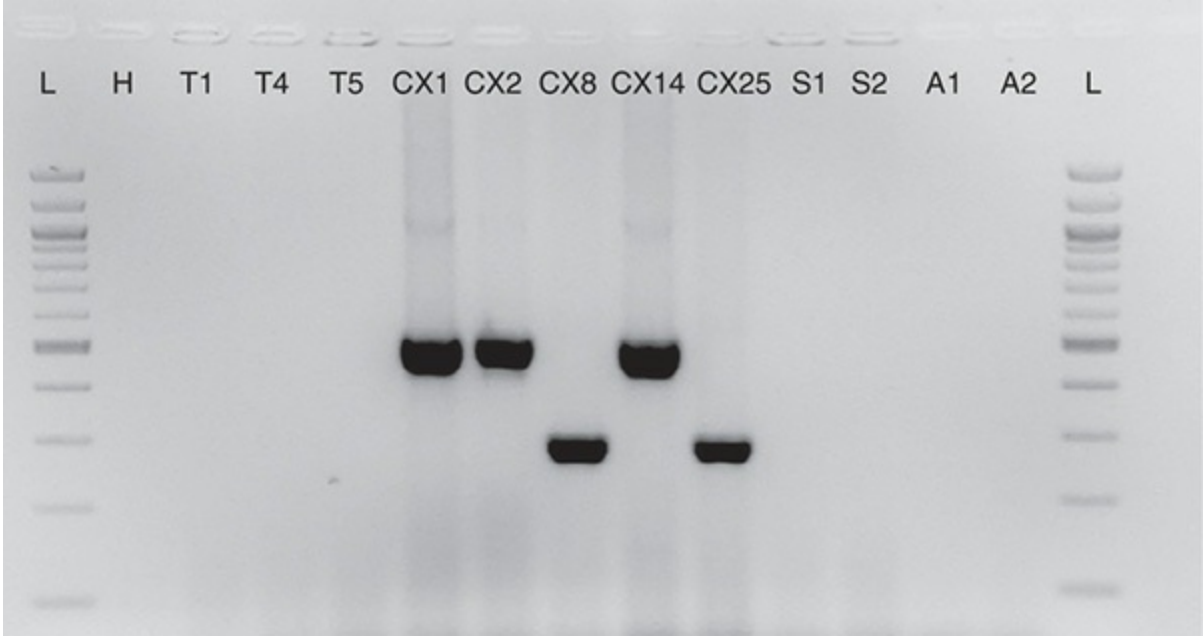


■ **FIGURE 17-33** Etest strip for demonstration of ESBL production in an isolate of *Klebsiella pneumoniae*. The MIC of the isolate for ceftazidime (TZ) is 6 μg/mL. (Note that the zone of inhibition intersects the strip closer to 4 μg/mL on the lower side, but the intersection is at 6 μg/mL on the upper side; the higher value is recorded.) At the other end of the strip, the MIC of the isolate for ceftazidime in the presence of clavulanic acid is 0.38 μg/mL. The reduction of the MIC is > fourfold, indicating the presence of an ESBL enzyme in the strain. Note the impression on the lawn of bacteria of the strip *(arrowhead)*, which was initially dropped upside down on the plate; because the side that was impregnated with antibiotic did not touch the agar, it was acceptable to reposition the strip and continue the test.

Reporting. ESBL-producing isolates of *E. coli*, *Klebsiella* spp., and *P. mirabilis* should be reported resistant to penicillins, except for the β-lactam/β-inhibitor combinations (amoxicillin/clavulanate and piperacillin/tazobactam), and for all cephalosporins, except for the cephamycins (cefoxitin and cefotetan). Laboratories will also encounter *Enterobacteriaceae* isolates that have a positive ESBL screen but a negative confirmatory test. Laboratories should have policies and procedures in place for referral of multidrug-resistant strains to a reference laboratory for molecular detection of β-lactamase genes because of the limitations of existing phenotypic methods (i.e., not all types of ESBLs will be detected and an AmpC enzyme is likely present). Other mechanisms of resistance such as the presence of another type of β-lactamase not inhibited by clavulanic acid (i.e., AmpC enzyme of TEM or SHV enzymes), or altered porins may also be present and effecting the results of the ESBL confirmatory test.

Molecular ESBL Detection. Many investigators have developed and evaluated primers to detect TEM, SHV, OXA, CTX-M, and GES enzymes in gram-negative bacteria.[471,609] Both conventional PCR as well as real-time PCR assays have been developed using primers for one or more ESBL enzymes, and several novel multiplex formats have recently been used including microarrays to simultaneously detect a large panel of β-lactamases.[126,143,338] Figure 17-34 shows a typical multiplex PCR results for detection of CTX-M-type ESBLs in *E. coli*. Although CLSI no longer recommends confirmatory testing for detection of ESBLs, both phenotypic and molecular tests may be required in order to deploy appropriate Infection Control measures and to define a nosocomial outbreak.[455,664] Polsfuss and colleagues recently published a diagnostic approach that combines ESBL screening with subsequent confirmation both by phenotypic and multiplex PCR for detection of CTX-M-type and multiplex PCR and sequencing for TEM and SHV ESBLs before reporting an isolate as "ESBL positive."[481] This article contains a comprehensive diagnostic flow chart that laboratories may wish to consult when having to confirm ESBL as the mechanism of resistance.

AmpC β-Lactamase Detection. Although AmpC detection may be important for clinical and epidemiologic purposes, there are currently no recommended assays for reliable detection of these enzymes published in antimicrobial susceptibility testing guidelines (CLSI or EUCAST). Inducible chromosomal AmpC enzymes occur in *Enterobacteriaceae* within the "SPICE" group of genera including *Serratia marcescens*, indole-positive *Proteus mirabilis*, *Citrobacter freundii,* and *Enterobacter cloacae*.[281] This enzyme confers broad resistance to β-lactams including β-lactam/β-lactamase inhibitor combinations such as piperacillin/tazobactam. More recently, multidrug resistance determinants have moved onto plasmids in other genera, including *Klebsiella* spp., *E. coli,* and *Salmonella* spp. and *P. mirabilis*.[281] Strains that carry an AmpC enzyme, which also have mutations in their porin structure, may also be resistant to carbapenems.[571] Isolates carrying a plasmid-mediated AmpC enzyme, however, are typically resistant to cephamycins such as cefoxitin or cefotetan (MIC ≥ 32 μg/mL), which is a clue to their presence.[281,472] However, second-generation cephalosporin resistance is not a definitive test because other resistance mechanisms may be operative (i.e., altered or lost porin structure).



■ **FIGURE 17-34** Multiplex PCR for detection of the ESBL type in *E. coli*. *H*; *H20*, *T1*; *TEM-3*, *T4*; *TEM-10*, *T5*; *TEM-50*, *CX1*; *CTX-M-1*, *CX2*; *CTX-M-2*, *CX8*; *CTX-M-8*, *CX14*; *CTX-M-14*, *CX25*; *CTX-M-25*, *S1*; *SHV-2*, *S2*; *SHV-7*, *A1*; *NMC-A*, *A2*; *KPC-1*. (Courtesy of Calgary Laboratory Services.)

Gram-negative isolates producing a high amount of AmpC enzyme will give a positive result in ESBL screening tests but fail to confirm. Readers are referred to an excellent review by Jacoby of all of the methods used for detection of AmpC enzymes,[281]only a brief description of those used clinically are outlined. Diagnostic tests for AmpC detection have been developed that use an enzyme inhibitor (e.g., 48-1220, LN-2-128, EDTA, cloxacillin, and various boronic acid derivatives),[45] but only 3-amino-phenylboronic acid (PBA) is commercially available. A caveat of using inhibitor-based methods to detect AmpC enzymes is that false-positive results may occur due to KPC enzymes.[45] Yagi and colleagues developed a DDT method that incorporates 300 µg-PBA into disks containing ceftazidime or cefotaxime.[674] Similar to the ESBL DDT test described above, a ≥5 mm increase of the zone of inhibition around the β-lactam/boronic acid disks compared to disk with the drug alone reliably detected the AmpC-types present but not strains with ESBLs or carbapenemases. Pitout and colleagues recently described a modification of the DDT method using cefotetan disks containing PBA to detect plasmid-mediated AmpC enzymes in *Klebsiella* spp., *E. coli*, *Salmonella* spp. and *P. mirabilis*.[472] This study tested 126 control strains and 29,840 nonrepeat clinical isolates that were initially screened using cefoxitin resistance followed by confirmation with the cefotetan DDT. A total of 623 (2%) of the isolates were found resistant on the screen test and 332(52%) were AmpC

positive. Cloxacillin (200 μg) may also be used as the AmpC inhibitor in a double-disk test using cefotetan disks (30 μg).[600] Several commercial methods are also available including a DDT and 4-disc tests for simultaneous AmpC and ESBL enzyme detection (MASTdiscs ID, MAST Group Ltd., Bootle, UK, and Rosco Diagnostics, Denmark), and Etest AmpC strips (contain cefotetan on one end and cefotetan–cloxacillin on the other). A double-disk synergy test that uses cefpodoxime disks alone and supplemented with PBA as the AmpC enzyme inhibitor is shown in Figure 17-35. The AmpC Etest strip contains a concentration gradient of cefotetan on one-half of the strip and cefotetan

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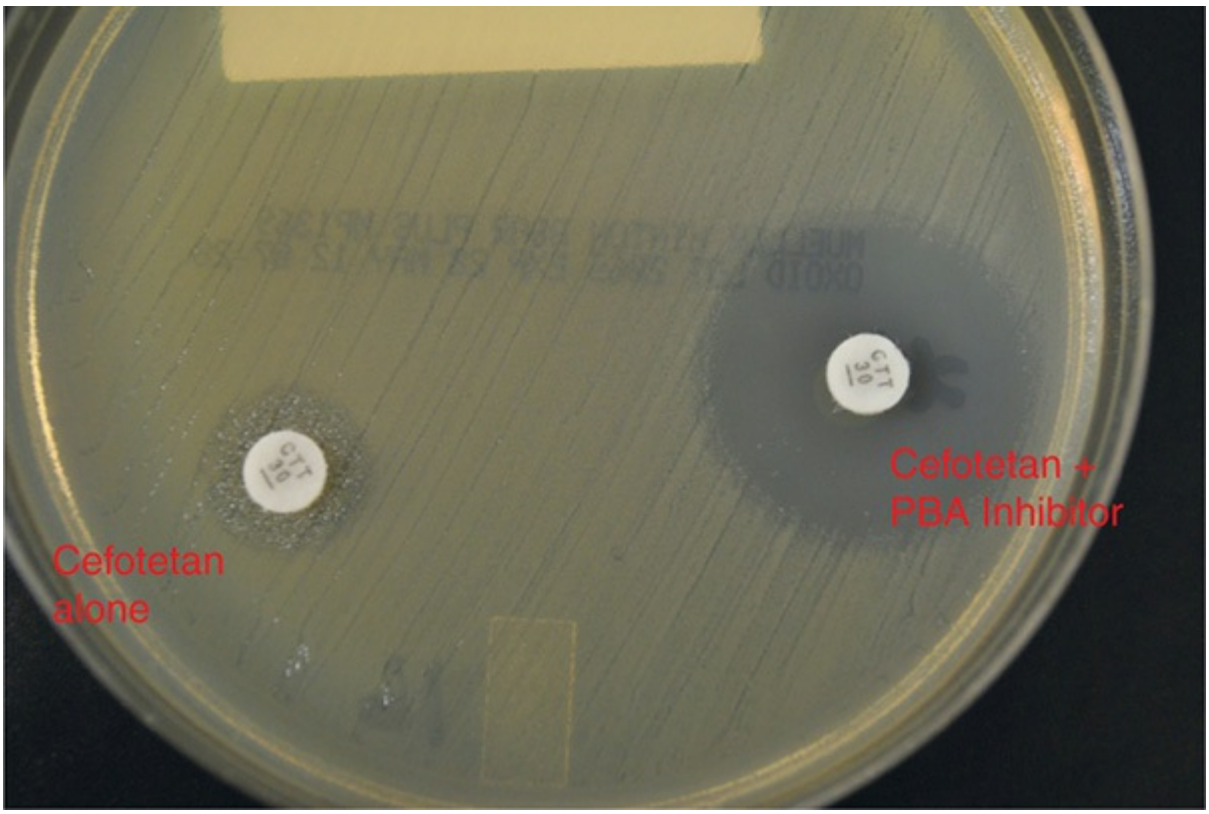
with cloxacillin on the other half of the strip; MIC ratios of ≥8 between cefotetan alone and cefotetan with cloxacillin are considered indicative of the presence of an AmpC enzyme. A recent evaluation of the AmpC Etest compared to two DDT methods showed that all three confirmatory tests were able to detect >90% of the strains that were AmpC producers that had been molecularly characterized.[459]



■ **FIGURE 17-35** AmpC enzyme confirmation in *E. coli* using a double-disk test. (Courtesy of Calgary Laboratory Services.)

Molecular detection methods are currently the "gold standard" for definitive detection and characterization of AmpC enzymes. Because of the plethora of AmpC enzymes carried by gram-negative bacteria, and the fact that phenotypic tests cannot distinguish between plasmid-mediated and/or chromosomal gene determinants, multiplex PCR is used to detect plasmid-mediated resistance. A multiplex PCR using six primer pairs has been shown to reliably detect most plasmid-mediate AmpC enzymes, except for CFE-1 β-lactamase.[457] However, a seventh set of primers may be used to detect the CFE-1 enzyme.[416] Polsfuss and colleagues have recently published a comprehensive diagnostic approach for reliable detection of AmpC enzyme producing *Enterobacteriaceae* that includes an initial screen for cefoxitin resistance followed by confirmatory testing using a DDT that include cefotetan/cloxacillin with subsequent multiplex PCR for plasmid-mediated AmpCs and/or AmpC gene promoter sequencing to detect chromosomal promoter mutations.[482] However, this extent of molecular analysis may not be feasible for many clinical laboratories, but commercial development of an automated microarray assay would enable reliable detection and tracking of AmpC-resistant determinants for epidemiologic purposes.

Reporting. Susceptibility reports should be based on the revised **CLSI** breakpoints for cephalosporins and aztreonam; it is no longer necessary to change results for β-lactams to resistant when a susceptible in vitro result is obtained on using the recommended susceptibility test methods. However, *Enterobacteriaceae* confirmed to carry an AmpC enzyme by phenotypic and/or molecular methods should have the penicillins and all cephalosporins except for fourth-generation agents (i.e., cefepime) reported as resistant including, and β-lactam/β-lactam inhibitors combination drugs.

Carbapenemase Detection (*Enterobacteriaceae*). *Enterobacteriaceae* that produce carbapenemases are becoming an increasing problem worldwide. Specific β-lactamase enzymes in molecular classes A, B, and D have carbapenemase activity as shown in Tables 17-29 and 17-30. *Enterobacteriaceae* carrying KPC (*K. pneumoniae*), SME (*S. marcescens*), NDM (*E. coli*, *Klebsiella* spp.), and the OXA enzymes which are widely distributed in many species are all clinically important. Detection of carbapenemase resistance according to CLSI relies on the revised MIC breakpoints and corresponding zone diameter sizes initially published in 2010. Table 17-30 compares the "previous" and revised CLSI MIC breakpoints for carbapenem drugs for *Enterobacteriaceae*. These new breakpoints were established after review of the pharmacokinetic/pharmacodynamic (PK-PD) properties of these drugs, limited clinical data, and the current MIC distribution of clinically relevant *Enterobacteriaceae* species including carbapenemase producers. Revised CLSI carbapenem breakpoints and disk diffusion zone diameters have been set to detect most carbapenemase producers in the *Enterobacteriaceae*. If carbapenem resistance is detected (i.e., meropenem MIC ≥ 1.0 μg/mL) then CLSI no longer recommends doing a phenotypic

confirmation test (CLSI M100-20-22).[120] If laboratories continue to use the "previous" CLSI carbapenem MIC breakpoints (Table 17-31) then a phenotypic confirmation test needs to be done before reporting the isolate as resistant to carbapenems (CLSI M100-S20-22). A Modified Hodge

Test (MHT) may be used to confirm the presence of class A carbapenemases (i.e., KPCs) as described by CLSI in M100-S22 Table 2A. Pasteran and colleagues described modifications to the MHT method by incorporating boronic acid (inhibits class A carbapenemases and AmpC enzymes) and oxacillin (inhibitor of AmpC enzymes) that had a high sensitivity (>90%) and specificity (100%) for distinguishing class A carbapenemases (KPC, GES, SME, IMI, and NMC-A) from other classes of β-lactamases (ESBLs, AmpC enzymes and MBLs).[448] The results of a positive MHT are shown in Figure 17-36. However, CLSI M100-S22 Table 2A indicates that it is important to recognize that not all carbapenemase-producing *Enterobacteriaceae* isolates will have a positive test, and MHT-positive tests may be encountered in isolates with carbapenem resistance mechanisms other than carbapenemase production (Table 17-32). There are currently no CLSI-validated phenotypic tests to confirm the presence of a metallo-β-lactamase in *Enterobacteriaceae*. *Enterobacteriaceae* isolates that demonstrate carbapenem resistance should be referred to a reference laboratory for confirmatory testing.

TABLE 17-29 Molecular Classification of β-Lactamases in Gram-Negative Bacteria

Class	Active Site	Examples
A	Serine Inhibitor susceptible β-lactamases	TEM-1, SHV-1, KPCs, OXY, and most ESBLs including CTX-M types
B	Zinc Metallo-β-lactamases	Metalloenzymes; VIM, IMP, SPM, NDM, GIM, SIM
C	Inhibitor-resistant β-lactamases	AmpC enzymes
D	Oxacillin-active β-lactamases that may be inhibitor susceptible	OXA (including rare ESBL phenotypes), PSE

Adapted from reference 122.

TABLE 17-30 β-Lactamases With Carbapenemase Activity

β-Lactamase Class[a]	Gram-Negative Bacteria Where Enzyme Detected	Examples
A	*K. pneumoniae* *Enterobacteriaceae*	KPC, SME, NMC-A, IMI, PER, GES, SFO, SFC, IBC
B	*Enterobacteriaceae* *P. aeruginosa* *A. baumannii*	Metallo-β-lactamases (MBLs) inhibited by EDTA (IMP, VIM, NDM), SPM, SIM, GIM
D	*Enterobacteriaceae* *A. baumannii*	OXA, PSE

[a] No class C enzymes have carbapenemase activity.
Adapted from references 90 and 285.

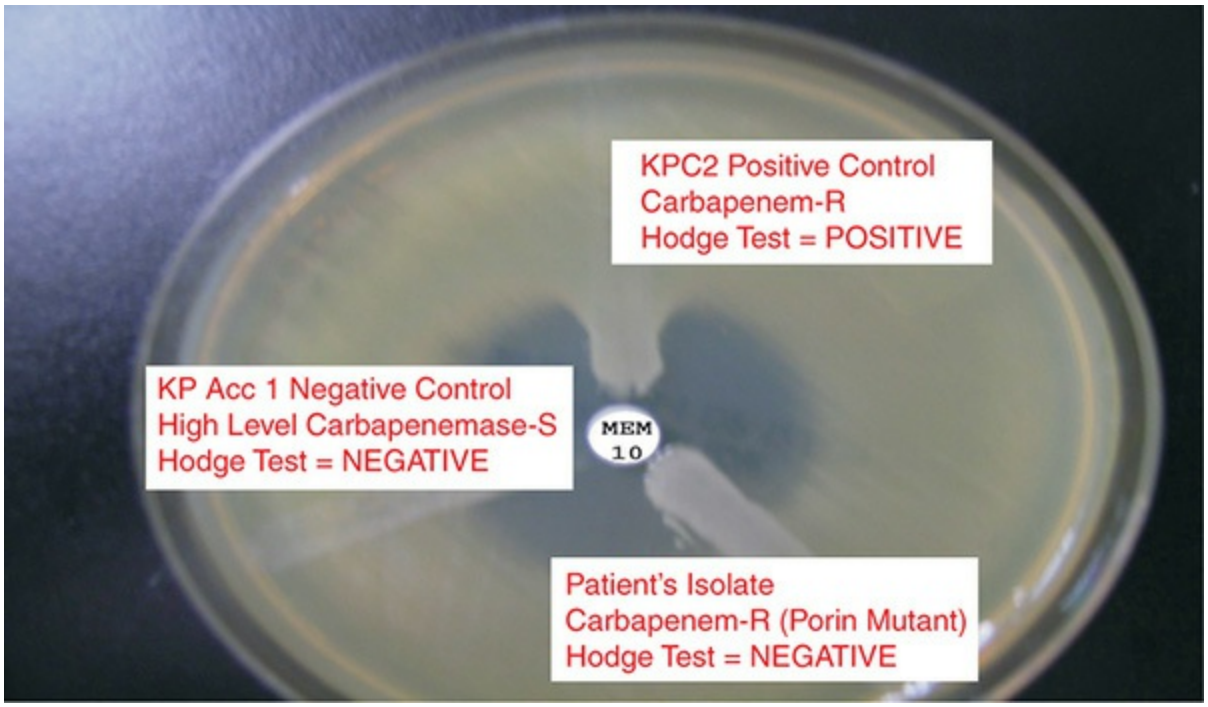
TABLE 17-31 CLSI Previous (M100-S19) and Revised (M100-S20) *Enterobacteriaceae* Breakpoints (MICμg/mL) for Carbapenems

	Previous (M100–S19)			Revised (M100–S20)		
Antimicrobial	**S**	**I**	**R**	**S**	**I**	**R**
Doripenem	NA	NA	NA	≤1	2	≥4
Ertapenem	≤2	4	≥8	≤0.25	0.5	≥1
Imipenem	≤4	8	≥16	≤1	2	≥4
Meropenem	≤4	8	≥16	≤1	2	≥4

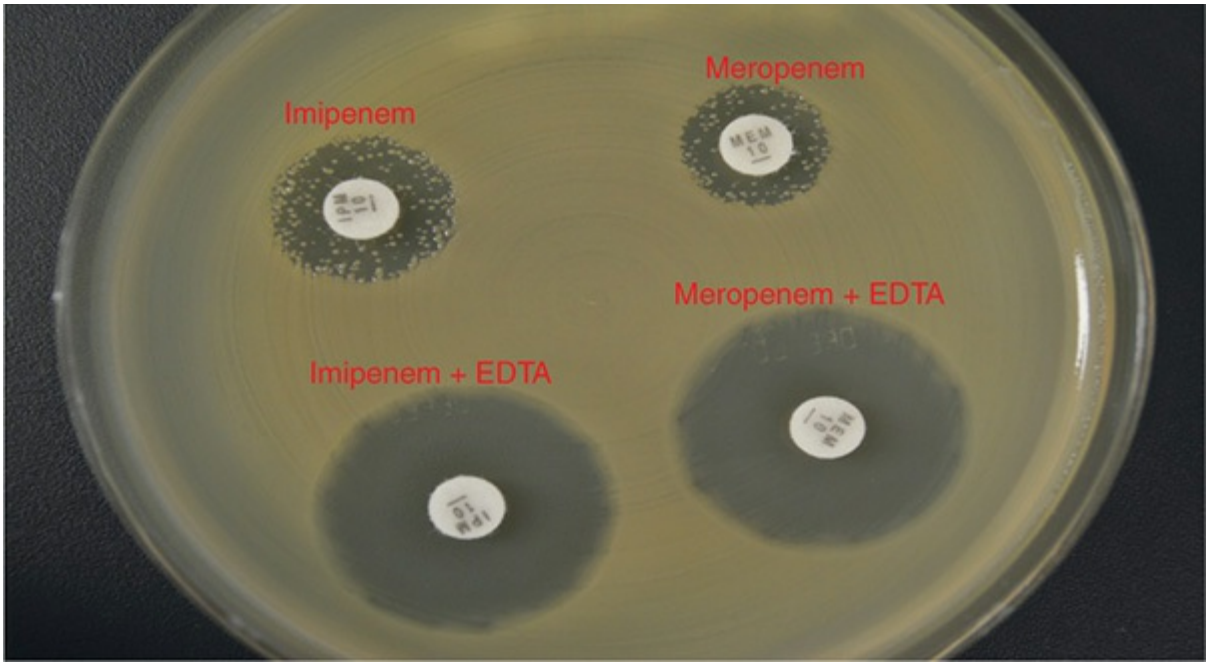
S, susceptible; I, intermediate; R, resistant.
Adapted from CLSI M100-S19 and CLSI M100-S20. For revised disk diffusion breakpoints, see CLSI M100-S20.

The recommended phenotypic confirmation tests for detection of class A, B, and D

carbapenemases in *Enterobacteriaceae* and their potential for false-positive tests due to the presence of other β-lactamases is outlined in Table 17-32. Cohen, Stuart, and colleagues have recently published guidelines for phenotypic screening and confirmation of carbapenemases in *Enterobacteriaceae*.[127] Besides the MHT test, various combination DDT assays are used that rely on testing of carbapenem susceptibility alone and in the presence of a specific carbapenemase inhibitor. Several different types of DDT assays have been developed for detection of MBLs that use meropenem disks (10 μg) supplemented with dipicolonic acid (DPA) as the enzyme inhibitor, or imipenim disks (10 μg) supplemented with EDTA as the enzyme inhibitor (Fig. 17-37). Other DDT assays have been developed to detect class A carbapenemases that use meropenem disks (10 μg) supplemented with boronic acid, and meropenem disks (10 μg) supplemented with oxacillin to help distinguish the presence of an AmpC enzyme.[218] The Carba NP TEST II identifies carbapenemase production in *Enterobacteriaceae* and *Pseudomonas* spp., and differentiates between classes A, B, and D enzymes.[168] This test is based on the detection of acidification that occurs with imipenem hydrolysis, coupled with tazobactam and EDTA as inhibitors. The Carba NP II test can be rapidly performed and has a reported sensitivity and specificity of 100%,[168] but it is not yet commercially available. Agar-based screening methods have also been described that perform well for detection of MBL and KPC-type enzymes where carbapenem disks are placed on inhibitor (EDTA, PBA, and cloxacillin) impregnated MHA; this method also distinguishes other types of β-lactamases.[42] Due to the limitations of phenotypic tests and the high diversity of genes encoding carbapenemases,[498] molecular analysis is considered the "gold standard" detection method. Genotypic confirmation is performed using multiplex PCR detection for carbapenemases, and sequencing of carbapenemase genes.[432,586] A commercial real-time PCR assays has been developed by Cepheid (Xpert MDRO) for the rapid, accurate screening of patients for the presence of enteric bacteria that carry a bla_{KPC}, bla_{NDM} and bla_{VIM} carbapenem resistance gene.[606] Evaluation of the Xpert MDRO assay compared to broth enrichment culture and sequencing of the target genes showed highly reliable detection of KPC and VIM enzymes from clinical samples, and similar performance for detection of the NDM enzyme in contrived stool samples.



■ **FIGURE 17-36** Modified Hodge test for detection of KPC-carbapenemase in *K. pneumoniae*. (Courtesy of Calgary Laboratory Services.)



■ **FIGURE 17-37** Confirmatory double-disk test for detection of NDM-carbapenemase in *K. pneumoniae*. (Courtesy of Calgary Laboratory Services.)

TABLE 17-32 Detection of Carbapenemases and Other β-Lactamases in *Enterobacteriaceae* and Gram-Negative Nonfermenters Using Phenotypic Confirmation Tests

Test	Class A	Class B	Class D	AmpC + Porin Mutation	ESBL + Porin Mutation
Modified Hodge	+	+	+	+/–	+/–
Meropenem + PBA	+	–	–	+/–	–
Meropenem + Cloxacillin	–	–	–	+/–	–
Meropenem + DPA	–	+	–	–	–
Imipenem + EDTA	–	+	–	–	–

Adapted from reference 127.

Reporting. *Enterobacteriaceae* should be reported as resistant to carbapenems based on the revised CLSI MIC breakpoints and corresponding disk diffusion zone diameters. *Enterobacteriaceae* isolates that demonstrate carbapenem resistance should be referred to a reference laboratory for confirmatory testing. Laboratories should have policies and procedures for extended susceptibility testing of alternate drug therapy (tigecycline, colistin) for multidrug-resistant strains, and/or immediate referral to a reference laboratory where such testing can be urgently done.

Detection of Fluoroquinolone Resistance in *Salmonella* spp. Salmonellosis is one of the main causes of bacterial enterocolitis transmitted through the fecal-oral route by ingestion of contaminated food or water. Nontyphoidal salmonella are estimated to cause approximately a million cases of bacterial enterocolitis per annum, and invasive cases are not infrequent, especially among infants <1 year of age, the elderly and those with immunosuppression (i.e., HIV, transplant recipients etc.).[139,367] Enteric fever is a serious illness with bacteremia caused by *Salmonella enterica* serovars Typhi and Paratyphi, which mainly occurs in underdeveloped countries with poor standards of hygiene and sanitation, but it is also a common cause of fever in the returned traveler in developed countries.[7,447] Antimicrobial treatment of salmonellosis is essential in these types of patients, but recently increased antimicrobial resistance to key therapeutic agents has been documented in *S.* Typhi and nontyphoidal salmonellae.[138,558] The WHO guidelines (2003) currently recommend the fluoroquinolones as the treatment of choice for uncomplicated enteric fever in adults (www.who.org). The reduced fluoroquinolone susceptibility (MICs of 0.12 to 1 μg/mL) of *S.* Typhi and extraintestinal *Salmonella* infections is a concern because the clinical response rates to ciprofloxacin are poor for these strains.[138]Most fluoroquinolone resistance in *Salmonella* was due to several single base pair mutations

in the DNA gyrase (*gyr*A) gene.[52] A recent study in 13 European countries of *Salmonella* and *E. coli* isolates from animals, humans, food, and the environment, however, showed that plasmid-mediated fluoroquinolone resistance genes (PMQR) were found in 59% of the *Salmonella* isolates.[641]

Previous methods recommended by the CLSI may not be optimal for detection of *Salmonella* strains that are fluoroquinolone resistant due to acquisition of a PMQR, particularly strains that have acquired aac(6′)-1B-cr determinants. Nalidixic acid testing has been used to screen for fluoroquinolone resistance in *Salmonella*, and is a reliable method for isolates that have decreased susceptibility because of one or more gyrase (*gyr*A, *gyr*B) mutations. But an MIC test should be done on *S.* Typhi and extraintestinal *Salmonella* isolates in order to enhance detection on strains carrying a plasmid-encoded fluoroquinolone resistance gene.[121,122]

CLSI currently recommends that susceptibility testing should be done for typhoidal *Salmonella* (*S.* Typhi and *Salmonella* Paratyphi A–C) and on isolates from extraintestinal and intestinal sources.[121,122] But routine susceptibility testing is not indicated for nontyphoidal *Salmonella* spp. isolated from intestinal sources because enterocolitis is a self-limited illness in healthy children and adults. Table 17-33 outlines the revised CLSI guidelines for fluoroquinolone testing of *Enterobacteriaceae* using either disk diffusion or an MIC method. The CLSI MIC breakpoints for *Enterobacteriaceae* susceptibility to fluoroquinolones other than *S.* Typhi and extraintestinal *Salmonella* spp. remain unchanged. However, CLSI has lowered the MIC breakpoints and corresponding zone diameters for fluoroquinolone (ciprofloxacin, levofloxacin, and ofloxacin) testing *S.* Typhi and extraintestinal *Salmonella* spp. in order to enhance detection of strains that may have acquired PMQR.[268] The revised ciprofloxacin breakpoints should also be used to test corresponding enteric *Salmonella* isolates recovered from patients with enteric fever or invasive salmonellosis. However, of the commercial antibiotic susceptibility testing systems, only Etest has incorporated the new CLSI fluoroquinolone breakpoints. CLSI, therefore, recommends the continued use of nalidixic acid to test for reduced fluoroquinolone susceptibility in *Salmonella* until laboratories can implement the MIC interpretive criteria for ciprofloxacin, levofloxacin, and ofloxacin resistance.[121,122]

TABLE 17-33 Revised CLSI Ciprofloxacin Zone Diameter and MIC Criteria Breakpoints for *Enterobacteriaceae* and *Salmonella typhi* and Extraintestinal *Salmonella* spp.

Antimicrobial	Disk Diffusion			MIC		
	S	I	R	S	I	R
***Enterobacteriaceae* and *Salmonella* spp.**						
Nalidixic acid	≥19	14–18	≤13	≤16	–	≥32
Ciprofloxacin	≥21	16–20	≤15	≤1	2	≥4
Levofloxacin	≥17	14–16	≤13	≤2	4	≥8
Extraintestinal *Salmonella* spp.						
Ciprofloxacin[a]	≥31	21–30	≤20	≤0.06	0.12–0.5	≥1

[a] *S. typhi* and extraintestinal *Salmonella* spp. should be tested against ciprofloxacin using the revised criteria. Nalidixic acid may not detect all mechanisms of fluoroquinolone resistance, and isolates that test resistant to nalidixic acid may be associated with treatment failure or delayed response in fluoroquinolone treated patients with extraintestinal infection.
S, susceptible; I, intermediate; R, resistant. Adapted from references 121 and 122.

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Reporting. Fecal isolates of *Salmonella* and *Shigella* spp. that are tested should only have ampicillin, a fluoroquinolone, and trimethoprim–sulfamethoxazole routinely reported. Extraintestinal isolates should also have a third-generation cephalosporin tested and reported, and chloramphenicol may also be tested and reported, if requested.[121,122] CLSI also recommends that laboratories continuing to screen for fluoroquinolone resistance using nalidixic acid alone should put a comment on their report that indicates that nalidixic acid-resistant strains may be associated with clinical failure or delayed response to fluoroquinolone-treatment in patients with extraintestinal salmonellosis.[121,122] First- and second-generation cephalosporins, cephamycins, and aminoglycosides are not clinically effective against *Salmonella* spp. and should not be reported although they may appear susceptible on in vitro tests. Multidrug-resistant *Salmonella* strains should immediately be referred to a public health or other reference laboratory for serotyping and extended antibiotic susceptibility testing using an MIC method.

Gram-Negative Nonfermenters

P. aeruginosa and other pseudomonads, along with *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex, and *Acinetobacter baumannii,* are the most

commonly isolated gram-negative nonfermenters worldwide. Members of this gram-negative nonfermenter group represent a unique and important threat because they are ubiquitous in the healthcare environment, cause opportunistic infections in patients who are critically ill and/or immunocompromised, and are frequently multidrug-resistant (i.e., resistant to ≥3 antibiotic classes).[307,357,386] Cystic fibrosis patients are also commonly colonized with *P. aeruginosa*, and/or less frequently *B. cepacia* complex, and multidrug-resistant strains result in a higher failure rate of initial therapy for exacerbations of infection, as well as decreased survival.[289,446] This section briefly describes the laboratory challenges of detecting recently emerging resistance problems in this nonfermenter group.

P. aeruginosa *(Other Pseudomonads)*

P. aeruginosa strains that are pan-resistant to multiple classes of antibiotics have recently developed due to several mutations that upregulate AmpC β-lactamase and efflux, decrease bacterial cell wall permeability through alterations or loss of porins, and alter the gyrase enzyme target to convey fluoroquinolone resistance.[355] However, *P. aeruginosa* clones have also disseminated to many parts of the world that have acquired plasmid-mediated resistance to several drug classes carried on an integrin gene cassette that encodes metallo-β-lactamases and multiple aminoglycoside modifying enzymes (e.g., MBL-producing *P. aeruginosa*).[130,316,330] As shown in Tables 17-29 and 17-30, class B MBL-β-lactamases are carbapenemases (i.e., IMP, VIM, GIM, SIM, SPM enzyme types) that have zinc at the active site, and these enzymes can be inhibited by EDTA.[647] In some locations, hospital outbreaks have occurred with high mortality in the affected patients due to the limited choices that remain for treatment of MBL-producing-*P. aeruginosa* infections.[445] Laboratories need to be able to reliably detect MBL-producing *P. aeruginosa* isolates so that appropriate antibiotic therapy and infection control measures can be promptly deployed.

CLSI (M100-S22) guidelines recently revised the *P. aeruginosa* MIC breakpoints for piperacillin and ticarcillin with or without a β-lactamase inhibitor to be the same as those for *Enterobacteriaceae* except for slight differences in the corresponding disk diffusion zone diameters.[120] Tam and colleagues recently studied mortality due to *P. aeruginosa* bacteremia due to isolates that had reduced susceptibility to piperacillin/tazobactam (PTZ) (MIC, 32 to 64 μg/mL) but reported as susceptible based on the "previous" CLSI breakpoints (Table 17-34); seven patients received empiric therapy with this drug.[597] Thirty-day mortality was 85.5% in the group treated with PTZ and 22.2% in the control groups who had received other drug therapy (p=0.004). Decreased susceptibility to PTZ and empirical use of this drug also remained the main risk factor for mortality in multivariate analysis after adjusted

differences in age and APACHE II score between the two groups. These investigators recommended that the old CLSI breakpoints for PTZ susceptibility for *P. aeruginosa* were too high, because strains with intermediate-level resistance were being interpreted as susceptible. CLSI originally set the breakpoints for ticarcillin, piperacillin, and their respective β-lactamase inhibitor combination drugs higher for *P. aeruginosa* than those recommended for *Enterobacteriaceae* because of the FDA drug label indications that they should be used in combination with an aminoglycoside. However, the revised CLSI breakpoints are for use of these drugs alone in a dosage regimen of at least 3 g every 6 hours.[120]

Detection of MBL-Producing *P.* aeruginosa. The CLSI has also revised the *P. aeruginosa* MIC breakpoints and corresponding zone diameters for susceptibility testing of carbapenems (doripenem, imipenem and meropenem) in 2012.[56,120]These new breakpoints were established after review of the PK/PD properties of these drugs, limited clinical data, and the current MIC distribution of clinically relevant *P. aeruginosa* species including MBL-β-lactamase producers. If laboratories have implemented the new MIC breakpoints shown in Table 17-34 or the revised zone diameters for disk testing of these drugs, confirmatory testing is no longer required before reporting carbapenem susceptibility results for *P. aeruginosa*. The revised breakpoints for these compounds have been based on specific drug dosage regimens outlined by CLSI in M100 S20-22, Table 2B-1.[120] These drug dosages are required to achieve plasma drug exposures in adults with normal renal and hepatic function, and laboratories using these criteria should provide this information to important stakeholders (i.e., infectious diseases physicians, pharmacists, infection control practitioners, and their respective committees).

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TABLE 17-34 CLSI Previous (M100-S21) and Revised (M100-S22) Breakpoints (MIC μg/mL) for *P. aeruginosa* β-Lactam/β-Lactamase Inhibitors and Carbapenems

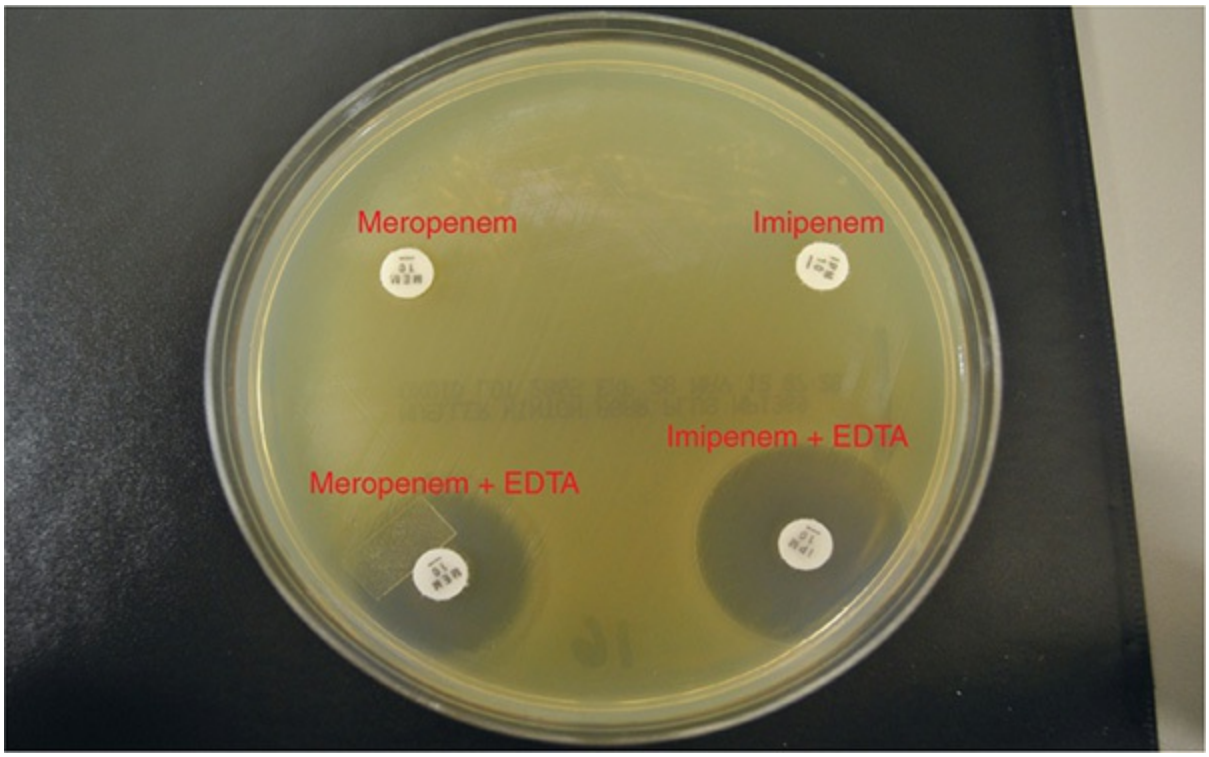
Antimicrobial	Previous (M100–S21)			Revised (M100–S22)		
	S	I	R	S	I	R
Doripenem[a]	NA	NA	NA	≤2	4	≥8
Imipenem[b]	≤4	8	≥16	≤2	4	≥8
Meropenem[b]	≤4	8	≥16	≤2	4	≥8
Piperacillin	≤64	–	≥128	≤16	32–64	≥128
Piperacillin/tazobactam	≤64/4	–	≥128/4	≤16/4	32/4–64/4	≥128/4
Ticarcillin	≤64	–	≥128	≤16	32–64	≥128
Ticarcillin/clavulanate	≤64/2	–	≥128/2	≤16/2	32/4–64/4	≥128/2

[a] Doripenem interpretive criteria for *P. aeruginosa* are based on a dosage regimen of 500 mg every 8 hour.
[b] Imipenem and Meropenem interpretive criteria for *P. aeruginosa* are based on high dose, frequent therapy (1 g every 6–8 hours).
S, susceptible; I, intermediate; R, resistant.
Adapted from CLSI M100-S21 (2011) and M100-S22 (2012)[120].

Although an MHT similar to the confirmation test described previously for detection of class B carbapenemases (i.e., KPCs) by CLSI in M100-S22 Table 2A (Fig. 17-36) may be done,[120] it performs poorly for confirmation of the presence of a MBL. Pasteran and colleagues described modifications to the MHT method by incorporating boronic acid (inhibits class A carbapenemases and AmpC enzymes) and oxacillin (inhibitor of AmpC enzymes) that had a high sensitivity (>90%) and specificity (100%) for distinguishing class A carbapenemases (KPC, GES, SME, IMI, and NMC-A) for other classes of β-lactamases (ESBLs, AmpC enzymes, and MBLs).[448] An MHT-positive test, however, may be encountered in *P. aeruginosa* isolates because of the presence of carbapenem resistance mechanisms other than carbapenemase production (Table 17-32). There are currently no CLSI-validated phenotypic tests to confirm the presence of a metallo-β-lactamase in *P. aeruginosa*. Isolates that demonstrate carbapenem resistance should be referred to a reference laboratory for confirmatory testing.

The recommended phenotypic confirmation tests for detection of class A, B, and D carbapenemases in *Enterobacteriaceae* and gram-negative nonfermenters, and their potential for false-positive tests due to the presence of other β-lactamases is outlined in Table 17-32. Besides the MHT test, various combination DDT assays are used that rely on testing of carbapenem susceptibility alone and in the presence of a specific carbapenemase inhibitor. Several different types of DDT assays have been developed for detection of MBLs, but the use of meropenem disks (10 μg) or imipenim disks (10

μg) supplemented with EDTA as the enzyme inhibitor appears to work well (Fig. 17-38).[207] The Carba NP TEST II identifies carbapenemase production in *Enterobacteriaceae* and *Pseudomonas* spp., and differentiates between classes A, B and D enzymes.[168] This test is based on the detection of acidification that occurs with imipenem hydrolysis, coupled with tazobactam and EDTA as inhibitors. The Carba NP II test can be rapidly performed and has a reported sensitivity and specificity of 100%,[168] but it is not yet commercially available. Agar-based screening methods have also been described that perform well for detection of MBL where carbapenem disks are placed on inhibitor (EDTA, PBA, cloxacillin) impregnated MHA; this method also distinguishes other types of β-lactamases.[42] An MBL Etest may also be used that has imipenem on one end of the strip and imipenem plus EDTA on the other (Fig. 17-39). However, due to the limitations of phenotypic tests and the high diversity of genes encoding carbapenemases, molecular analysis is considered the "gold standard" detection method. Genotypic confirmation is performed using multiplex PCR detection for carbapenemases, and sequencing of carbapenemase genes (Fig. 17-40).[177] A commercial real-time PCR assays has been developed by Cepheid (Xpert MDRO) for the rapid, accurate screening of patients for the presence of carbapenemases (i.e., KPC or NDM enzymes) in enteric bacteria as well as a VIM enzyme (i.e., metallo-β-lactamase) in *P. aeruginosa*.[606] Evaluation of the Xpert MDRO assay compared to broth enrichment culture and sequencing of the target genes showed highly reliable detection of VIM enzymes from clinical samples.



■ **FIGURE 17-38** Confirmatory double-disk test for detection of MBL-carbapenemase in *P. aeruginosa*. (Courtesy of Calgary Laboratory Services.)

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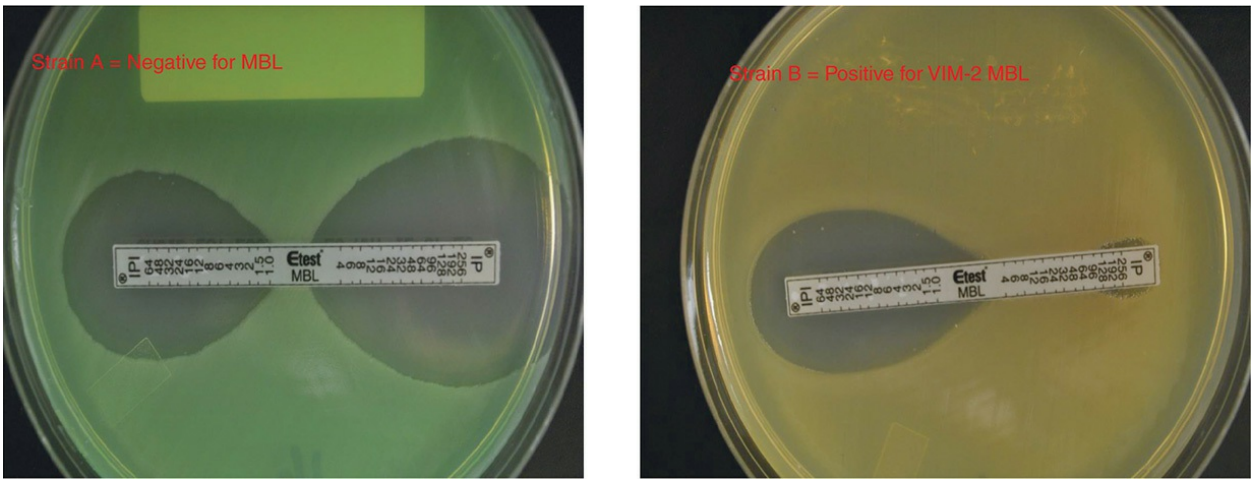
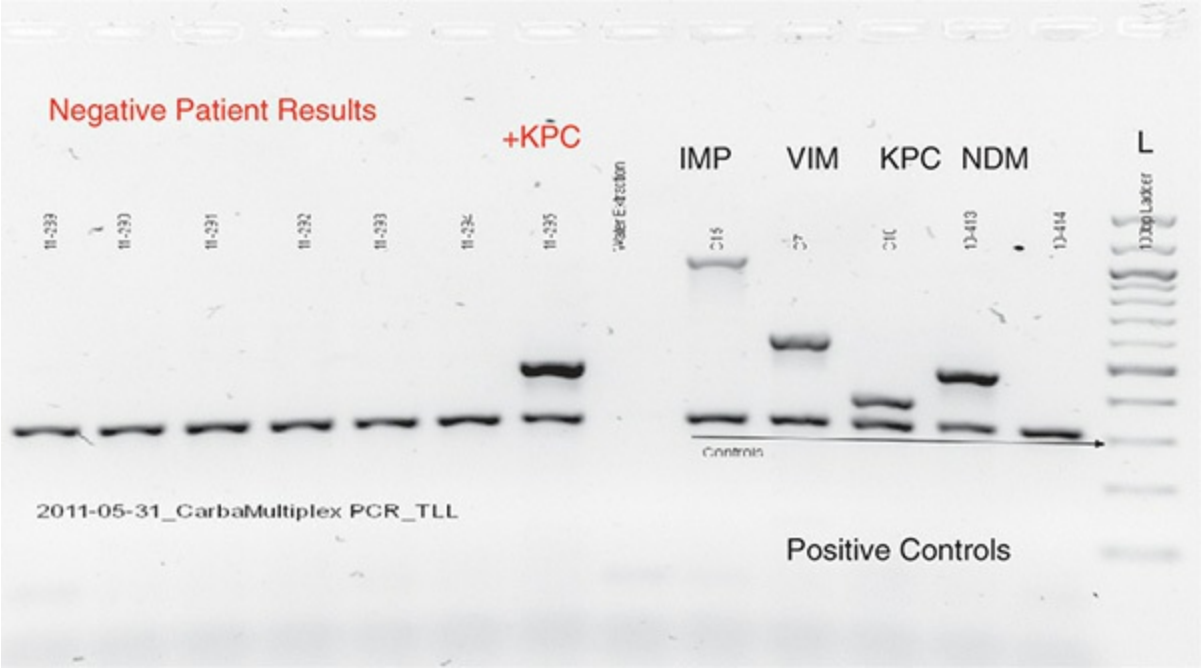


■ **FIGURE 17-39** Etest MBL confirmation in *P. aeruginosa* with a VIM-2 enzyme. (Courtesy of Calgary Laboratory Services.)



■ **FIGURE 17-40** Multiplex PCR for detection of carbapenemases. (Courtesy of Calgary Laboratory Services.)

Reporting. *P. aeruginosa* should be reported as resistant to carbapenems based on the revised CLSI MIC breakpoints and corresponding disk diffusion zone diameters. Isolates that demonstrate carbapenem resistance should be referred to a reference laboratory for confirmatory testing. Laboratories should have policies and procedures in place for extended susceptibility testing of alternate drug therapy (tigecycline, colistin) for multidrug-resistant strains, and/or immediate referral to a reference laboratory where such testing can be promptly performed.

Acinetobacter baumannii

Acinetobacter baumannii is an opportunistic pathogen that has emerged as a major cause of nosocomial pneumonia, particularly ventilator-associated pneumonia in ICU patients, bacteremia and burn wound sepsis.[312] This organism also has a propensity for transmission resulting in nosocomial outbreaks because of its resistance to desiccation and inherent resistance to disinfectants.[50,411] *A. baumannii* has transformed from being susceptible to most antibiotics three decades ago, to the current global situation where some strains are now pan-resistant to almost all available agents including carbapenems, colistin, and tigecycline. Carbapenem and pan-resistant *A. baumannii* is a major threat to patient care in many hospitals, as mortality is understandably higher for serious infections due to these strains.[312]

A. baumannii are almost universally resistant to oxyimino-cephalosporins, primarily

because of an intrinsic chromosomal AmpC β-lactamase, although a large outbreak due to ESBL-producing strains (VEB-1 type) has been reported from France.[411] Although the AmpC enzyme is only constitutively expressed at a low level, insertion of an upstream ISAab1 promoter sequence from the β-lactamase gene can result in over expression.[627] Classical TEM-1 penicillinases are also widespread in *Acinetobacter* spp.[358] All *A. baumannii* isolates also carry the gene for OXA-51-like enzymes, and intrinsic molecular class D carbapenemase, which has low-level expression, so that these strains appear susceptible to carbapenems. However, high-level carbapenemase resistance can be "switched" on by a similar mechanism as that described above for AmpC enzymes. Some clones of *A. baumannii* have been transferred from casualties coming back to hospitals in both the United Kingdom and the United States that are now endemic.[123,662] Several other virulent *A. baumannii* clones carrying other OXA carbapenemases have arisen in at least three clusters (OXA-23-like, OXA-(24)-40-like, and OXA-58) and have now widely disseminated in different parts of the world.[124,479,668] Of concern is a more recent report of NDM-1-producing *A. baumannii* isolated from a patient repatriated to the Czech Republic from Egypt.[265] Although treatment for carbapenemase-resistant *A. baumannii* is limited, colistin and tigecycline have been used.[312] Colistin is, however, the drug of choice for carbapenemase-producing *A. baumannii* infections because tigecycline achieves suboptimal serum levels and resistance may develop on therapy. Tigecycline-resistant *A. baumannii* (typed as OXA-23 epidemic clone) has recently been recovered in the United Kingdom from a patient on therapy; resistance was mediated by an Ade-ABC-mediated efflux

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mechanism.[263] Colistin-resistant *A. baumannii* isolates have also been reported initially from the Czech Republic in 1999, but have since widely spread.[92] Colistin resistance in *A. baumannii* may be difficult to accurately detect in the clinical laboratory because heteroresistance is common.

Laboratories can use either microdilution or disk diffusion to perform susceptibility testing of *A. baumannii*. The considerable differences that existed in susceptibility to carbapenem drugs based on whether one is using the EUCAST (www.eucast.org) or CLSI guidelines have been eliminated. CLSI now recommends carbapenem drug zone diameters for *A. baumannii*, and lowered MIC breakpoints for *A. baumannii* that are very similar or identical to those used by EUCAST (Table 17-35).[121,122] Similar phenotypic confirmation methods can be used to detect an MBL-type carbapenemase in *A. baumannii* as described earlier for *P. aeruginosa* including a MHT and a DDT using meropenem and imipenem disks with and without EDTA. Currently, there are no

CLSI-validated methods to reliably detect the presence of an OXA-type carbapenemase in *A. baumannii*. Detection of OXA-type enzymes relies on molecular detection of the bla_{OXA} determinant and sequencing of the gene.[668]

Susceptibility Testing of Cystic Fibrosis Isolates

Cystic fibrosis (CF) patients have chronic pulmonary colonization with a complex microbial flora that is composed of various gram-negative nonfermenters including *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex,*Achromobacter xyloxidans,* and other bacterial species. Many of these co-colonizers are also inherently resistant to antibiotics,[22,71] and in the case of *P. aeruginosa*, resistance increases after repeat exposure to broad-spectrum antibiotics during exacerbations.[180] *S. anginosus* group may also be an important emerging pathogen in some CF patients.[555] During pulmonary sampling, particularly during exacerbations, most patients have one or more morphotypes of *P. aeruginosa* isolated from lower respiratory specimens (i.e., sputa and bronchoalveolar lavages). Much effort and laboratory resource goes into isolating and performing susceptibility testing on individual CF lung isolates but this is far from being a precise science. CLSI breakpoints that were developed for parenteral antibiotic therapy for example, may not accurately predict susceptibility of *P. aeruginosa* to the same agents given by inhalation.[401] Although CLSI recommended methods and guidelines should be used to interpret the results of individual isolate testing, these results may not accurately predict clinical outcome in CF patients for several reasons outlined below.[272]

The complex microbial community inhabiting the lungs of CF patients mainly exists within biofilms, and not as planktonic forms. Studies of *P. aeruginosa* biofilms in the respiratory tract of CF patients clearly show that aggressive antibiotic therapy restrains, but doses not eradicate infection from the inflammatory conductive zone.[44] Methods have been developed for biofilm susceptibility testing (BST) that were recently evaluated in a multi-centered randomized trial that assessed the clinical outcomes of an antibiotic regimen based on the results of the BST compared to conventional susceptibility testing.[402] Study groups showed no significant difference in mean decreases in bacterial density in sputa or mean increases in forced expiratory volume in 1 sec (FEV1), but only a small number of patients were enrolled. Larger numbers of patients need to be assessed in an adequately powered clinical trial before discounting the predictive value of BST methods in this unique clinical situation.

Because *P. aeruginosa* and other gram-negative nonfermenters are also frequently multidrug-resistant, most CF patients are empirically treated with combination antibiotic therapy administers both in parenteral formulation and via inhalation into the lungs. Specialized laboratories have, therefore, developed methods to perform multiple

combination bactericidal synergy tests (MCBT) on CF isolates in order to determine which combination regimen may provide synergistic or additive activity. Only one randomized, double-blinded, controlled trial has, however, been recently completed to assess the clinical outcomes of CF patients treated with antibiotic therapy based on conventional versus MCBT susceptibility testing.[1] Antibiotic therapy directed by MCBT did not result in improved clinical or bacteriologic outcomes compared to conventional susceptibility methods.

Most clinical laboratories that perform routine susceptibility testing on CF pulmonary isolates do so using conventional methods. Conventional susceptibility tests of CF *P. aeruginosa* isolates is particularly challenging because they have a highly mucoid phenotype, and often grow insufficiently to determine a valid result unless incubation is extended for a full 48 hours. When the disk diffusion test was compared to an agar dilution method, poor correlation was observed when mucoid strains were tested against some antibiotics although the interpretive category agreed well between the two methods.[84] For these reasons, disk diffusion testing is not recommended for susceptibility testing.

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Although the Etest (AB Biodisk, Solna, Sweden) correlated well with the microdilution method in one study for both for mucoid and nonmucoid isolates, although a small number of very major errors occurred.[84] A recent study compared both the CLSI disk diffusion method and the Etest to broth microdilution for testing a large cohort of isolates from CF patient and non-CF patients.[62] An unacceptable rate of very major and major errors was found for various antimicrobials tested against both CF and non-CF isolates using either the disk diffusion method or Etest.

TABLE 17-35 Comparison of Old and Current CLSI Versus EUCAST Breakpoints (MIC μg/mL) for *Acinetobacter* spp.

	Old			*Current*			*EUCAST*		
Antimicrobial	**S≤**	**I**	**R≥**	**S≤**	**I**	**R≥**	**S≤**	**I**	**R>**
Doripenem	–	–	–	2	4	8	1	–	4
Imipenem	4	8	16	2	4	8	2	–	8
Meropenem	4	8	16	2	4	8	2	–	8

S, susceptible; I, intermediate; R, resistant.
Adapted from references 121 and 122.

Broth microdilution or agar dilution should be used for testing CF isolates, although multiple methods may need to be compared when major discrepancies occur in the susceptibility profile of an isolate from a patient from one visit to the next. Reference methods may also not be reliable for every CF patient isolate. Although broth microdilution compared well with a reference agar dilution technique for *Pseudomonas aeruginosa*, discordant results were found significantly more frequently in isolates from patients with CF than in other patients.[539] Vitek 2 nonfermenting gram-negative cards and version 4.02 software was recently validated for routine clinical use for susceptibility testing of nonfermenting gram-negative rods from patients with CF.[440] The overall essential and categorical agreement of Vitek 2 susceptibility testing was 97.6% and 92.9%, respectively. Direct comparison of the BD Phoenix system with the MicroScan Walkaway system for susceptibility testing of nonfermentative gram-negative bacteria showed an overall agreement of 89.1%, and although there were no very major errors, the rate of major and minor errors was 0.5% and 7.7%, respectively.[561]

Both *B. cepacia* complex species and *S. maltophilia* have inherent high-level resistance to several major classes of antimicrobials. *B. cepacia* complex species are intrinsically resistant to aminoglycosides and polymyxins. Active efflux also mediates resistance to fluoroquinolones, chloramphenicol and trimethoprim, and the presence of inducible β-lactamases in concert with altered PBPs confers resistance to most β-lactams.[22,484] CLSI MIC breakpoints are therefore only available for minocycline, ceftazidime, meropenem, and trimethoprim–sulfamethoxazole. *S. maltophilia* is intrinsically resistant to β-lactam agents due to the presence of two β-lactamases, one of which is a metallo-enzyme (L1) that confers resistance to imipenim. Broad resistance to aminoglycosides and fluoroquinolones also occurs due to decreased permeability through the OM.[71] CLSI MIC criteria are therefore only available for trimethoprim–sulfamethoxazole, minocycline, ceftazidime, ticarcillin clavulanate, and fluoroquinolones. Although many laboratories have not routinely performed susceptibility testing on CF *B. cepacia* or *S. maltophilia* isolates other than TMP-SMX as the drug of choice, increasing resistance to this agent dictates the need for broader testing.[22,617] All of the CLSI recommended antibiotics should be tested and reported on either of these organisms when isolated from CF patients with pulmonary exacerbations. *S. maltophilia* isolates from CF patients with hypersensitivity to TMP-SMX or those found to be resistant to this agent should also be tested against tigecycline and colistin.[187,403] Colistin may need to be tested against *S. maltophilia* using agar dilution as broth microdilution and Etest have high rates of major error.[403] Few agents are left that effectively treat *B. cepacia* complex infections due to TMP-SMX-resistant strains, but β-lactams alone or in combination may be the most effective regimens.[22]

Bacterial Agents of Bioterrorism

Although CLSI has published guidelines for susceptibility testing of potential bacterial agents of bioterrorism (i.e., *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Burkholderia mallei*, *Burkholderia pseudomallei,* and *Brucella* spp.) public health should be immediately notified as soon as these isolates are presumptively identified, and confirmation of their identification and susceptibility testing is only available in reference or public health laboratories.[114] Wherever there is a potential for aerosolization to occur when working with these organisms in the clinical laboratory, one should work in a BSL-3 cabinet and use BSL-3 containment practices including personal protective equipment.

Antibiotic Susceptibility Reporting

The timely and effective communication of antimicrobial results is a critical stage in the susceptibility testing process. Box 17-7 outlines some of the clinical principles that should govern the development of laboratory policy and procedure for the testing and selective reporting of antimicrobial agents from clinical specimens from all sites/sources. These general guidelines can be applied to implementing detailed reporting protocols for specific pathogens isolated from different sites/sources, and the cascading or selective reporting of agents within a given antibiotic class based on the susceptibility profile of the isolate. Laboratories play a critical role in directing the appropriate use of antibiotics. Major time and effort should be go into the development of clinically relevant policies and procedures that ensure that appropriate effective guidance is provided by the laboratory in all clinical situations. This section outlines the formatting, selective reporting and generation of reporting comments that will assist in development of such procedures.

Formatting Antibiotic Susceptibility Reports

Antibiotic susceptibility results should be reported in a standardized format. Smaller laboratories that manually report results should use a standardized form, transcription format and ink color that can be legibly read when the report is faxed and printed. Most laboratories use a laboratory information system for generating susceptibility reports. Decisions need to be made about the format for the susceptibility reports at the time the LIS is implemented. Although most systems allow both the quantitative results (i.e., zone diameter or MIC value) and the interpretive result of susceptible (S), intermediate (I), and resistant (R) to be reported, most stakeholders only need to know the interpretive result to select appropriate therapy. The quantitative result should

never be reported without an interpretation because most clinicians are not familiar with CLSI interpretive guidelines, and do not have time to look up and interpret the quantitative values for each drug on each isolate. In most cases, the preferred report format is to only report the interpretative results (S, I, or R) for each drug for each pathogen isolated from a given specimen. However, the laboratory should be able to provide the quantitative results upon request to clinicians, particularly the Infectious Diseases service.

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BOX 17-7

Guidelines for Antibiotic Reporting

- CLSI designated Group A (Primary test and report) agents should be routinely tested and reported.
- Antibiotic susceptibility reports **should include** an interpretation for each drug of S, I, or R.
- Antibiotics **should not be reported** to which the patient is allergic.
- Specific antibiotics **should not be reported** in neonates, infants, and young children who are potentially toxic (i.e., trimethoprim-sulfamethoxazole, chloramphenicol, fluoroquinolones).
- Antibiotics that do not cross the blood–brain barrier **should not be reported** on isolates from cerebrospinal fluid samples.
- CLSI designated Group U (only for treatment of urinary tract infections) **should not be reported** on isolates recovered from any other source except urine.
- Policies and procedures for cascade reporting of antibiotics within each drug class should be implemented and based on these factors:
  - Site/source of the infection
  - Resistance profile of the isolate
  - Available antibiotic formulary and recommended utilization guidelines
- Isolate testing of extended antibiotic panels for CLSI designated Group B and C agents should routinely be performed in the following situations:
  - Patient is allergic to one or more Group A drugs;
  - Group A drugs are not in your formulary; or
  - Isolate is multiple resistant to one or more Group A agents and/or MDR strain (≥3 antibiotic classes).
  - Upon clinical request
- Policies and procedures for either in-house testing or immediate referral to a reference laboratory should be implemented for confirmation of the isolate identification, and resistance mechanism in MDR isolates.

The report should also clearly label the antibiotic susceptibility results with the pathogen identification so there is no clinical confusion as to which results align with which isolate. The report should be formatted in such a way that the antibiotics and interpretive results are aligned for different types of pathogens isolated from a polymicrobial sample. Laboratories should also validate that the report format stays integral when sent across an electronic interface between the LIS and a hospital or of physician-office information system. Antimicrobial reporting format error or clinical confusion in reading a complex susceptibility report may be a major source of inappropriate antibiotic prescription.

Clinically Relevant Antibiotic Reporting

Antibiotic susceptibility results should only be tested and reported on the organisms considered to be pathogens. In most clinical situations, the reporting of an isolate with a susceptibility result indicates to the clinician that the laboratory considers the isolate to be clinically relevant, and treatment may be required. Reporting of antibiotics on commensal bacteria may therefore lead to inappropriate antibiotic prescription.

Automated broth microdilution methods allow the laboratory to test many more antibiotics than appropriate for routine reporting. The CLSI guidelines should be used to direct the reporting of appropriate agents based on the organism and the site/source of infection (Tables 17-7 and 17-8).[114,121,122] Antibiotics not recommended for reporting on specific organisms should not be automatically reported even though an in vitro result was generated by an automated system or panel (Table 17-7). Tables 17-7 and 17-21 also outline situation where a surrogate antibiotic may be used to predict susceptibility to alternate agents within a drug class (i.e., β-lactams). A few examples of inappropriate antibiotic reporting of this type are provided. Although TMP-SMX is included in many test panels for gram-negative nonfermenters, this agent should not be reported on *P. aeruginosa*. *Enterobacteriaceae* known to carry an AmpC β-lactamase should have ampicillin results suppressed regardless of the in vitro susceptibility result. TMP-SMX and clindamycin should also not be reported on enterococci. Antibiotic susceptibility reports in total, or in part should be audited by clinical microbiologists and/or medical staff and pharmacy to ensure that laboratory antibiotic reporting policies are being followed.

Only antimicrobial agents appropriate for the site/source of infection should be included in a susceptibility report. This means that a substantially different antibiotic panel of drugs will be tested and potentially reported for invasive isolates from sterile fluids, tissues and blood cultures than for localized infections. Mainly potent parenteral antibiotics will be reported on isolates from invasive infections because the use of an oral agent would be clinically inappropriate for initial therapy. Oral drugs that have

been designated as Group U by CLSI for use against localized urinary tract

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infection should only reported on isolates from urine cultures. Drugs that are active only in the urinary tract should not be reported on isolates from other sites. Antimicrobial agents that do not penetrate into the site of infection should not be reported for organisms isolated from that site. Tigecycline, for example, does not penetrate well into the urinary tract and should not be reported on urine culture isolates. Daptomycin does not work well in the lung due to inactivation by surfactant, and this drug should not be reported on MRSA isolated from lower respiratory specimens. Similarly, first- and second-generation cephalosporins do not cross the blood–brain barrier and should not be reported on *Streptococcus pneumoniae* isolated from CSF; in this clinical situation, CLSI recommends testing and reporting penicillin, cefotaxime, ceftriaxone, meropenem, and vancomycin.[121,122]

There are also some general principles that should be medically followed in selectively reporting antibiotics in specific patient circumstances (Box 17-7). Antibiotic reporting should be tailored to drugs appropriate for the patient. If the laboratory is provided with a clinical history of antibiotic allergy to a specific agent or agents, then reporting of these drugs should be suppressed for that individual. For example, penicillin should not be reported on β-hemolytic streptococci isolated from a patient with a reported penicillin allergy. Some drugs such as fluoroquinolones should also not be prescribed in infants and young children, and routine reporting of these agents should be suppressed. Finally, many drugs may be teratogenic or toxic in pregnant women, especially in the first trimester.[415] Laboratories may decide to suppress reporting of these agents when an indication of pregnancy and/or gestation is provided.

Selective Reporting of Antibiotics

Laboratories should only test and report antibiotics that are locally available on formulary in consultation with members of the medical staff and pharmacy. Laboratories should then work in consultation with local medical experts such as infectious diseases physicians and pharmacists to decide which antibiotic susceptibility results to routinely report for a given isolate and site/source of infection, and which to suppress. The purpose of selective antibiotic reporting is to preserve the clinical utility of broad-spectrum or newer agents when an isolate is susceptible to narrower spectrum agents. Some laboratories may also elect to report the cost per dose either on each susceptibility report, or as part of a local guide to antibiotic therapy. In general, antibiotics that have been available for a longer period of time are less expensive, and selective reporting is also cost-effective. Aside from the antibiotic susceptibility rules provided and flagged by automated broth microdilution systems, laboratories can also

build additional rules either into the artificial intelligence software of the instrument or the LIS, to assist with tailoring antibiotic reporting to conform to local policies.

Table 17-36 shows a selective antibiotic reporting policy for *S. aureus* isolated from a blood culture or central nervous system (CNS) site. In the case of a fully susceptible strain, the report would only include the primary agents listed. However, for an MRSA isolate, extended reporting of newer agents such as linezolid and daptomycin may also be released. Table 17-37 shows a selective antibiotic reporting policy works for *Enterobacteriaceae* isolated from a nonurine site/source. In the case of a fully susceptible strain, the report would only include the primary agents listed. However, for more resistant strains, secondary drugs would also be reported, and in the case of a multidrug- or pan-resistant strain, the report would also include most select agents depending on the site/source of infection. Selective testing and reporting policies must be constantly reviewed and clinically updated as new antibiotic resistance trends evolve, or the local formulary is changed.

TABLE 17-36 Selective Reporting of Antimicrobial Agents for an *S. aureus* Isolate From a Blood or Central Nervous System Culture

Antibiotics	Primary	Secondary	Selective	Comments
Penicillin	X		X	If S, then do not report without performing a β-lactamase test. May only be reported if requested by the physician.
Oxacillin	X			OXA result may be based on Cefoxitin result. If R, then Cefazolin is R. Report additional secondary and selective agents.
Cefazolin	X			Test and report on all isolates. Report as S if OXA is S.
Vancomycin	X		X	Test on all isolates. May only report on MSSA isolates from β-lactam allergic patients and MRSA isolates.
Trimethoprim–sulfamethoxazole		X		Do not report on CNS isolates
Tetracycline		X	X	Do not report on CNS isolates. Report only on MRSA isolates from blood cultures. Do not report on children, pregnant women.
Linezolid			X	Test and report on MRSA isolates
Daptomycin			X	Test on MRSA isolates. Report only on request of the infectious diseases physicians.
Ceftaroline			X	Test and report on MRSA isolates. Report only on request of the infectious diseases physicians.

TABLE 17-37 Selective Antibiotic Reporting for *Enterobacteriaceae* Nonurine Isolates

Antibiotics	Primary	Secondary	Selective	Comments
Ampicillin	X			If I/R, then test AMX/CLA.
Amoxicillin clavulanate		X		If AMP I/R, then test. Report selectively by site. Do not report on CNS isolates.
Piperacillin–tazobactam		X		If AMP I/R, then test. Report selectively by site. Do not report on CNS isolates.
Cefazolin	X			If I/R, then report AMP I/R. Do not report on CNS sites.
Gentamicin	X			If I/R, then test Tobramycin.
Tobramycin		X		If I/R, then test Amikacin.
Cefoxitin	X			Test to screen for AmpC β-lactamase. Do not report.
Ceftriaxone	X			If I/R for *E. coli, Klebsiella,* or *P. mirabilis,* then may be ESBL-producer.
Ceftazidime	X			If I/R for *E. coli, Klebsiella,* or *P. mirabilis,* then may be ESBL-producer.
Trimethoprim-sulfamethoxazole	X			Do not report on CNS sites.
Meropenem	X			If I/R, then may be a carbapenemase-producer.
Ciprofloxacin	X			Report on blood only. Do not report on children.
Colistin			X	Test and report on MDR isolates.
Tigecycline			X	Test and report on MDR isolates. Do not report on blood isolates.

Cumulative Antibiogram Reporting

Most clinical decisions about initial antibiotic prescription are made empirically, because the decision cannot await isolation and testing of a bacterium. To improve the adequacy of empiric prescription pending a detailed susceptibility report, knowledge of local susceptibility patterns is essential. Cumulative antibiotic susceptibility reports for various locations should be compiled and published on an annual basis but interim updates may also be provided for critical care units. Although national surveillance databases track important resistance trends in major pathogenic bacterial species,[101] their results may not accurately reflect what is happening locally, particularly in ambulatory practice.[230]

The CLSI has published recommendations for the methodology and format of compiling cumulative antibiotic susceptibility summaries (i.e., antibiogram).[112,252] Recommendations for the preparation and use of cumulative antibiogram are summarized in Table 17-38. Several additional factors worth noting are summarized in Box 17-8. If a local website or shared access to an electronic file is available, posting of the spreadsheet is a convenient way to provide general access. It may be possible to download the information into personal data assistants (PDAs). Examples of CLSI M39-A2 compliant antibiograms are shown from a large tertiary acute-care hospital (Fig. 17-41) versus all ambulatory care locations (Fig. 17-42) for a large Canadian regional health authority (www.calgarylabservices.com). It may also be useful to provide a hard and electronic copy to clinicians; a useful format is a pocket card that can be carried by the physician and/or downloaded onto an electronic device for easy access.

Antibiotic Stewardship

Antibiotic stewardship is the prudent and efficacious use of these agents by healthcare professionals including the use of the most appropriate antibiotic given in the correct dose and route, administered for a long enough duration to treat infection in an individual patient. Inappropriate antibiotic use including overuse or misuse (e.g., suboptimal dose) is a major factor contributing to the rapid rise in drug resistance and healthcare costs, and suboptimal patient outcomes.[101,131,372] Antibiotics are also not without risk, and patients may experience adverse drug-related complications and/or the development of *C. difficile*-mediated antibiotic diarrhea. Antibiotic stewardship program committees (ASPs) function as a specialized multidisciplinary subcommittee of Pharmacy & Therapeutics (P&T) programs within hospitals, whose aim is to determine antibiotic formulary, to improve clinical outcomes, and to reduce costs by minimizing the inappropriate use of antibiotics. The Infectious Disease Society of America (IDSA) recently published guidelines for the development of a hospital-based ASP.[153] Core membership of the multidisciplinary ASP committee should include as a minimum, an infectious diseases physician and a clinical pharmacist with infectious diseases training who should direct the program. Ideally, a clinical microbiologist, an information system specialist, and infection control practitioner and a hospital epidemiologist should also be recruited. Collaboration of the ASP committee members with infection control and the P&T committee is essential. Hospital administration must also support and collaborate with the ASP so that the necessary resources can be put in place to implement various elements of the program. Similar to infection control programs, the ASP should function within the hospital under the auspices of a quality assurance program within the areas of patient safety and risk management. Specific expectations should be set for the ASP, including both clinical and economic outcomes, and the necessary infrastructure must be put in place to prospectively measure antimicrobial use and to monitor quality outcomes. Creation of relational laboratory and healthcare system databases allows the evaluation of process interventions and outcome measurements as a routine quality indicator. Without access to an electronic medical record, compilation and evaluation of program outcomes is less feasible.

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TABLE 17-38 CLSI M39-A3 Recommendations for Cumulative Antibiogram Preparation and Use

Recommendations	Comments

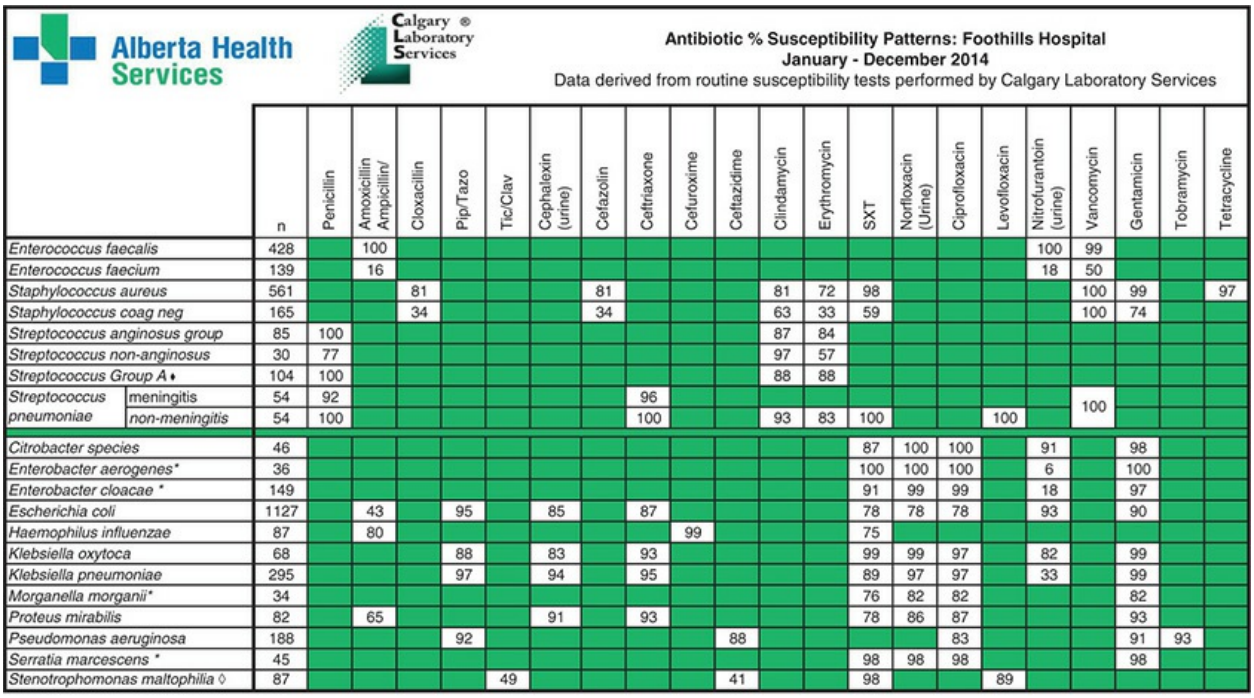
Use CLSI M39-A2 to compile antibiogram	Published guideline should be used because data will be used to guide initial empiric therapy.
Analyze and present data at least annually	Small isolate sample size may not provide an accurate antibiogram profile.
Include diagnostic, not surveillance isolates	Surveillance isolates may skew the data (i.e., tend to be more resistant).
Include results only for drugs that are routinely tested	If some drugs included in the cumulative antibiogram are only tested on selected isolates, the data will be skewed.
Include the first isolate per patient in the period analyzed, irrespective of the body site from which the specimen was obtained or the AST pattern	If repeat isolates are not eliminated from the analysis, the percentage susceptible will in most cases be lower than if repeat isolates are eliminated.
Calculate the percentage susceptible. Do not include the percentage of isolates with intermediate susceptibility	The percentage susceptible for a specific drug–pathogen combination will be impacted by culturing practices, patient populations, specimen collection practices, and laboratory antimicrobial susceptibility testing policies. Because the data will be used to guide initial empiric therapy, only drugs that are susceptible should be chosen.
For *Streptococcus pneumoniae*, calculate and list both the percentage susceptible and the percentage of isolates with intermediate susceptibility for penicillin; calculate and list the percentage susceptible for cefotaxime or ceftriaxone using both the meningitis and nonmeningitis breakpoints	Assists with empiric antibiotic therapy based on site/source of infection; CLSI M100 S23 breakpoints differ for penicillin, cefotaxime, or ceftriaxone based on diagnosis.278
For viridians streptococci, calculate and list both the percentage susceptible and the percentage of isolates with intermediate susceptibility for penicillin	In addition to the percent S to penicillin (e.g., 80%), include the percent I in a footnote (e.g., "For the 20% of nonsusceptible isolates, 15% were intermediate [MIC 0.25–2 μg/mL] and 5% were resistant [MIC 4 μg/mL] to penicillin").
For *Staphylococcus aureus*, calculate and list the percentage susceptible for all isolates, as well as for the subset of MRSA	Many MRSA isolates will have lower percent S to non–β-lactam antistaphylococcal agents than MSSA isolates.

Adapted from CLSI M39-A3 and reference 252.

BOX 17-8

Guidelines for Compiling a Cumulative Antibiotic Susceptibility Report

- Duplicate results should clearly be excluded; only the first isolate of a particular bacterium from a particular patient in a given time period (e.g., 1 year) be included in the analysis, regardless of other characteristics of the isolates.
- If possible, outbreak isolates that are clonally related should be excluded in order to not bias the antibiogram profile of a particular pathogen.
- Group A and B agents for each organism should be included; exclude selectively tested drugs.
- Differentiate the cumulative antibiogram profile for important isolates by invasive versus localized body sites and/or specimens sources for all locations and patient groups.
- Compile antibiogram profiles for different facility locations (i.e., ICU vs. other hospital wards), and different regional locations (i.e., different hospitals vs. ambulatory care) if there is a sufficient number of isolates to do so.
- Analyze isolate data separately for the elderly (i.e., long-term-care facilities), and hospitalized adults and pediatric patients to determine difference in resistance trends for these patient groups.
- A concise format should be used that is easy for clinicians and other stakeholders to access and read (i.e., electronic reporting is preferred).
- Data should be updated at regular intervals; a cumulative antibiogram should be published annually, or more frequently for ICUs.



Alberta Health Services

Calgary Laboratory Services

Antibiotic % Susceptibility Patterns: Foothills Hospital
January - December 2014
Data derived from routine susceptibility tests performed by Calgary Laboratory Services

	n	Penicillin	Amoxicillin Ampicillin/	Cloxacillin	Pip/Tazo	Tic/Clav	Cephalexin (urine)	Cefazolin	Ceftriaxone	Cefuroxime	Ceftazidime	Clindamycin	Erythromycin	SXT	Norfloxacin (Urine)	Ciprofloxacin	Levofloxacin	Nitrofurantoin (urine)	Vancomycin	Gentamicin	Tobramycin	Tetracycline
Enterococcus faecalis	428		100															100	99			
Enterococcus faecium	139		16															18	50			
Staphylococcus aureus	561			81				81				81	72	98					100	99		97
Staphylococcus coag neg	165			34				34				63	33	59					100	74		
Streptococcus anginosus group	85	100										87	84									
Streptococcus non-anginosus	30	77										97	57									
Streptococcus Group A ♦	104	100										88	88									
Streptococcus pneumoniae meningitis	54	92							96										100			
Streptococcus pneumoniae non-meningitis	54	100							100			93	83	100			100					
Citrobacter species	46													87	100	100		91		98		
*Enterobacter aerogenes**	36													100	100	100		6		100		
Enterobacter cloacae *	149													91	99	99		18		97		
Escherichia coli	1127		43		95		85		87					78	78	78		93		90		
Haemophilus influenzae	87		80							99				75								
Klebsiella oxytoca	68				88		83		93					99	99	97		82		99		
Klebsiella pneumoniae	295				97		94		95					89	97	97		33		99		
*Morganella morganii**	34													76	82	82				82		
Proteus mirabilis	82		65				91		93					78	86	87				93		
Pseudomonas aeruginosa	188				92						88					83				91	93	
Serratia marcescens *	45													98	98	98				98		
Stenotrophomonas maltophilia ◊	87					49					41			98			89					

* These organisms usually produce inducible ß-lactamase which cause failure of 3rd generation ß-lactam therapy, despite in vitro susceptibility
♦ Susceptibility testing not routinely performed. Results based on all GAS isolated from adult throat cultures at CLS where susceptibility testing was indicated for treatment purposes.
◊ Stenotrophomonas maltophilia results represent the whole Calgary in-patient population.
Note: Percent susceptible for each organism/antimicrobial combination was generated by including the first isolate of that organism recovered from a given patient. Only those species with at least 30 isolates are included in the antibiograms.
Note: Please refer to the Community antibiogram for any isolate not listed.

■ **FIGURE 17-41** Cumulative antibiogram for a large tertiary care hospital. (Courtesy of Calgary Laboratory Services.)

Table 17-39 outlines several elements of an effective ASP that should be implemented in whole or in part depending on local policy, needs, and resources.[153] However, the optimal strategies for the prevention and control of antimicrobial resistance within a hospital have not been fully established as yet because few randomized, controlled trials have been done for various interventions. As a minimum, as ASP should actively monitor rate of resistance for key organisms (i.e., MRSA, VRE, multidrug-resistant gram-negatives), implement strategies to minimize inappropriate drug use and work closely with infection control to minimize the secondary spread to resistance. Most physicians do not receive adequate training in the diagnosis of infectious diseases and the prescription of antibiotics. Education is, therefore, one of the most essential components to an ASP.

The clinical microbiology laboratory plays a critical support role to both the hospital infection control and antimicrobial stewardship programs. As discussed earlier in the chapter, the timely and accurate identification of key bacterial pathogens and clinically important antibiotic resistance profiles are central to the effective delivery of both of these quality assurance programs. Generation of local antibiogram(s) with pathogen-specific susceptibility data is also critical to the development of appropriate treatment

recommendation for empirical therapy of various types of infection. Genotyping of important antibiotic-resistant strains to track clonal spread is also a key function of the laboratory. Although not required for routine patient care, the molecular characterization of specific mechanisms of resistance in important pathogens is a key requirement for effective surveillance and the investigation of outbreaks. For example, molecular typing of all MRSA isolates within a healthcare jurisdiction can determine whether the strains are hospital-acquired or community-acquired, or community-acquired but endemic in a given facility due to secondary spread.[149] Likewise, epidemiologic investigation of the spread of ESBL-producing *E. coli* in a large regional health authority identified travel to Asia as one of the major causes for the introduction and spread of this agent as a cause of urinary tract infections.[470]

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Alberta Health Services — Calgary Laboratory Services

Antibiotic % Susceptibility Patterns: Calgary Community
January – December 2013
Data derived from routine susceptibility tests performed by Calgary Laboratory Services

	n	Penicillin ♦	Ampicillin/ Amoxicillin	Cloxacillin	Pip/Tazo	Cephalothin/ Cephalexin	Cefazolin	Ceftriaxone	Ceftazidime	Ceftazidime	Clindamycin	Erythromycin	SXT	Norfloxacin (Urine)	Ciprofloxacin	Levofloxin	Nitrofurantoin	Vancomycin	Gentamicin	Tobramycin	Gent synergy †	Tetracycline
Streptococcus anginosus group	93	100									87	79										
Group A Streptococcus #	210	100									94	89										
Enterococcus faecalis	250		100														98	100			81	
Enterococcus species ¥	1345		99												84		97	100			84	
Staphylococcus aureus	2932	0		81			81				83	70	94					100	99			95
Staphylococcus coag neg	281	13		78			78				80	73	85					100	95			
Streptococcus pneumoniae ‡♦	125	81						79				76	61			100						
*Citrobacter freundii complex**	223												91	94	94		92		97			
*Citrobacter kosari**	195												99	100	100		61		100			
*Enterobacter aerogenes**	174												99	99	99		8		100			
*Enterobacter cloacae**	283												92	99	99		21		99			
Escherichia coli	16641		50		98	65	93	95					78	88	88		95		93			
Haemophilus influenzae	101		79						100				74									
Klebsiella oxytoca	257				96	81	82	96					99	100	100		80		100			
Klebsiella pneumoniae	1415				98	96	97	98					94	98	98		38		98			
*Monganella morganii**	75									96			73	89	88				91			
Proteus mirabilis	499		81			91	93	97					85	96	96				93			
Pseudomonas aeruginosa	351				96										89				90	97		
*Serratia marcescens**	64												98	100	100				95			
Stenotrophomonas maltophilia	57												100			84						

† Synergy with cell wall active antibiotics predicted from susceptibility to 500 mg/L gentamicin.
* These organisms usually produce inducible ß-lactamase which cause failure of 3rd generation ß-lactam therapy, despite *in vitro* susceptibility
# Susceptibility testing not routinely performed.
¥ Isolated from urine. Not speciated.
♦ Pneumococcus Penicillin and Ceftriaxone susceptibilities based on meningitis breakpoints.
≈ Refer to Hospital In-patient antibiograms
Note: Percent susceptible for each organism/antimicrobial combination was generated by including the first isolate of that organism recovered from a given patient. Only those species with at least 30 isolates are included in the antibiograms.

■ **FIGURE 17-42** Cumulative antibiogram for community care locations in a large Regional Health Authority. (Courtesy of Calgary Laboratory Services.)

Clinical microbiologists should be part of an effective ASP program and ensure that laboratory procedures are in place to provide the various types of support outlined. In particular, antibiotic susceptibility testing and reporting procedures should be reviewed annually, and updated guidelines published by CLSI should be promptly implemented in order to accurately detect emerging types of resistance.

Future Perspectives

Newer technology may increasingly be used to rapidly detect specific types of resistance in key bacterial pathogens. Recently, it has been demonstrated that carbapenemase activity could be rapidly (4 hours) detected in *Enterobacteriaceae*, *P. aeruginosa,* and *A. baumannii* by MALDI-TOF spectrometry.[81,266,311] This assay has many several advantages over other conventional techniques for detection of carbapenem resistance, such as phenotypic methods and PCR, because it was able to detect low-level carbapenemase activity at a low cost even when the type of enzyme in a particular strain was unknown. MALDI-TOF could potentially be used to detect resistance due to other types of enzymes in key bacterial pathogens.

Molecular detection of several resistance determinants using a Luminex xMAP (Luminex, Texas) microarray assay is also feasible although the rate-limiting step is identification of all of the possible genes involved in various types of resistance. A Luminex assay has been used to detect fluoroquinolone resistance in *Campylobacter jejuni* and *Salmonella* spp.[26,564] Other commercial molecular multiplex PCR systems have more recently become available for the rapid simultaneous identification of major gram-positive and gram-negative organisms along with key antibiotic resistance genes. The Verigene Gram-positive Blood Culture Test (Nanosphere Inc., Northbrook, Ill) has recently been FDA-cleared for the rapid identification of pathogens from blood cultures (i.e., ~2.5 hours), and includes detection of the *mec*A and *van*A/B genes (www.nanosphere.us/product/gram-positive-blood-cultures).[582,663] Minimal hand-on time is required for sample preparation, inoculation of the test cartridge and loading the cartridge into the Verigene reader and processor SP instrument. Recent evaluations of this assay show a high degree of overall accuracy compared to routine blood culture methods for rapid pathogen identification, and use of this assay may have allowed appropriate antibiotic treatment to be started earlier for AROs. The Biofire FilmArray is an FDA-cleared multiplex PCR test that integrates sample preparation, amplification, detection, and analysis in one system (www.filmarray.com/the-panels). The FilmArray blood culture identification panel currently tests for a total of 27 targets including 24 pathogens and three antibiotic resistance genes associated with bloodstream infections. This rapid identification test can identify the most commonly detected bacterial pathogens and two *Candida* species in a positive blood culture in approximately an

hour with a limited amount of hands-on time. Few evaluations comparing the FilmArray BCID with routine blood cultures or other molecular methods have so far been reported in the literature.[8,46]

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TABLE 17-39 Key Elements of an Effective Antimicrobial Stewardship Program

Elements	Strategies	Comments
Monitoring antimicrobial resistance	Prospective audit of agent use and feedback to the prescriber	Results in reduced inappropriate use of antimicrobials. Monitoring should be done routinely in institutions using formulary restrictions to determine if prescribing shift to alternative agents is resulting in increased resistance.
Pharmacy interventions	Formulary restriction and preauthorization requirements for specific agents	Results in significant reductions in antimicrobial use and costs. May be beneficial to a multifaceted response to a nosocomial outbreak. Long-term benefit on controlling antimicrobial resistance is not established.
	Antibiotic order forms	Results in decreased antimicrobial consumption through the use of automatic stop orders requiring physician adjustment. Can also facilitate implementation of practice guidelines.
	Dose optimization	Adjustment of dosing based on patient characteristics, causative organisms and site of infection, and PK/PD drug characteristics is essential.
	Conversion from parenteral to oral therapy	Systematic plan for step-down to oral therapy, when the condition of the patient allows; this can decrease the length of hospital stay and healthcare costs. Institutional practice guidelines facilitate implementation.
	Education is essential to	

Fostering appropriate antibiotic use	influence prescribing behavior and enhances the acceptance of other strategies	Education without active intervention is only marginally effective in changing behavior.
	Development and implementation of multidisciplinary practice guidelines and clinical pathways	Guidelines and clinical pathways that incorporate local microbiology and resistance patterns can improve antimicrobial utilization. Guideline implementation is facilitated by education and feedback on antimicrobial use.
Microbiology laboratory	• Provides patient-specific culture and susceptibility data • Provides cumulative antibiotic susceptibility data (i.e., antibiogram)	Optimizes patient management and assists infection control in the effective surveillance of resistance. Assists physicians with initial empiric antibiotic choice and assists infection control in the effective surveillance of resistance.
Infection control program	Collaboration with an effective infection control program	Prevents the secondary spread of resistance.

Adapted from reference 112.

Newer technology are allowing the cycle time from collection of a clinical sample to reporting or clinically relevant identification and antibiotic susceptibility results to be significantly reduced compared to routine culture and phenotypic identification and antimicrobial susceptibility test methods. Reduction in the microbiology test cycle, particularly for patients with serious invasive infections (i.e., bacteremia), may ultimately result in improved clinical outcomes because appropriate antibiotic therapy is started as early as possible, and subsequently healthcare costs may be reduced because of decreased length of stay.

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CHAPTER 18

Mycoplasmas and Ureaplasmas

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Introduction

Mycoplasmas and ureaplasmas are organisms that differ from other bacteria in that they lack a rigid cell wall. Individual cells are bound only by a trilaminar unit membrane. In addition, the amount of genetic material comprising the genome of these organisms is quite small (approximately 500 kb). These organisms have limited biosynthetic capabilities, and consequently, the cultivation of mycoplasmas and ureaplasmas requires an enriched medium that contains precursors for nucleic acid and protein (inclusion of basal peptone and yeast extract), and lipid biosynthesis (serum is usually included). In fact, one of the principal criteria used in the taxonomic classification of these organisms is the requirement for the complex lipid cholesterol in the growth medium by certain mycoplasma and mycoplasma-like organisms.[472,505] All *Mycoplasma* species produce adenosine triphosphate (ATP) by substrate-level phosphorylation effected by phosphoglyceric acid kinase and pyruvate kinase, two

enzymes of the glycolytic pathway from glucose to pyruvate.[392] These two enzymes appear to be the major source of most of the ATP synthesized by organisms in the class *Mollicutes.*

These organisms are much smaller than most bacteria and are able to pass through bacteriologic filters. *M. hominis* (MH) and *Ureaplasma* spp. are coccoid cells, about 0.5 μm in diameter; *M. pneumoniae* appears as spindle-shaped cells, about 1 to 2 μm long and 0.1 to 0.2 μm in diameter.[524] The generation time for these organisms varies from 1 to 1.5 hours for *Ureaplasma* spp. and MH to ~6 hours for *M. pneumoniae*. There are also more fastidious species that have been recently isolated from clinical disease in humans, such as *M. genitalium*, that have a generation time of ~16 hours.[524] The lack of a typical bacterial cell wall containing peptidoglycan renders these organisms insensitive to cell wall active antimicrobial agents, such as penicillins and cephalosporins. Because of

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this, the recovery of these organisms from clinical specimens may have significant therapeutic implications.

Mycoplasmas and ureaplasmas have been recovered from humans, animals, birds, insects, and plants, and new species are being continually identified and reported in the taxonomic literature.[505] Some species also have a free-living existence in soil and water. The human mycoplasmas belong to the genus *Mycoplasma* and to the genus *Ureaplasma,* the latter of which contains mycoplasmas that are able to hydrolyze urea. Several species in the genus *Mycoplasma* and two species in the genus *Ureaplasma*, *U. urealyticum* (UU) and *U. parvum,* are found in human clinical specimens. For purposes of simplicity, *Ureaplasma* spp. will be used in this chapter when referring to these genital mycoplasmas rather than to individual species names. MH, *M. genitalium*, and *M. fermentans* will be discussed as separate species, however.

With the exception of *M. pneumoniae*, the role of other mycoplasmas—specifically MH, UU, and *U. parvum*—in human disease has been controversial. *M. pneumoniae* is the well-recognized cause of atypical pneumonia, whereas MH, UU, and *U. parvum* are associated primarily with genital tract colonization and infection in adults and respiratory tract colonization and disease in newborns.[121,435] These species are associated with and implicated as causative agents in a wide variety of pathologic conditions. However, these same species may also be isolated from asymptomatic individuals, suggesting that they may behave principally as opportunistic pathogens.[321,323,324] *M. genitalium* has been isolated from both the genital and

respiratory tracts, and its role as a sexually transmitted agent causing nongonococcal, nonchlamydial urethritis (NGNCU) and other genital tract infections is currently being elucidated.[23,312,509] However, the presence and behavior in the respiratory tract is not clear. Other *Mycoplasma* species are found as part of the normal flora of the mouth, particularly in the gingival areas surrounding the teeth. In the late 1980s, another "new" mycoplasma, termed "*M. incognitus*" at that time, was detected in autopsy specimens from patients with AIDS, suggesting that this organism may be either another opportunistic infection or a sexually transmissible cofactor that influenced disease progression.[290,345] This organism was subsequently identified as a strain of the fastidious mycoplasma, *M. fermentans*. Several other newly described mycoplasmas have been isolated from HIV-infected patients, and these discoveries have spurred a renewed interest in virulence factors of mycoplasmas, as well as in the methods for their detection and identification.[546] In this chapter, the human mycoplasmas will be addressed, with emphasis on the clinical significance of *M. pneumoniae* and the genital mycoplasmas (MH, UU, and *M. genitalium*) and the methods used for the isolation, identification, and susceptibility testing of these bacteria in the clinical laboratory.

Taxonomy of Mycoplasmas and Ureaplasmas

The first published report referring to the genus *Mycoplasma* came in 1898 by the French scientists Nocard and Roux when they described an unusual organism associated with contagious bovine pleuropneumonia.[418] Mycoplasmas involved in human infection were not described until 1942 when Eaton et al. recovered a filterable agent from the sputum of patients with "atypical pneumonia." The term *Eaton agent* was later used when referring to these small organisms that were initially thought to be viral particles. By 1962, the Eaton agent was isolated in cell-free cultures, demonstrating that it was actually a bacterium that did not possess a cell wall and had specific requirements for growth in culture.[418]

Mycoplasmas and ureaplasmas are classified in the class *Mollicutes* (Box 18-1). The name *Mollicutes* means "soft skin," referring to the lack of a rigid bacterial cell wall. Analysis of ribosomal RNA (rRNA) sequences has revealed that the *Mollicutes* are most closely related to gram-positive bacteria, and their closest relatives are the streptococci.[407,418] The class *Mollicutes* contains 4 orders (*Mycoplasmatales*, *Entomoplasmatales*, *Acholeplasmatales,* and *Anaeroplasmatales*; Box 18-1), 5 families, 8 genera, and >150 species, of which about 16 have been isolated from human specimens.[524] The order *Entomoplasmatales* was created to accommodate the families *Entomoplasmataceae* and *Spiroplasmataceae*. The genera *Entomoplasma* and *Mesoplasma* in the family *Entomoplasmataceae* are found in insects and plants.[506]

The genus *Entomoplasma* contains sterol-requiring species that grow optimally at 30°C, whereas the genus *Mesoplasma* contains species that do not require cholesterol but grow best at 30°C in cholesterol- or serum-free media supplemented with Tween 80 (0.04%). The family *Spiroplasmataceae* includes the single genus *Spiroplasma*, which are spiral-shaped *Mollicutes* that require cholesterol for growth and are found in plants and insects. Members of the order *Acholeplasmatales* do not require sterols for growth and are found predominantly in plants, animals, and insects.[16,511] The presence of these organisms in animal tissues is documented by the occurrence of acholeplasmas as contaminants in tissue culture media that are supplemented with animal serum.[16] Two species, *Acholeplasma laidlawii* and *Acholeplasma oculi*, have been isolated from humans.[314,400,536] *A. laidlawii* is immunologically related to both *M. pneumoniae* and *M. genitalium*.[72] Because sterol compounds are not required for growth, acholeplasmas may be cultivated in medium lacking exogenous serum. The order *Anaeroplasmatales* comprises a single family (*Anaeroplasmataceae*) containing two species,*Anaeroplasma* and *Asteroleplasma*.[506] These two species are strictly anaerobic in their metabolism. *Anaeroplasma* species require cholesterol for growth, whereas members of the genus *Asteroleplasma* do not. Both of these species are found in the rumens of cattle and sheep. The family *Mycoplasmataceae* now includes the hemotrophic mycoplasmas that were previously classified in the genus *Eperythrozoon* and the genus *Haemobartonella* (Box 18-1). These organisms parasitize the erythrocyte surfaces of various animals.

Members of the order *Mycoplasmatales* require sterols, such as cholesterol, for cultivation in vitro. The sole family in the order—*Mycoplasmataceae*—contains two genera: *Mycoplasma* and *Ureaplasma*. The genus *Mycoplasma* contains over 100 species that inhabit a wide variety of plants and animals, including mammals, insects, birds, and reptiles, and may exist as commensals, parasites, and/or pathogens.[320,505,517] The *Mycoplasma* species that have been isolated from humans are listed in Box 18-2.*Mycoplasma hominis*, *M. genitalium*, *M. fermentans*, *M. primatum*, *M. spermatophilum*, and *M. penetrans* are isolated primarily from the human genital tract, whereas *M. pneumoniae*, *M. salivarium*, *M. orale*, *M. buccale*, *M. faucium*, and *M. lipophilum* may be recovered from the human respiratory tract. *M. salivarium* is found in the gingival crevices and may play a role in certain types of periodontal disease. *M. orale*, *M. faucium*, *M. buccale*, and *M. lipophilum* are considered part of the normal upper respiratory tract microbiota and are nonpathogenic. *M. genitalium* has garnered much scientific interest in recent years as the etiologic agent of acute and chronic nongonococcal urethritis (NGU) and a host of other genital tract infections.[312,517] *M. fermentans* was first isolated from the human urogenital tract and is postulated to play roles in urethritis, rheumatoid arthritis, and progression of HIV infection. This species is the same as "*M. incognitus*," recovered

from patients with AIDS (see discussion below).[413] *M. penetrans*, a relatively new addition to the genus, has been isolated from the urine of patients with AIDS and, like *M. fermentans*, may act primarily as an opportunistic infectious agent in immunocompromised hosts. The ecologic niche of *M. pirum* in humans is uncertain.

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BOX 18-1

Taxonomy of the Phylum *Firmicutes*, Class *Mollicutes*

Order	Family	Genus	Sterols Required for Growth	Habitat	Comments
Mycoplasmatales	*Mycoplasmataceae*	*Mycoplasma*	Yes	Humans, animals	Optimal growth at 35°C–37°C; metabolize glucose or arginine or both
		Ureaplasma	Yes	Humans, animals	Optimal growth at 35°C–37°C; metabolize urea
		"*Eperythrozoon*" (now members of the genus *Mycoplasma*)		Animals, including primates	Noncultivable, cell wall-less, parasitic bacteria found on the membrane surfaces of red blood cells
		"*Haemobartonella*" (now members of the genus *Mycoplasma*)		Animals; the possibility of human infections has been raised.	Noncultivable, cell wall-less, parasitic bacteria found on the membrane surfaces of red blood cells
Entomoplasmatales	*Entomoplasmataceae*	*Entomoplasma*	Yes	Insects, plants	Optimal growth at 30°C
		Mesoplasma	No	Insects, plants	Optimal growth at 30°C; grow in serum-free medium with 0.04% Tween 80
	Spiroplasmataceae	*Spiroplasma*	Yes	Insects, plants	Optimal growth at 30°C–37°C
Acholeplasmatales	*Acholeplasmataceae*	*Acholeplasma*	No	Animals, insects, plants	Optimal growth at 30°C–37°C
Anaeroplasmatales	*Anaeroplasmataceae*	*Anaeroplasma*	Yes	Rumen of cattle, sheep	Anaerobic
		Asteroleplasma	No	Rumen of cattle, sheep	Anaerobic

BOX 18-2

Mycoplasmas and Ureaplasmas Isolated From Humans

Species	Common Site(s) of Isolation	Comments (i.e., Other Sites of Isolation, Associated Diseases)
Mycoplasma pneumoniae	Respiratory tract, genital tract (very rare), joint fluid aspirates (very rare)	Pneumonia, bronchitis, bronchiolitis, pharyngitis, croup; meningoencephalitis; rare isolate from genital tract and joint fluid aspirates
Mycoplasma orale	Oropharynx and nasopharynx	No disease association; has been isolated from leukemic bone marrow and lymph nodes
Mycoplasma salivarium	Oropharynx and nasopharynx	No disease association, questionable role in periodontal disease; rare isolates from cervix/vagina and arthritic joints
Mycoplasma buccale	Oropharynx and nasopharynx	No disease association
Mycoplasma faucium	Oropharynx and nasopharynx	No disease association
Mycoplasma lipophilum	Oropharynx and nasopharynx	No disease association
Mycoplasma spermatophilum	Cervix, sperm	No disease association
Mycoplasma primatum	Oropharynx and nasopharynx, female urethra	No disease association; recovered from umbilicus site
1175 *Mycoplasma hominis*	Female genitourinary tract, oropharynx	Blood cultures (postpartum septicemia); bacterial vaginosis and other genital tract infections; lung and pleural effusion specimens; organ and tissue transplant infections; surgical wound infections; prostheses-associated infections; neonatal infections; amnionitis
Mycoplasma genitalium	Genitourinary tract, oropharynx	Some cases of nongonococcal, nonchlamydial urethritis; role in respiratory tract infection unknown; presence in joint fluid (rare) probably results from bacteremia

		and seeding of joint spaces
Mycoplasma fermentans	Genitourinary tract, respiratory tract	Peripheral blood lymphocytes and urine in AIDS patients; arthritic joints; bone marrow; possible role in pathogenesis of HIV infection
Mycoplasma penetrans	Genitourinary tract, urine	Urine in patients with AIDS; possible role in HIV pathogenesis; opportunistic infectious agent
Mycoplasma pirum	Blood (rare)	Peripheral blood lymphocytes; a contaminant in tissue culture cells of human origin; previously proposed role in HIV pathogenesis
Ureaplasma urealyticum	Genitourinary tract; oropharynx, lower respiratory tract, placental tissues	Lower respiratory tract, blood (neonatal sepsis); cerebrospinal fluid; surgical sites; possible role in nongonococcal urethritis; genital tract infections; neonatal infections
Ureaplasma parvum	Genitourinary tract	Genital tract infections

As with other bacteria, the human mycoplasmas recovered from clinical material differ in certain phenotypic characteristics that are exploited for their isolation and identification (Table 18-1). *M. pneumoniae* ferments glucose with the production of acidic end products, whereas MH uses arginine with the formation of basic end products. Cultivation of these organisms is dependent on the use of special agar and broth media that are enriched with factors required for mycoplasmal growth in addition to specific growth substrates, such as glucose and arginine. *M. pneumoniae* strains are antigenically homogeneous, with only one recognized serovar with all strains having a similar rRNA composition. The number of different MH serovars is unknown, but heterogeneous rRNA patterns have been demonstrated.[71,563] In addition to certain ultrastructural similarities, both serologic cross-reactivity and genetic homologies have been described between *M. pneumoniae* and *M. genitalium*.[72,280,564]

The genus *Ureaplasma* includes mycoplasmal organisms that are specifically able to hydrolyze urea. The genus currently contains seven species: UU, *U. parvum*, *U. diversum*, *U. gallorale*, *U. felinum*, *U. cati*, and *U.*

canigenitalium.[19,167,168,194,249,438] *Ureaplasma* was formerly known as the "T-strain" mycoplasma because of the small ("T" for "tiny") size of their colonies on solid media.[438] Prior to 1999, 14 serotypes of UU had been described in humans, nonhuman primates, and other animals. These 14 serotypes were defined by reactivity with a panel of polyclonal antisera.[19,405] These serotypes were clustered into two biovars: Biovar 1 was composed of serotypes 1, 3, 6, and 14, and biovar 2 included serotypes 2, 4, 5, and 7 through 13.[166] Members of these two biovars also have differences in other properties, including restriction-fragment-length polymorphisms, whole-cell protein patterns on polyacrylamide gel electrophoresis, and sensitivity to manganese salts.[19,166,399,405,463] Within biovars, analysis of rRNA with nucleic acid probes demonstrated significant nucleotide sequence homology among constituent serotypes. The percentage of DNA–DNA hybridization among serotypes 1, 3, and 6 in biovar 1 was 91% to 92%, whereas that among serotypes 2, 4, 5, 7, and 8 in biovar 2 ranged from 69% to 97%. However, monoclonal antibodies developed against biovar 1 serotypes also reacted on Western immunoblots with clinical biovar 2 isolates, indicating considerable antigenic cross-reactivity within the polyclonally defined serotypes.[64] Polymerase chain reaction (PCR) techniques that detect biovar-specific 16S rRNA were developed and have been used to examine reference strains and clinical isolates to determine relationships between biovars and specific disease entities associated with UU.[406,491] In 1998, UU biovars 1 and 2 were formally classified as *Ureaplasma parvum* and *Ureaplasma urealyticum*, respectively.[248]

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TABLE 18-1 Characteristics for the Identification of Human *Mycoplasma* and *Ureaplasma* Species

Site of Isolation Species	Utilization of			Optimal pH	Time to Recovery	Growth in			Serovars
	Glucose	Arginine	Urea			Air	CO_2	Anaerobic	
Respiratory Tract									
Mycoplasma pneumoniae	+	–	–	6.5–7.5	4–21 days	4+	4+	1+	1
Mycoplasma salivarium	–	+	–	6.0–7.0	2–5 days	2+	NA	4+	1
Mycoplasma orale	–	+	–	7.0	4–10 days	2+	NA	4+	1
Genital Tract									
Mycoplasma hominis	–	+	–	5.5–8.0	1–5 days	4+	4+	4+	Unknown
Ureaplasma urealyticum/ U. parvum	–	–	+	5.5–6.5	1–4 days	4+	4+	4+	14
Mycoplasma fermentans	+	+	–	7.0	4–21 days	2+	NA	4+	1
Respiratory/Genital Tracts									
Mycoplasma genitalium	+	–	–	7.0	SLOW	2+	3–4+	1+	Unknown
Acholeplasma laidlawii	+	–	–	6.0–8.0	1–5 days	4+	4+	4+	1

+, positive; –, negative; NA, data not available.

The other *Ureaplasma* species are found exclusively in animals. *U. diversum* (three serovars) is found in the bovine and ovine respiratory and genital tracts; it is the etiologic agent of maternal and fetal infections in these animals, including amnionitis, abortion, low birth weight, and neonatal pneumonia and death.[194] *U. gallorale* (one serovar) is a nonpathogenic species found in the respiratory tract of chickens and other fowl.[19,225,249] Two species—*U. felinum* and *U. cati*—have been recovered from the respiratory tracts of healthy domestic cats.[167] *U. canigenitalium*, the most recently described species, was isolated from oral, nasal, and prepuce cultures of dogs and includes four serovars.[168] In addition to these seven formally described species, genetically and antigenically distinct avian, porcine, simian, bovine, and caprine/ovine ureaplasmas have also been described but have not been assigned definitive species status or epithets.[19,169]

Virulence Factors of Human Mycoplasmas

Mycoplasma and *Ureaplasma* are primarily mucosa-associated bacteria that reside in the respiratory and urogenital tracts of the host in close association with epithelial cells, but are located extracellularly.[195] Potential virulence factors in human pathogenic mycoplasmas have been discovered, and some proposed mechanisms that may explain their pathogenicity have been suggested. These include competition for biochemical precursors, adherence to cells, fusion to cell membranes, cell invasion, and

cytotoxicity.[418] *M. pneumoniae* possesses an attachment organelle, which is a tapered extension at one pole of the cell that has an electron dense core and a terminal "button."[256,432] These structures are part of the mycoplasmal cytoskeleton, which is composed of a network of various proteins. Clustered at the tip of the attachment organelle is a membrane-associated, 169-kDa protein called P1, which is the major cytoadhesin that mediates the adherence of *M. pneumoniae* to host cells.[206,524] Following adherence P1 attachment induces ciliostasis, local inflammation, and tissue destruction that is further mediated by the liberation of peroxides.[524] In addition to P1, other membrane proteins that are associated with cytoadherence include P30, P116, and HMW1-3. Alterations or absence of these proteins renders *M. pneumoniae* avirulent.[418] Because cytoadherence is a necessary first step for infection of susceptible mucosal surfaces, these proteins represent true virulence factors in this organism. Individuals infected with *M. pneumoniae* produce a vigorous antibody response against the P1 adhesin, suggesting that purified adhesin proteins may be useful in serologic tests for diagnosis of *M. pneumoniae* infections.[109,205,206] Cytopathic changes can be related to the local damage following cytoadherence of *M. pneumoniae* proteins. *M. pneumonia*e can penetrate cell membranes and invade host cells. Hydrolytic enzymes and cytolytic metabolites are released directly into the cell once the attachment and invasion has occurred.[409,561]Substantial literature also exists on the induction of multiple cytokines (interleukins, interferon, tumor necrosis factor [TNF], and colony-stimulating factors)

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resulting from infection with *M. pneumoniae* and the role of these cytokines in the pathogenesis and mechanisms of mycoplasmal disease.[354,418,560]

Another possible virulence factor, referred to as MPN372, has been identified that could be responsible for airway cellular damage and other sequelae associated with *M. pneumoniae* infections in humans. MPN372 elicits extensive vacuolization and ultimate cell death of mammalian cells, including distinct and progressive patterns of cytopathology in tracheal rings in organ culture that had been infected with virulent *M. pneumoniae*.[228] *M. pneumoniae* evades the host immune response due to survival within host cells, phenotypic plasticity, and molecular mimicry. In addition, *M. pneumoniae* may induce transient depression of T-lymphocyte function and depletion of the host's CD4+ T cells.[418]

Cytoadherence proteins responsible for the initiation of colonization and infection have also been demonstrated in MH. P50 and P100, two surface-localized polypeptides of MH, function in adherence of this species to eukaryotic cells.[178] MH also binds sulfated glycolipids in a time-, temperature-, and dose-dependent manner

that can be inhibited by preincubation of the organisms with high-molecular-weight dextran sulfate.[369] Binding of recombinant P50 protein to HeLa cells is also inhibited by high-molecular-weight dextrans, indicating that P50 cytoadherence protein binds to sulfatides on the host-cell membrane.[244] Sulfated glycolipids and other glycoconjugates are present in high concentrations in the male and female urogenital tracts, and the specific interaction of MH with these molecules may help explain the urogenital tissue tropism of MH. The P100 cytoadherence lipoprotein of MH also possesses a substrate-binding domain for peptide transport across the cell membrane.[179] MH strains also possess another surface-exposed, adherence-associated protein called Vaa (variable adherence-associated) antigen, which is encoded by six distinct *vaa* gene types.[43,44] Expression of these genes can be switched on and off; this ability has been postulated to promote spread of the organisms from one cell to another.[569]

UU also produces a variety of virulence-associated factors. Isolates of UU produce three phospholipase enzymes (A1, A2, and C) that are localized in the plasma membrane.[103] These enzymes hydrolyze phospholipids, with the release of arachidonic acid. Because UU is associated with amnionitis and perinatal morbidity and mortality (i.e., spontaneous abortions, prematurity, stillbirth), it has been postulated that infection of the female genital tract may initiate a sequence of pathologic events related to phospholipase production.[121] Release of arachidonic acids from amniotic membranes may lead to production of prostaglandins, which can trigger premature labor.[29] UU has also been shown to induce the production of inflammatory cytokines (i.e., TNF-α, interleukin-6 [IL-6]) in both human and rat macrophage cell lines, and to stimulate the release of nitric oxide by cultured alveolar macrophage.[274,276] The extensive release of these cytokines may contribute to the inflammatory response and to the pathology of chronic lung disease seen in very low-birth-weight premature infants. UU has also been demonstrated to induce apoptosis in human lung epithelial cells and macrophages; cell death was abrogated by anti-TNF-α monoclonal antibodies.[275]These data suggest that UU lung infection in these neonates may be involved in the chronic impairment of lung tissues both directly and indirectly by the production of inflammatory cytokines that are known to play roles in chronic lung disease of premature infants.[251,504] The three phospholipases described above may also contribute to fetal lung disease related to respiratory tract infection with UU.[417] Both MH and UU also produce immunoglobulin A (IgA) proteases, which may facilitate mucosal invasion by hydrolyzing mucosal IgA.[229]

M. fermentans, *M. penetrans*, and *M. pirum* have been associated with disease in both healthy individuals and patients with AIDS. These organisms have been implicated as opportunistic agents or cofactors that contribute to the pathology and

pathogenesis of HIV-related disease.[345] The mechanisms involved in these postulated roles include specific activation of the cellular immune system, production of superantigens that stimulate the release of various immunomodulating lymphokines and cytokines, and the generation of free radicals that contribute to oxidative stress observed in HIV infection. All of these "AIDS-associated" mycoplasmas use glucose and hydrolyze arginine. Mycoplasmal arginine deaminase may cause depletion of arginine in infected macrophages. Arginine is a precursor for a molecule that is directly involved in macrophage-mediated cytotoxicity; therefore, depletion of arginine by mycoplasmal infection may result in diminished macrophage cytotoxicity. Mycoplasmas also release into the growth medium nucleases that degrade host-cell nucleic acids to generate precursors for synthesis of their own nucleic acids. *M. fermentans*, *M. penetrans*, and *M. pirum* are also able to invade cells and survive as intracellular pathogens.[84]The association of these organisms with intracellular locations in lymphocytes and macrophages helps explain the recovery of these organisms, especially *M. fermentans* from the blood of patients with and without AIDS.

Clinical Significance of the Human Mycoplasmas

Mycoplasma pneumoniae

M. pneumoniae is a frequent respiratory pathogen in children, as well as in adults. It infects the upper and lower respiratory tracts and is one of the many causes of the pneumonic process called atypical pneumonia; other causes of atypical pneumonia include several viruses (e.g., influenza, respiratory syncytial virus, adenoviruses), fungi (e.g., *Pneumocystis jirovecii*), and bacteria (e.g., *Chlamydophila pneumoniae*, *Legionella pneumophila*).[42,132,450] *M. pneumoniae* is transmitted from person to person by airborne transfer of droplets containing the organisms, with transmission facilitated by coughing or sneezing. About 20% of community-acquired pneumonias that require hospitalization are caused by *M. pneumoniae*; however, these cases represent only 3% to 10% of all *M. pneumoniae* infections, since tracheobronchitis or minor upper respiratory tract infections, not pneumonia, develop in most infected individuals, or else they remain asymptomatic.[132,418,453,455,524] The incubation period is 1 to 3 weeks, but symptoms can occur in as little as 4 days after infection.[418] In a report from Seattle, WA, in 1977, the rates of infection with *M. pneumoniae* varied from 2% in endemic years to 35% in epidemic years, with epidemics occurring

every 4 to 7 years. Children aged 5 to 9 years developed more pneumonia than did

adolescents aged 15 to 19 years.[137] In closed populations (e.g., summer camps, military bases, schools, and college dormitories), *M. pneumoniae* may result in outbreaks of pneumonia. Upper respiratory tract infections tend to develop in children younger than 3 years of age, whereas tracheobronchitis or pneumonia more often develops in older children and adults. Interestingly, studies in pediatric populations have not demonstrated a role for *M. pneumoniae* in acute myringitis or otitis media with effusion.[252,459] Most infections caused by *M. pneumoniae* are relatively mild and include pharyngitis, tracheobronchitis, bronchiolitis, and croup; up to one-fifth of infections are actually asymptomatic.[132]

Disease is generally not seasonal and may occur year-round, but the incidence of overt disease is usually highest in late fall and winter, when young people are going back to school. A study in China, however, found the incidence to be highest in the summer as compared with *C. pneumoniae,* which did not have a seasonal occurrence.[63]

The presenting symptoms of mild *M. pneumoniae* illness are sore throat, coughing, and wheezing and hence, infection can be mistaken for asthma. Fulminant respiratory infections are uncommon, but severe infections requiring hospitalization may develop in elderly and immunocompromised patients, and death may result from some of these infections.[316,383,418] For a more recent historical review of *M. pneumoniae* and its relevance in clinical disease, see Cunha.[82]

After exposure in susceptible hosts, the organism attaches to epithelial cells in the respiratory tract and multiplies. Organisms may be recovered in culture during the incubation period and for several weeks during and after clinical illness, even in the presence of specific antibodies. Following a 2- to 3-week incubation period, the clinical presentation of mycoplasmal pneumonia is usually insidious, rather than abrupt, with the gradual onset of constitutional and pneumonic symptoms that mimic influenza. Fevers of 101°F to 102°F will develop over a few days in most patients, with chills, malaise, headache, sore throat, nasal congestion, and a dry, nonproductive cough appearing early in the clinical course. The leukocyte count is often normal or only slightly elevated. In 5% to 10% of patients, progressive lower respiratory tract symptoms develop; the sputum becomes more mucoid or mucopurulent and blood may be noted in the sputum. Occasionally, Gram-stained smears of sputum at this point usually show few to moderate polymorphonuclear leukocytes and no organisms. With the onset of pneumonic symptoms, patients may feel as if they have a severe cold or the "flu," yet will continue to function relatively normally, hence the application of the term *walking pneumonia* to this disease. On chest examination, localized rhonchi and scattered rales are usually detected, and findings on chest roentgenograms are consistent with a diffuse bronchopneumonia, generally involving multiple lobes of the lung without consolidation. With *M. pneumoniae* pneumonia, chest roentgenogram patterns vary widely and may show peribronchial pneumonic

infiltrates, atelectasis, nodules, and hilar lymphadenopathy. Radiologic findings usually appear more extensive than the physical examination of the patient would suggest. Pleural effusions are relatively rare with mycoplasmal pneumonia, occurring in up to 20% of patients.[379] Other pleuropulmonary complications, such as pneumothorax and lung abscess, are also uncommon. However, there was a report of a 10-year-old with a 15-day history of fever and cough, who did not respond to amoxicillin and ceftriaxone for presumed pneumococcal disease, who showed a rapid response when treated with clarithromycin; serologic evidence suggested *M. pneumoniae* infection with lung abscess.[268] A few other patients with lung abscesses have been reported, and like this patient in whom macrolide therapy was delayed, the *M. pneumoniae* disease progressed.

M. pneumoniae pneumonia is generally self-limited, with resolution of most constitutional symptoms in 3 to 10 days without antimicrobial therapy. Abnormalities on chest films generally resolve more slowly, and complete resolution may take anywhere from 10 days to 6 weeks. Recurrences and relapses of pneumonia despite appropriate antimicrobial therapy may also occur. Using serologic methods and molecular detection techniques, several studies have now shown that *M. pneumoniae* may play a significant role in acute and chronic asthma in children and adults, exacerbations of chronic obstructive pulmonary disease particularly among older adults, and adult respiratory distress syndrome.[34,131,255,332,524,552] Data also suggest that *M. pneumoniae* may be implicated in acute chest syndrome, a pneumonia-like disease that manifests in patients with sickle cell disease.[360]

Respiratory infections with *M. pneumoniae* are associated with a wide range of extrapulmonary manifestations, including neurologic, cardiac, dermatologic, musculoskeletal, hematologic, and gastrointestinal symptoms.[156,502,532] As many as 25% of patients with *M. pneumoniae* infection may experience dermatologic complications. Most commonly, erythema multiforme major (Stevens–Johnson syndrome) is seen, presenting as vesicular lesions in mucocutaneous areas of the body[272,412,418]; various types of cutaneous macular, morbilliform, and vesicular eruptions; bullous erythema multiforme; and erythema nodosum.[66,272,428] In one instance, a patient with *M. pneumoniae* urticarial vasculitis was mistaken for adult Stills' disease in the United Kingdom but fortunate for the patient, once appropriate treatment was given, the patient did recover.[110] The mechanism of skin and mucosal disease is unknown but postulated to be due to immune complex vascular injury or autoimmune mechanisms.[418]In adults and pediatric patients with mucositis, *M. pneumoniae* infection should be included in the differential diagnosis.[130]

Cardiovascular manifestations may occur in as many as 10% of patients and include conduction defects, cardiac arrhythmia, pericarditis, tamponade, and congestive heart failure.[236,335,419] Myopericarditis has been described in a child who presented with

fever, lethargy, oliguria, and dyspnea.[376]

Central nervous system (CNS) manifestations are the most common extrapulmonary complications of *M. pneumoniae* infections. In patients with serologically confirmed infections that require hospitalization, as many as 10% may develop neurologic complications, most commonly encephalitis and meningoencephalitis, followed by polyradiculitis and aseptic meningitis.[156,418] *M. pneumoniae* is a major cause of encephalitis in children, especially those <10 years, often resulting in the need for intensive care management. A study in children with acute encephalitis documented *M. pneumoniae* infection in 31% of the patients, with *M. pneumoniae* being the probable cause of the encephalitis in at least 7% of

the patients, based on PCR cerebrospinal fluid (CSF) results and positive serologic tests.[36,537] Acute transverse myelitis also occurs because of *M. pneumoniae* infections, including those associated with Guillain–Barré syndrome; this is most common in individuals <35 years old.[156,418,447,500] The pathogenesis of CNS complications is not completely understood. It may be due to direct invasion of the bacteria, especially in patients with an early onset of encephalitis, as suggested by the presence of PCR-positive findings of *M. pneumoniae* in the CSF.[269,354,503] It is likely, however, that postinfectious neurologic syndromes probably occur via an autoimmune mechanism induced by the mycoplasmal infection, and/or via thromboembolic events leading to microangiopathy, cerebral vasculitis, and demyelination in perivascular areas in the CNS.[31,35,156,354,386,503] Severe CNS complications of the latter type have been associated with strokes following acute *M. pneumoniae* infection.[372,454]

Urologic and hepatobiliary complications are manifested as acute nephritis and cholestatic hepatitis, respectively.[155,448] Hemolytic uremic syndrome (HUS) has been described in a one-year-old boy as a complication of *M. pneumoniae* respiratory illness.[144] An article from China has described an increased seroprevalence of and infection with *M. pneumoniae* in patients with hepatitis C who were receiving hemodialysis.[260]

Ocular complications reported in association with *M. pneumoniae* infection include optic disk swelling, optic nerve atrophy, optic papillitis, retinal exudation, hemorrhage, and bilateral uveitis.[340,415,547] Various types of hematologic complications have also been reported (i.e., hemolytic anemia secondary to the formation of cold agglutinins, intravascular coagulopathy, neutropenia, thrombocytopenia, paroxysmal cold hemoglobinuria), which may result from the generation of specific antibodies and autoantibodies directed against lung, tissue, cardiolipin, and muscle tissues.[62,65,92]

Humoral and cellular immunodeficiency states also predispose individuals to more serious disease with *M. pneumoniae,* as well as other mycoplasmas.[132,375,408] Individuals with hypogammaglobulinemia may suffer repeated bouts of *M. pneumoniae* pneumonia and have difficulty eliminating the organism from the respiratory tract despite adequate therapy. *M. pneumoniae* organisms persist in most individuals with respiratory tract infection for a few months after infection, whereas they may persist for years in a patient with hypogammoglobulinemia.[524] These patients often have severe upper and lower respiratory tract symptoms with few or no infiltrates observed on chest radiography, and have significant complications, including rashes, joint pain, septic arthritis, and osteomyelitis.[224,263,485,489] *M. pneumoniae* may also cause severe disease in patients with conditions that abrogate cellular immunity, including HIV infection and sickle cell disease.[217,243,360,375] A PCR assay of bronchoalveolar specimens was found positive of *M. pneumoniae* in a 10-year-old renal transplant patient who had chronic respiratory symptoms and a new mass lesion in the upper lobe of his lung. The young child responded well to macrolide therapy.[430]

Although arthralgias are a common manifestation of mycoplasmal infection, definitive arthritis, with positive cultures from joint fluids, has been reported, but is considered very uncommon.[61,88] Joint infections, however, have been associated with many of the *Mycoplasma* spp., including *M. pneumoniae. M. pneumoniae* and *M. salivarium* have been found in synovial fluid of patients with arthritis in a UK study, but not in fluid that developed after traumatic injury. *M. fermentans* has likewise been found in cellular infiltrates of inflamed joints.[223] In addition, more recently, PCR and sequencing have led to the finding of *M. pneumoniae* in periprosthetic joint infections.[161] With better laboratory assays for detection of *Mycoplasma* spp., the detection of *M. pneumoniae* in these clinical situations may increase.

Fulminant, disseminated *M. pneumoniae* infection with multisystem involvement is rare, but it has been reported. Kountouras and colleagues reported on a previously healthy 50-year-old man who presented with progressive respiratory distress, aseptic meningitis, cholestatic hepatitis, renal failure, and disseminated intravascular coagulation.[253] *M. pneumoniae* infection was diagnosed serologically; the patient was treated with erythromycin and experienced an uneventful recovery. Takiguchi and colleagues reported on a previously healthy 64-year-old woman in whom progressive respiratory distress developed, with widespread bilateral alveolar infiltrates on chest radiographs.[467] Acute respiratory distress syndrome and disseminated intravascular coagulation then developed. *M. pneumoniae* infection was diagnosed serologically. Despite intensive antimicrobial and corticosteroid therapy, mechanical ventilation, hemodialysis, and plasma exchanges, the patient died of multiple-organ system failure. Similarly, Koletsky and Weinstein reported a 30-year-old woman who developed rapidly fatal, disseminated *M. pneumoniae* infection during which severe respiratory

disease, pneumonia, cardiovascular collapse, and renal failure developed after a 9-day clinical course.[246] Unfortunately, the patient died within 24 hours of admission to the hospital. On autopsy, the organism was recovered from the lungs, kidneys, and brain. Histopathologic examination revealed bilateral consolidated pneumonia and disseminated intravascular coagulation.

M. pneumoniae may occasionally be isolated from unusual extrapulmonary sites other than those listed above. Goulet and coworkers isolated *M. pneumoniae* from urogenital tract specimens of 22 female patients over a 2-year period.[145] In addition, the organism was recovered from the urethra of one of three male sexual partners of a woman who harbored the organism in her genital tract. *M. pneumoniae* has also been isolated from a tubo-ovarian abscess.[495] An association with Kawasaki's disease has been reported in four Italian children in which serology confirmed *M. pneumoniae* infection; however, there was no other evidence of a causal relationship.[520]

Mycoplasma hominis *and* Ureaplasma urealyticum

Both MH and UU may be isolated from the genital tract of asymptomatic men and women.[321,323,324] Publications in 1972 and 1986 by McCormack and colleagues demonstrated that rates of colonization with MH and UU in men varied from 0% to 13% and 3% to 56%, respectively.[321,322] The same authors reported data in women that vaginal colonization rates for MH and UU range from 0% to 31% and 8.5% to 77.5%, respectively, depending on age, race, sexual experience, and socioeconomic status.[321,322,324] Rates of genital

tract colonization in both men and women are related to sexual activity, and individuals with multiple sexual partners are more likely to be colonized. Therefore, the epidemiology of organism acquisition suggests that these mycoplasmas are likely sexually transmitted. Additional evidence for sexual acquisition is suggested by much lower isolation rates among women who use barrier contraceptives.[324] Given these data, it is clear that these organisms are exceedingly prevalent, particularly in the lower genital tracts of sexually active women.

Over the past several years, both UU and MH have been implicated in a variety of clinical conditions primarily related to lower genital tract colonization and infection, upper genital tract infections in women, and, rarely, upper genital tract infection and prostatitis in men.[121,170,209,381] Both organisms have been postulated to play roles in early and late endometritis, chorioamnionitis, and premature rupture of membranes.[106,121,151] The presence of UU in the genital tract, in particular, has been

associated statistically with prematurity, low-birth-weight infants, and infertility.[124,151,170,230] Controlled studies on the pathogenicity of the genital mycoplasmas are complicated because both organisms are highly prevalent in sexually active asymptomatic adults. To further confound issues of etiology, genital mycoplasmas are often recovered in culture along with other recognized genital tract pathogens, such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.[330] These associations make it difficult to determine whether a pathologic condition is attributable solely to the presence of the genital mycoplasmas. An excellent example of this dilemma is the clinical condition of bacterial vaginosis. MH is often isolated from women with this infection.[182,234,259] However, bacterial vaginosis is now known to result from a complex interaction among aerobic and anaerobic bacteria. The roles of genital mycoplasmas in this condition have not been elucidated or defined, and a causal relationship between MH and bacterial vaginosis has not been proven. Studies on the pathogenicity of the genital mycoplasmas are further confounded by recovery of the organisms during or after the administration of antimicrobial agents for other genital tract pathogens.[79] Antimicrobial therapy for these other organisms may act to select for mycoplasmas that are resistant to the agents being used (e.g., penicillins and cephalosporins). After their recovery from a genital culture or a site of infection, it becomes difficult to ascribe an etiologic role for the organisms in the context of previous or ongoing antibiotic administration.

MH and UU have been isolated from blood cultures of women with postpartum fever.[94,121,123,261,325,353] About 10% of women with fever postpartum or post abortion will have either MH or UU isolated from blood cultures.[524] In some cases, these bacteria may be isolated with other genital tract microorganisms, again confounding the etiologic role of the genital mycoplasmas. Postpartum bacteremia results from the ascension of the microorganisms from the vagina into the endometrium, where the organisms cause endometritis. Infection of the placental membranes and amniotic fluid with mycoplasmas most frequently occurs in colonized women with premature rupture of the fetal membranes and preterm or prolonged labor.[136] From these sites, the organisms enter the bloodstream during and after labor and delivery, or after a cesarean section. UU bacteremia has also been documented as a complication in women undergoing hysterectomy.[90]

Isolation of mycoplasmas, especially UU, from the lower genital tracts of pregnant women is associated with several adverse outcomes of pregnancy, including the delivery of low-birth-weight infants. In a study conducted by Kass and coworkers, women whose genital tracts were colonized with UU and who demonstrated a fourfold or greater antibody response to the organism showed a 30% rate of low-birth-weight infants, whereas only 7.3% of colonized women who did not generate this antibody response had low-birth-weight infants.[230] Furthermore, this antibody response was

serotype-specific, suggesting that the additional rate of low birth weight was related to recent infection with a new serotype of UU that differed from the serotype or serotypes already present in the vagina. A study that examined the relationship between the two UU biovars and pregnancy outcomes found that the *urealyticum* biovar was associated with more adverse outcomes with regard to birth weight, gestational age, and preterm delivery than the *U. parvum* biovar.[1] Therapeutic trials with tetracycline or erythromycin have resulted in decreased rates of low-birth-weight infants, implying that eradication of mycoplasmas (or other susceptible organisms) from the genital tract may have a direct, beneficial effect on certain adverse outcomes of pregnancy.[230,486] Review articles concerning the association of mycoplasmas and adverse outcomes during pregnancy are recommended for those interested in additional reading.[262,487]

Amniotic infections with UU may be demonstrated in asymptomatic women with intact fetal membranes, and these infections are frequently associated with adverse pregnancy outcomes.[57,152,193,565] UU is capable of initiating an intense inflammatory tissue response, with no associated symptoms; the serologic evidence of an antibody response to these organisms and simultaneous recovery of ureaplasmas from the blood of these patients appears to confirm the possibility of silent amniotic infections.[57,138] Gray and associates studied two groups of asymptomatic pregnant women who were matched for maternal age, gestational age, and indications for amniocentesis.[152] Transabdominal amniocentesis specimens were cultured for mycoplasmas. Among the 86 women in the *Ureaplasma*-negative group, there was a 1.2% incidence of spontaneous second trimester abortion and a prematurity rate of 9.3%, for an overall adverse outcome rate of 10.5%. However, the rate of adverse outcomes (six spontaneous abortions and two premature deliveries) among the eight *Ureaplasma*-positive women was 100%, representing an 8.6-fold greater risk of adverse outcome. One of the premature deliveries survived but with the complication of hyaline membrane disease. Histologic examination of placental tissues from the eight infected women revealed chorioamnionitis in all of them, and all seven lung tissue specimens from the infants showed pneumonia. Four of five placentas and three of five fetal lung tissue specimens that were cultured grew UU.[152] Horowitz and colleagues also showed that 50% of six women with midtrimester amniotic cultures for UU that were positive had adverse pregnancy outcomes, as compared with 12% of 123 women with negative amniotic fluid cultures.[193] In another study that examined endocervical colonization with and serologic responses to UU, Horowitz and colleagues found that cervical colonization, when

associated with increased titers to UU, identified a population of women who were at risk for complications of pregnancy.[191,192] Eschenbach reviewed the evidence relative to the contribution of UU to adverse outcomes of pregnancy and concluded that, although the presence of UU in the lower genital tract was not associated with premature birth, the presence of the organism in the chorioamnion was strongly associated with histologic evidence of chorioamnionitis and weakly associated with premature birth as an outcome.[124] Although it has been well established that healthy women who are colonized with MH in the genital tract usually deliver healthy colonized infants, MH has also occasionally been associated with chorioamnionitis, abortion, stillbirth, and intrauterine fetal death.[331]

In some cases, placental infection with ureaplasmas has been shown to contribute a priori to subsequent endometritis, premature rupture of membranes, and premature labor. Colonization of the placenta permits infection of the endometrium, from which the organisms can gain access to the bloodstream. The recovery of UU and MH from endometrial tissue sometime after delivery has established that mycoplasmas and ureaplasmas could be the probable causes of early- and late-onset endometritis in some cases. Postpartum endometritis is a major complication of pregnancy and occurs more frequently after cesarean section than after vaginal delivery. In one study, endometritis occurred in 28% of women with UU present in the chorioamnion at cesarean delivery, compared with only 8.4% if the culture was negative and 8.8% if only bacteria other than UU were present.[15] These workers concluded that chorioamniotic colonization with UU in women with intact membranes being delivered by cesarean section was an independent predictor of subsequent endometritis. A subsequent randomized clinical trial of extended-spectrum antimicrobial prophylaxis using agents active against UU in women undergoing cesarean delivery was found to reduce the frequency of postdelivery endometritis.[14] Although it has been suggested by some studies, a definite causal relation between genital mycoplasmas and pelvic inflammatory disease (PID; salpingitis) has not been unequivocally demonstrated.[121,279,435,462]

Because of the high colonization rates of genital mycoplasmas in both men and women, these organisms have also been evaluated for relationships with involuntary infertility. Although some studies suggest a possible role for these organisms in this condition, there is no definitive proof indicating that these organisms cause either male or female infertility.[471] MH was found in 1.3%, UU in 20%, *N. gonorrhoeae* in 0.4%, and *C. trachomatis* in 2.2% of 230 women undergoing infertility evaluations in the United States.[202] In a 2002 study of 50 infertile women and 46 fertile women, MH was isolated from the genital tracts of 8% of the infertile women and was not isolated from any of the controls.[128] UU was isolated from 56% of the infertile women and from 39% of the control women; this difference was not statistically significant, but

the authors suggested that an association between infertility and UU may exist. In a study of 92 asymptomatic male partners of infertile couples, 12 (13%) men had UU in their ejaculates. Again, these workers suggested that UU may play a role in infertility in the absence of other conditions.[273] In vitro, UU has been shown to adhere to sperm cells, resulting in significant reductions in sperm motility and causing alterations in cell membranes, which could suggest an in vivo role for UU in infertility.[364] However, another study found no difference in sperm quality in men with and without positive cultures for MH or UU, as assessed by sperm density, vitality, motility, and morphology.[13] More recently, an Italian study of unselected men attending an infertility clinic showed an elevated prevalence of UU and MH infection and demonstrated that the presence of these microorganisms was associated with a higher percentage of patients with abnormal sperm parameters. Of the 250 infertile men, 16% had cultures positive for UU and 4% had cultures that contained MH.[414]

UU may play a role in spontaneous abortion through its ability to cause chorioamnionitis, but like the other conditions described above, definitive proof has not been obtained. Joste and associates cultured and examined the histopathology of specimens from 42 first-trimester spontaneous abortions, 21 first-trimester elective abortions, and 32 third-trimester, preterm deliveries, 11 of which were culture-positive for UU.[226] Specimens from 26% of the first-trimester spontaneous abortions grew ureaplasmas, whereas none of the 21 elective abortion specimens contained UU. Histologic evidence of chorioamnionitis did not correlate with culture positivity for the spontaneous abortion specimens, but did correlate with positive UU cultures for the 11 third-trimester, preterm deliveries. These workers postulated that early changes caused by UU infection other than histologically evident chorioamnionitis may be responsible for the pathogenesis of spontaneous abortions related to ureaplasmal infection.[226] A study conducted from 1989 to 1994 in Belgium noted a strong association between bacterial vaginosis and miscarriage.[107] After multivariate analysis, MH and UU, but no other microorganisms, remained significantly associated with increased risk of miscarriage. In contrast, another study published in 2004 found no association between first-trimester miscarriages and the presence of mycoplasmas or ureaplasmas in expelled placental specimens.[319]

Urinary tract infections caused by mycoplasmas have also been reported in pregnant women. Determination of the significance of mycoplasmas in clean-catch urine specimens from women is difficult, owing to the likely contamination of the specimen from organisms colonizing the vagina and the distal urethra. Savige and coworkers collected suprapubic bladder aspirates for culture from 72 healthy pregnant women and 51 women with preeclampsia.[425] UU was isolated from the urine of 7% of the healthy women and from 20% of those with preeclampsia. In a subsequent study of bacteriuria in 340 pregnant women, the presence of UU in the urine during the first

trimester correlated with the development of preeclampsia during the third trimester of pregnancy.[141] Among the 21 women in whom preeclampsia developed, 29% had ureaplasmas present in urine during the first trimester, compared with only 10% of the patients in whom preeclampsia did not develop. Therefore, the development of preeclampsia was three times more likely in women who had ureaplasmas in the urine at the start of pregnancy than in women who were culture-negative. Although the mechanisms involved in the pathogenesis of preeclampsia are unclear, it is suggested that ureaplasmas, similar to other bacteria involved in bacteriuria, may also contribute to adverse pregnancy outcome. In another study of mycoplasmas in 48 women with symptoms

of chronic urinary tract infection, 23 (48%) had positive cultures (22 UU and 1 MH).[393] Cultures from all the women became negative after targeted therapy, and 91% of the infected women experienced alleviation of their chronic symptoms. These investigators concluded that, following exclusion of common agents of urologic infections, anatomic abnormalities, and neurologic dysfunctions, culture for mycoplasmas and subsequent targeted antimycoplasmal chemotherapy is warranted before other diagnostic procedures are entertained.[393] A study from Greece examined 153 nonpregnant women with chronic voiding symptoms and found that 52% of the women had UU isolated from urethral, vaginal or urine specimens and 3.3% of women had both MH and UU isolated. After therapy for the supposed MH/UU infections, 92% of the women cleared their MH/UU cultures and all had significant improvement in symptoms posttherapy.[18]

Genital mycoplasmas have been associated with NGNCU. *C. trachomatis* is well established as the etiologic agent responsible for 30% to 50% of acute urethritis not attributable to *N. gonorrhoeae*. A significant association has also been made between NGNCU and *Mycoplasma genitalium*, which may account for 15% to 25% of cases of acute NGNCU.[96,97,186] Although several studies have been done, the role of UU in male urethritis is not entirely clear. Although earlier data suggested that UU is the cause of some NGNCU, it is not known what proportion of infections is due to the organism; even if the organism is present in the urethras of symptomatic men, its role as the causative agent has not been established. Studies of symptomatic men treated with antibiotics having differential activity between *C. trachomatis* and UU, and cultures performed on men with persistent symptoms of urethritis following antigonococcal and antichlamydial therapies suggest that some patients with NGU are caused by UU.[79,476] Use of highly sensitive molecular approaches along with serologic responses has already provided more complete information regarding the role of UU in

NGU. A study conducted by Horner and colleagues found that UU was not associated with acute NGNCU in multivariate analysis, but was associated with chronic NGNCU, which was defined for the study as urethritis occurring 30 to 92 days after commencement of treatment.[187,188] These workers concluded that ureaplasmas were a probable cause of chronic NGNCU that occurs after treatment. A 2004 study conducted in Japan reported a significant association between UU and NGNCU and suggested that the presence of *U. parvum* in the urethra may reflect colonization and not infection.[99]Other recent studies using sensitive molecular techniques have established that UU is associated with some cases of NGNCU and that *U. parvum* is seen more often in asymptomatic infections, although some patients with *U. parvum* may be symptomatic.[395,567] The relationship of MH and NGNCU is much less defined in men and has not been found to be involved in women with urethral syndrome.[524]

The role of MH and UU as an etiology of chronic prostatitis has been investigated by many researchers. Using classical techniques for localizing infections to the prostate, several investigators have documented UU as being responsible for a small proportion of cases of chronic prostatitis.[365,366,381,449] In a 2002 study from Croatia, UU was the sole agent isolated from the prostatic fluids of 2.54% of 276 patients with chronic prostatic infection.[449] In a study from Japan, UU was recovered from prostatic fluid secretions of 18 of 143 patients with prostatitis.[366] Targeted anti-*Ureaplasma* treatment eradicated the organisms and resolved symptoms, suggesting that UU was the causative agent in these patients. UU is rare as a cause of epididymitis; a single patient infection has been reported wherein the organisms were isolated from an epididymal aspirate in association with a significant rise in the specific antibody titer.[209] In a PCR-based Russian study in 2011, men with chronic prostatitis were found to harbor MH at a rate three times higher than those with benign prostatic hyperplasia, and there was no evidence of the organism in men without prostate disease. In addition, the study found higher titers of antibody against MH and increased prostate-specific antigen (PSA) levels in patients who were positive for MH. The authors felt that MH might potentially be used as a marker of early prostate disease and as a target for its improved prevention and treatment.[21]

Infants born to mothers who are colonized with genital mycoplasmas are also frequently colonized. In survey studies, 18% to 45% of neonates delivered to colonized mothers were also colonized with MH, with positive cultures being obtained from the throat, the genital tract, or the urine.[106,121] The rate of vertical transmission of UU ranges from 18% to 55% among full-term infants and from 29% to 55% among preterm infants.[416] Colonization usually decreases after about 3 months of birth; in addition, less than 10% of older children and non–sexually experienced adults are

found to be colonized.[524] Syrogiannopoulos and associates cultured the throat, eyes, and vaginas of 193 full-term infants born to women colonized vaginally with UU.[465] Of these, 107 (55%) had UU present in at least one of the sites cultured. Colonization may persist in these babies for prolonged periods with no ill effects to the child. In the latter study, 68%, 33%, and 37% of the infants who had colonization at birth in the throat, eyes, and vagina, respectively, still had colonization on follow-up at 3 months of age. Among the children who had colonization in the respiratory tract, there was no increased incidence of respiratory tract illnesses when compared with children who did not have colonization in this area. In a study performed in Israel, 24% of 99 preterm infants were colonized with mycoplasmas; UU was isolated from 21 infants and MH was isolated from three infants.[204] In this study, the colonization rate was inversely correlated with gestational age, with 80% of infants younger than 28 weeks of gestation having colonization, as opposed to 17.9% of infants at 28 to 36 weeks of gestation.[204] Of the 27 infants requiring ventilatory assistance in this study, 22% had UU isolated from lower respiratory tract secretions. Ureaplasmas can invade the amnionic sac and induce an inflammatory response, resulting in chorioamnionitis, preterm labor, and neonatal lung injury. The ability of UU and MH to cause pneumonia, bacteremia, and meningitis in newborns can no longer be questioned.[537a] Future investigations to prevent prematurity should be directed toward identification and localization of specific microorganisms combined with targeted antibiotic trials to determine whether such interventions can improve long-term infant outcomes.[537a]

Several studies have suggested that UU is associated with chronic lung disease, bronchopulmonary dysplasia, persistent pulmonary hypertension, and systemic infection in premature infants.[32,56,58] Prospective studies and animal model

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studies have now shown a significant relation between UU respiratory tract infection and the development of chronic lung disease of the newborn.[80,410] Cassell and others found that the recovery of UU from the tracheas of infants weighing less than 1,000 g, who also required ventilatory assistance, was associated with the development of bronchopulmonary dysplasia and associated conditions (e.g., hyaline membrane disease, patent ductus arteriosus, and pneumonia).[56] A subsequent study by Crouse and associates showed that low-birth-weight infants with respiratory distress who had UU recovered from tracheal cultures were more likely to show dysplasia and evidence of pneumonia on chest radiography than those without UU.[80] Nasopharyngeal or tracheal colonization of preterm infants with UU is also associated with an elevated peripheral white blood cell count.[367] Sanchez and Regan found that, among infants colonized with ureaplasmas in the respiratory tract, chronic lung disease requiring

mechanical ventilatory assistance developed in 30%, whereas respiratory tract illness developed in only 8% of noncolonized neonates.[417] Bloodstream infection with UU has been documented in newborn infants, and these infections are often associated with coexisting respiratory tract infections.[32,49,528] These systemic infections are associated with urinary tract infection, preterm or prolonged membrane rupture, preterm labor, chorioamnionitis, low birth weight, and the presence of congenital anomalies and perinatal asphyxia in the newborn.[528] The pathogenesis of this syndrome in the neonate has been supported by animal models and reflects both the nature of these bacteria as opportunistic agents and the immunocompromised status of the premature infant.[531] Studies with neonatal lung fibroblast cultures have demonstrated induction of cytokine release from these cells by infection with UU in vitro, suggesting a role for cytokines in the pathogenesis of bronchopulmonary dysplasia in prematurity.[457]Serologic studies indicate that the neonate or fetus systemically infected with UU generates serovar-specific anti-*Ureaplasma* IgM and IgG responses, providing additional evidence for the pathogenic potential of these organisms.[398]

Several reports and studies have also shown that both UU and MH may be recovered infrequently from the CNS as a cause of silent or clinically symptomatic meningitis in the newborn period.[518] Several cases of MH CNS infections in the neonate have been described; most of these occurred in premature infants for whom prolonged rupture of membranes had been documented, although cases have also been described in full-term infants.[11,326,531,550] In a prospective study of 100 preterm infants, Waites and coworkers isolated UU from the CSF of eight babies and MH from the CSF of five babies.[529] Among the babies with UU infection, six had intraventricular hemorrhage, three had hydrocephalus, and three died. None of the infants with MH infections died, and only one had neurologic signs of meningitis. Gilbert and Drew, however, reported a case of meningitis in a premature infant caused by MH that had a chronic course, with the development of intraventricular hemorrhage.[140] In all of these infants, eradication of the organisms with antimicrobial therapy proved difficult. In a study of 318 predominantly full-term infants with signs of suspected sepsis or meningitis born to primarily low-risk women, MH was isolated from the CSF of nine infants and UU was recovered from the CSF of five infants.[528] Of these 14 infected infants, 12 recovered without therapy and 2 died. Of these two infants, one with MH died of *Haemophilus influenzae* sepsis, whereas the other, infected with UU, had intraventricular hemorrhage. This study suggested that mycoplasmas are more common in neonatal CNS infections than had been appreciated previously. A case of MH meningitis subsequent to neurosurgery in an adult has been described, detected using PCR of the CSF, since culture, and other nonmolecular

techniques were not sufficient for the diagnosis.[264] Although CNS infections with MH usually resolve spontaneously, ureaplasmas apparently can elicit an inflammatory response that is associated with intraventricular hemorrhage.[176]

In addition to CNS infections, mycoplasmas, particularly MH, have also been isolated from other extragenital infections in both children and adults. Perinatally acquired MH has been recovered from subcutaneous scalp abscesses at the site of forceps or monitor injuries, conjunctival infections, and submandibular lymph nodes in the newborn.[47,143,396] MH was also isolated in multiple blood cultures from a 10-month-old child who had suffered severe burns.[87] In adults, MH has been cultured from patients with a wide variety of infections, including bacteremia, postoperative and posthysterectomy wound infections, hematomas, septic arthritis, aspiration-associated empyema, septic thrombophlebitis, peritonitis, periorbital abscesses, intra-abdominal and retroperitoneal abscesses, and brain abscesses.[48,75,137,177,242,271,309,317,327,340,341,380,402] MH bacteremia and postoperative sternal wound infection have occurred in patients following cardiothoracic surgery.[347,352,451] In another report of a MH sternal wound infection, UU was also recovered.[390] MH prosthetic valve endocarditis has been reported, and both MH and UU have been isolated from pericardial tissues or fluids of patients with pericardial effusions.[40,135,236]

Patients with these infections had histories of recent cardiac surgery (e.g., coronary artery bypass surgery, aortic valve replacement, heart transplantation secondary to idiopathic dilated cardiomyopathy) or were immunocompromised (e.g., systemic lupus erythematosus, chronic obstructive pulmonary disease).[236] MH was also isolated from a surgical wound infection following insertion of a silicone breast prosthesis.[420]

MH and UU can behave as opportunistic infectious agents in those with underlying diseases or conditions, such as hypogammaglobulinemia, Hodgkin's disease, systemic lupus erythematosus, renal transplantation, heart disease, leukemia, lymphoma, rheumatoid arthritis, and severe trauma.[75,227,306,339,342,370,378,480,497] There is a patient with perihepatitis associated with a perinephric abscess due to MH that occurred 4 months postrenal transplantation.[53] In an otherwise immunocompetent Australian patient, a parapharyngeal abscess was identified following an acute Epstein–Barr virus infection.[235] MH and UU septic arthritis have been reported in patients with hypogammaglobulinemia, hematologic malignancies, massive trauma, postpartum bacteremia, lupus erythematosus, and common variable immunodeficiency; this clinical entity likely occurs following hematologic seeding of the joint spaces.[51,75,225,497] In 1994, Kane and associates reported the first patient with a respiratory tract infection with diffuse alveolar hemorrhage caused by MH; the patient was a bone marrow transplant recipient.[227] MH has been isolated from postoperative

perihepatic

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hematomas that developed in the abdomen of liver transplant recipient a month after the transplant surgery.[208] Both MH and UU were isolated from a retroperitoneal hematoma 12 days after liver transplantation in a 45-year-old woman with fulminant hepatic failure.[160] Infections of prosthetic joints by MH have also occurred, and MH wound infections following open reduction of fractured mandibles and after cesarean sections have been reported as well.[451] A dual infection with antibiotic-resistant MH and *U. parvum* progressed from septic arthritis to a fatal disseminated infection in an immunocompromised patient in Germany.[307] Infections in the head, neck, and chest areas and in tissue contiguous with the genital tract may reflect the presence of these organisms in the adjacent respiratory and urogenital tracts, respectively.[341,402] For example, Kayser and Bhend reported a 45-year-old woman with paraspinal and intervertebral soft tissue infection that developed 16 days after she underwent an abdominal hysterectomy.[233]

MH has also been isolated from the urine of patients with HIV infection. In a study by Chirgwin and colleagues at the State University of New York in Brooklyn, MH was isolated from 18% of the urine specimens obtained from 180 HIV-positive individuals and from 21% of urine specimens from 38 HIV-negative individuals.[68] In this study, an additional 30 glucose-using mycoplasmas were recovered only from the HIV-positive individuals.[68] Growth inhibition with species-specific antisera allowed for the identification of 18 of these isolates: 14 were identified as *M. fermentans* and 4 were identified as *M. pirum*. The remaining 12 isolates were nonviable on subculture.

Mycoplasma genitalium

In 1981, Tully and coworkers isolated a previously undescribed and unusually fastidious mycoplasma from urethral specimens of 2 of 13 homosexual men with NGNCU.[509] Although several subsequent attempts to isolate this new organism failed, several isolates of the same organism were recovered from urogenital specimens in China.[304] In 1983, this new species was named *M. genitalium*.[510] Ultrastructural analysis of these isolates indicated that the new organism shared several characteristics with *M. pneumoniae*. These characteristics included the tapering flask-like shape of the individual cells and the presence of a specialized apical structure that facilitated the adherence of the organisms to tissue cells, erythrocytes, and inert material such as plastic and glass.[510] *M. genitalium* possesses a species-specific, 140-kDa protein called P140 that is a structural and functional counterpart of the 170-kDa P1

cytoadhesin protein of *M. pneumoniae*.[346] Nonadherent variants of *M. genitalium* have an altered P140 adhesin or have lost the membrane-associated adhesin molecule.[334] *M. genitalium* and *M. pneumoniae* contain a 43-kDa cross-reactive protein that is also detectable on isolates of *Acholeplasma laidlawii*.[72] These shared antigenic epitopes are likely to be responsible for the serologic cross-reactions observed with *M. genitalium* in most tests for *M. pneumoniae* antibodies, including complement fixation (CF), indirect immunofluorescence assays, and metabolic inhibition and growth inhibition tests using heterologous antisera.[85,86,280] DNA hybridization studies have shown that the two organisms have 6.5% to 8.1% nucleotide sequence homology, the latter probably reflecting those genes that code for the antigenically cross-reactive proteins.[280,564] Other gene sequences are indeed unique for the two species, providing evidence that *M. pneumoniae* and *M. genitalium* are distinct species.[510] There have been many studies since the 1990s implicating *M. genitalium* with male and female reproductive tract syndromes. Since the organisms are very difficult to culture in routine laboratories, and serology is not often useful for diagnosis, dependence on PCR and other molecular methods for enhanced detection is necessary for research and diagnosis.[312] In a study by Taylor-Robinson and colleagues, rises in antibody titers to *M. genitalium* were detected in 4 of 14 patients with NGU and in 2 of 17 patients without urethritis, but only 1 of the 4 patients who seroconverted had a positive culture.[478] Failure to isolate the organisms in studies on urethritis probably reflected the lack of adequate techniques for reliable culture of this fastidious species. To circumvent the inherent problems of culture, other investigators have used species-specific nucleic acid probes prepared from genomic DNA of *M. genitalium* with or without simultaneous PCR.[185] These workers have detected the organism in 14% of men with gonococcal urethritis, 10% of men with chlamydial urethritis, 14% to 28% of men with NGU, and 13% to 23% of men with NGNCU. One study, in addition, detected *M. genitalium* DNA in 27% of 37 men with recurrent or persistent urethritis. However, the organism was also detected in 6% to 12% of men who had no genitourinary tract symptoms in these studies. The presence of this organism in the male urogenital tract was independent of the presence of *Chlamydia trachomatis* in many of these studies, although others have suggested an increased incidence with *C. trachomatis* infection.[206,207,216,218,219]Using PCR techniques, one of the above research groups failed to detect *M. fermentans*, *M. penetrans*, or *M. pirum* in urethral specimens from patients with NGNCU.[95] These workers concluded that *M. genitalium* was the etiologic agent in many cases of NGNCU; this was also supported by the clinical response of the infected men to treatment with doxycycline.[186]

Several studies using molecular techniques have now established the incidence of

M. genitalium as the cause of acute NGU and NGNCU. It is estimated that between 18.4% and 45.5% of all cases of NGNCU in men are due to this organism, and that persistence of the organisms in the urethra after antimicrobial therapy is associated with persistent or recurrent NGU.[97,212,310,333,484,501] Molecular approaches for the detection of *M. genitalium* include conventional and real-time PCR assays that amplify the *M. genitalium*-specific adhesin (MgPa) gene or species-specific regions of the 16S rRNA gene.[100,119,215,566] The use of real-time PCR assays for detection of *M. genitalium* in first-voided urine specimens has resulted in quantitative results, showing that the organism loads of *M. genitalium* in these specimens of symptomatic men are much higher than those seen in asymptomatic men. Organism loads in men with *M. genitalium*-positive NGU were suppressed during levofloxacin therapy, and the patients became asymptomatic. Following treatment, symptoms often resolved, but the organisms remained detectable in the urethra. The reappearance of symptoms was associated with an increase in organism load in patients in whom treatment failed. These studies have suggested that antimicrobial therapy that results in the elimination of the organisms from

the genital tract is required for the management of *M. genitalium*-associated urethritis to prevent subsequent recurrence of NGNCU.[100]

Blanchard et al. recently published on the evolution of *M. genitalium* and reported that *M. genitalium*, MH, and *Ureaplasma parvum* share a core genome of ~250 protein-encoding genes that correspond to their basic cellular metabolism, but also show a striking difference in their energy-generating pathways. They concluded that *M. genitalium* is a sexually transmitted organism associated with NGU in men and several inflammatory reproductive tract syndromes in women, such as cervicitis, PID, and infertility.[37] A review of 34 studied by Manhart et al. in a 2011 Centers for Disease Control and Prevention (CDC) publication "Sexually Transmitted Guidelines," which included >7,100 men between 1993 and 2011 using PCR as method of detection, concluded that *M. genitalium* can indeed cause acute urethritis. Twenty-eight of the 34 studies were from resource-rich countries, including the United States, and four were from China and Africa. Across all studies, 13% of men with NGU were positive for *M. genitalium* (incidence range of 5% to 42%), and in 11 studies that included NGNCU data, 25% were positive with a range of 10% to 38%.[312] Taylor-Robinson et al. have described the proposed pathogenesis of *M. genitalium* in a review paper.[486] A human endocervical epithelial cell model system has been used to demonstrate that *M. genitalium* infection can activate cellular host defense and inflammation pathways.[329]

A few studies have addressed the detection and significance of *M. genitalium* in the female genital tract.[22,481] In a study performed in a sexually transmitted disease (STD) clinic in London, *M. genitalium* was detected by PCR in genital tract specimens from 18% of 57 female patients.[374] Another study, done in Copenhagen, reported finding of *M. genitalium* in endocervical specimens from 5 of 74 women.[219] Neither of these studies addressed the association of these isolates with the presence of disease. Although definitive evidence on the pathogenicity of *M. genitalium* in the female genital tract is lacking, indirect evidence suggests that it may also be involved in both lower and upper genital tract infection. A study conducted in Japan found *M. genitalium* in 7.8% of 64 women with purulent cervicitis, and studies conducted in the United States found that, once gonococcal and chlamydial infections were excluded, patients with purulent cervicitis were more than three times as likely to have *M. genitalium* in the cervix than those without purulent cervicitis.[313,514] In a study comparing the prevalence of *M. genitalium, C. trachomatis, N. gonorrhoeae,* and *T. vaginalis* in adolescent girls, using transcription mediation amplification, *M. genitalium* was detected in 22% of the girls as compared with 24% *C. trachomatis* and had a lower prevalence compared with the other two agents. There was no association of *M. genitalium* with vaginal or cervical symptoms; however, there was a higher incidence of *M. genitalium* when *C. trachomatis* was present and there was a history of recent sexual contact.[198] Similarly, Casin and colleagues examined 170 women with genital tract symptoms attending a STD clinic in Paris and found no correlation between mucopurulent cervicitis and the presence of *M. genitalium*, despite the fact that 38% of the women had positive PCR results for *M. genitalium* at one or more genital sites (usually vaginal).[55] A more recent study of 261 women in New Zealand also showed no correlation between clinical symptoms of cervicitis and detection of *M. genitalium* in endocervical swabs (8.4% prevalence), by which they concluded that presentation of cervicitis should not necessarily elicit a screen for *M. genitalium*.[368] Although there is continued controversy regarding the association of *M. genitalium* and clinical genital disease in women, a recent study reviewed epidemiologic evidence in over 27,000 women with a range of prevalence of *M. genitalium* from 2% to 7% and demonstrated strong correlations between the presence of *M. genitalium* and lower genital tract infections (urethritis, cervicitis, and vaginal discharge), as well as convincing evidence for upper genital tract inflammation (i.e., PID and infertility).[328] The CDC publication referenced above also reviewed data concerning cervicitis and urethritis. In the 14 studies of cervicitis, 57% found an association, whereas 43% did not. However, they did find that the studies that used only clinical criteria for decisions about the diagnosis were less likely to find an association with *M. genitalium* as compared with studies that defined cervicitis as ≥30 leukocytes per high-power field. The conclusion of the CDC group was that there was

existing evidence to support the hypothesis that *M. genitalium* may cause cervicitis, although data are conflicting at this point. Only a few studies addressed female urethritis. In these studies, *M. genitalium* was found in 4% to 9% of women with urethritis. Their conclusion was that *M. genitalium* may cause female urethritis, but data are presently too limited to draw any definite conclusions.[312]

Evidence also exists for *M. genitalium* being involved in endometritis and acute PID. Serologic studies have documented elevated antibody titers to *M. genitalium* in some women with PID that was not attributable to *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, or MH; experimental studies in chimpanzees have demonstrated the ability of *M. genitalium* to cause upper and lower genital tract disease in this animal model.[344,479] Serologic studies also suggest that *M. genitalium* infection may be an independent risk factor in the pathogenesis of an inflammatory process that may lead to scarring of the uterine tissues, resulting in infertility.[73] A study in Nairobi, Kenya, that examined 115 women who presented with persistent pelvic pain found that 16% of 58 women with histologically confirmed endometritis were PCR-positive for *M. genitalium* in the cervix, the endometrium, or both locations.[76] In another study conducted in London, 13% of 45 women with PID had PCR-based evidence of *M. genitalium* in the endocervix, compared with none of the control patients.[446] A US study showed that 682 women with suspected endometritis and PID were treated with cefoxitin and doxycycline; those positive by PCR for *M. genitalium* had a higher risk of short-term failure and were more likely to develop recurrent pelvic pain and PID, as well as infertility, and decreased pregnancy and live births. The authors suggest that the recommended antimicrobial regimen is not adequate for patients with *M. genitalium* endometritis.[159] The association of *M. genitalium* and PID and subsequent reproductive sequelae, independent of *C. trachomatis* and *N. gonorrhoeae* infection, has been affirmed in a review article that examined studies in which PCR detection methods were used.[158] The Manhart CDC publication reviewed nine studies that examined the association with PID by serology or PCR, but in many of the studies, the absence of a negative control group was a limitation. Their conclusion

was that the data provide some support for a relationship, but at the present time, data remain conflicting.[312] Taylor-Robinson (2011) has implicated *M. genitalium* as a potential cause of adverse outcomes in pregnancy and felt that if it was detected, then the patient should be treated due to its pathogenic potential.[486] The Manhart review, however, on the basis of five published studies, suggested that *M. genitalium* was not common in women who have preterm births, at least in resource-rich countries, and

the data from resource-poor countries are conflicting. In this same review, there was no sufficient evidence from few studies that addressed ectopic pregnancies that implicated *M. genitalium.*[312]

In 1988, Baseman and coworkers recovered *M. genitalium* along with *M. pneumoniae* from 4 of 16 frozen specimens collected from the upper respiratory tracts of military recruits during an *M. pneumoniae* vaccine trial in 1974 and 1975.[23] The role of *M. genitalium* in respiratory tract disease is not clear, but it has been suggested that the association with *M. pneumoniae* may result in a synergistic infection that causes more severe pneumonia or may contribute to the pathogenesis of extrapulmonary complications of *M. pneumoniae* infection.[212] The presence of *M. genitalium* in the respiratory tract, with or without *M. pneumoniae*, and the immunologic cross-reactivity between the two organisms may not only complicate the infectious process in coinfected individuals, but may also affect the immunologic response to infection. Such interactions, consequently, may interfere with the interpretation of serologic tests that are frequently used to diagnose *M. pneumoniae* infections. The use of species-specific PCR assays for *M. pneumoniae* and *M. genitalium* may provide a solution to clinical and diagnostic problems by circumventing the need to cultivate these agents and clarifying the cross-reactive immune responses that occur with these organisms.[200]

Besides the respiratory and genital tracts, *M. genitalium* has also been recovered from joint fluid aspirates of patients with arthritis. *M. genitalium* was detected by PCR analysis in the knee joint of a 25-year-old man with Reiter's syndrome and has been detected in the knee joint of a 58-year-old man with seronegative rheumatoid arthritis.[482] Both *M. pneumoniae* and *M. genitalium* were detected in a specimen of knee joint fluid from a patient with hypogammaglobulinemia and polyarthritis following a bout of mycoplasmal pneumonia.[507]

M. genitalium has also been associated with HIV infection, although not to the extent that other species, such as *M. fermentans* and *M. penetrans,* have been associated. Montagnier and Blanchard detected *M. genitalium* in the blood of a patient with AIDS in 1993 by PCR.[345] However, a 1996 search for the presence of six *Mycoplasma* species in peripheral blood mononuclear cells (PBMCs) of 154 HIV-seropositive patients, 40 HIV-seronegative STD clinic patients, and 40 HIV-seronegative blood donors failed to detect *M. genitalium* by PCR in any of them.[254] Another search for mycoplasmas in the urine of 15 HIV-seropositive children conducted in 1999 found only a single specimen positive for *M. genitalium* in 1 of 9 HIV-infected children classified as having severe HIV disease.[199] *M. genitalium* has also been detected by PCR in urethral and rectal specimens from homosexual men with and without NGNCU.[483] An Australian study of the prevalence of genital mycoplasmas in women attending STD clinic using a PCR method of detection found

M. genitalium in only 4% of 527 women, as compared with prevalences of 17%, 14%, and 52% for MH, UU, and *U. parvum*, respectively; in this study, *M. genitalium* was significantly associated with women who were HIV-positive and women who presented with symptoms of cervicitis, as compared with the other mycoplasmas. These authors recommended routine screening for *M. genitalium* in women who are HIV-positive and/or present with cervicitis symptoms.[305]

Mycoplasma fermentans

M. fermentans is a fastidious human *Mycoplasma* species that has been recognized as a tissue culture contaminant for many years.[515] The increased usage of human-derived lymphocytes and macrophages in immunologic and viral research was likely responsible for recognition of this agent as a tissue culture contaminant. *M. fermentans* has been detected by PCR in salivary samples of healthy individuals and has also been detected in the genitourinary and respiratory tracts of humans.[67,439] In a PCR-based study conducted in London, *M. fermentans* was sought in throat swabs and urine specimens from healthy university students and from patients with congenital immunodeficiencies.[7] Twenty-seven percent of the students who submitted throat and urine specimens had *M. fermentans* in at least one of these specimens. *M. fermentans* was detected in one throat swab and three urine specimens from the 19 patients with congenital immunodeficiencies tested. A hamster model has demonstrated the ability of two different strains of *M. fermentans* and a strain of *M. pneumoniae* to cause interstitial pneumonitis, suggesting the pathogenic potential for *M. fermentans* for respiratory disease and perhaps other chronic infections in humans.[559]

In 1986, interest in *M. fermentans* as a human pathogen was stimulated when Lo and coworkers at the Armed Forces Institute of Pathology (AFIP) and the National Institutes of Health (NIH) reported the isolation of a novel mycoplasma, provisionally named *M. incognitus,* from Kaposi's sarcoma (KS) tissues of patients with AIDS.[281,290,291] Using immunohistochemical techniques employing monoclonal antibodies against this new agent, Lo and coworkers detected *M. incognitus* antigens in thymus, liver, spleen, lymph nodes and brain tissues of patients with AIDS, and in placental tissues delivered by pregnant women with AIDS.[284] The histopathology of the infected tissues showed a range of responses; in some tissues, no histologic changes were observed, whereas in others fulminant necrosis with inflammation was noted. Intraperitoneal inoculation of four silver leaf monkeys with this agent resulted in a wasting syndrome and death within 7 to 9 months.[294,296] These same workers also reported on six patients, from six different geographic areas, who presented with an acute flu-like illness and a syndrome of persistent fevers, lymphadenopathy, diarrhea, and multiple-organ failure, resulting in death within 1 to 7 weeks. Autopsies revealed fulminant necrosis of lymph node, lungs, liver, adrenal gland, heart, and brain tissues.

Immunohistochemical techniques and electron microscopy revealed mycoplasmal antigens in the areas of necrosis, and a labeled, specific DNA probe detected the genetic material of the agent in the infected tissues.[283] None of these patients were infected with HIV-1 or HIV-2. Nucleic acid hybridization profiles, restriction

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endonuclease mapping, and antigenic analyses indicated that *M. incognitus* was not a new mycoplasma, but rather a strain of *M. fermentans*, a fastidious, "nonpathogenic" mycoplasma found in the genital tract.[54,413]

The "incognitus" strain of *M. fermentans* was subsequently found to be associated with other histopathology in patients with AIDS. Bauer and colleagues examined kidney tissues of 15 patients with AIDS with light microscopic evidence of AIDS-associated nephropathy.[24] Tissues from these patients showed immunofluorescence reactions with *M. fermentans* incognitus strain-specific monoclonal antibodies in glomerular endothelial and epithelial cells, glomerular basement membranes, tubular epithelial cells and casts, and mononuclear interstitial cells. Mycoplasmal structures were also seen by immunoelectron microscopy in kidney tissues of these patients. Renal tissues from patients with AIDS without renal involvement and from non–HIV-infected patients with and without nephropathy did not reveal any organisms on histopathologic examination.[24] Ainsworth and associates also reported a patient with HIV-associated nephropathy in whom *M. fermentans* was detected in renal tissues by PCR when nephropathy first became clinically apparent; 18 months later, as the disease progressed and renal function deteriorated, PCR detected *M. fermentans* in the patient's urine, throat, and peripheral blood.[8] *M. fermentans* DNA was also detected in lymph node specimens from 4 of 7 patients with AIDS.[423] Lo and colleagues reported fulminant adult respiratory distress syndrome associated with *M. fermentans* in three patients without AIDS; immunohistochemical and electron microscopic studies revealed evidence of the organisms in the lungs and liver of these patients.[295] Disseminated *M. fermentans* infection has also been documented in HIV-positive patients with non-Hodgkin's lymphoma.[5]

Others also reported on the association of the *M. fermentans* incognitus strain with ultimately fatal disease in patients without AIDS. Macon and colleagues described a 35-year-old homosexual man with KS and T-cell lymphoma with a peripheral CD4 cell count of 43 per cubic millimeter.[308] Fatal *Pneumocystis* pneumonia and disseminated cryptococcal infection subsequently developed. Multiple enzyme immunoassays (EIAs) and Western immunoblots for HIV-1, HIV-2, human T-cell lymphotropic virus (HTLV)-I, and HTLV-II antibodies were negative, as were retroviral cultures and reverse transcription-PCR studies. Systemic *M. fermentans* infection was documented

by immunohistochemical and PCR methods in premortem and postmortem tissue specimens. Beecham and coworkers reported a similar instance, wherein a previously healthy 28-year-old nonimmunocompromised man presented with a 7-day history of fever, abdominal pain, diarrhea, rash, and shortness of breath. *M. fermentans* was detected in a bone marrow biopsy specimens by PCR and electron microscopy.[27] In another report of a previously healthy HIV-seronegative man with AIDS-like symptoms of fever, malaise, weight loss, diarrhea, and extensive liver and spleen necrosis, treatment with doxycycline (300 mg/day, orally for 6 weeks) resulted in resolution of the symptoms and full recovery.[282] These studies suggested that *M. fermentans*, or a specific strain of this species, may be a systemic pathogen in non–HIV-infected patients.

Using PCR technology, Dawson and associates detected *M. fermentans* in 23% of 43 urine sediment specimens from patients with AIDS-associated nephropathy.[89] Some of these urines were culture-positive for MH and/or UU. None of 50 urine sediment specimens from 50 HIV-negative, healthy patients were culture- or PCR-positive for *M. fermentans*, although 23 specimens grew UU and one specimen grew MH.[89] Katseni and coworkers examined blood, throat, and urine specimens from 117 HIV-positive patients and 73 HIV-seronegative patients attending an STD clinic in London.[231] *M. fermentans* was detected by PCR in 10% of peripheral blood mononuclear specimens, 23% of throat specimens, and 8% of urine specimens from the HIV-positive patients. Among the 73 HIV-seronegative patients, *M. fermentans* was detected in PBMCs, throat, and urine specimens of 9%, 20%, and 6%, respectively. Hawkins and colleagues at the NIH detected *M. fermentans* DNA sequences in 11% of blood specimens from 55 HIV-seropositive patients, but detected none in 26 HIV-seronegative, low-risk individuals.[172] In France, Bebear and coworkers performed culture and PCR on throat, endocervical, urethral, urine, and PBMC specimens from 105 HIV-positive individuals.[25] Although culture and PCR assays for *M. pneumoniae* and *M. genitalium* were negative, *M. fermentans* was detected by PCR in a least one specimen site in 26.7% of the 105 patients. The presence of *M. fermentans* in these patients was not associated with the stage of HIV disease, the viral load, or the CD4 count.

M. fermentans is able to actively invade cultured cells, a property not previously associated with *Mycoplasma* species. Although an *M. fermentans* reference strain and the incognitus strain were able to invade HeLa cells, *M. fermentans* incognitus strains were more invasive when tested in tracheal explant cell cultures. Incognitus strain organisms could be observed intracellularly, whereas the reference strain was observed adhering to only between cells.[456,488] Intranasal infection of rats and subsequent explant cultures of the tracheal tissues of these animals also revealed an intracellular localization of these organisms within the cultured cells.[456] *M. fermentans* incognitus

strain and the other *M. fermentans* strains cause ciliostasis and cytopathology in tracheal explant cultures, but the extent and severity of the cytopathology varied considerably from strain to strain. Studies in animals also indicated that immunosuppression may allow *M. fermentans* to grow and flourish. Intravenous administration of *M. fermentans* incognitus strain killed BALB/c nude mice, but did not kill immunocompetent BALB/c mice.[477]

Although some investigators have noted differences between the *M. fermentans* incognitus strain and other *M. fermentans* strains, others have noted no differences. Sasaki and coworkers compared the original incognitus strain with three other reference and clinical isolates of *M. fermentans*. Restriction-fragment-length polymorphism analysis of the DNA from these strains showed similar patterns on gel and immunoblot profiles of cellular proteins, indicating a high degree of antigenic homogeneity among the four strains examined. Exposure of PBMCs to these mycoplasmas resulted in significant increases in the production of IL-1β, IL-6, and TNF-α; these increases were observed after exposure to all four *M. fermentans* strains and were not restricted to the incognitus strain. Lastly, exposure of HIV-infected PBMCs to any of the *M. fermentans* strains resulted in a 1.8- to 4.3-fold enhancement of reverse transcriptase activity and a 3.3- to 7.0-fold increase in the production of p24 antigen in the

culture. These workers concluded that the incognitus strain of *M. fermentans* was not unique when compared with other *M. fermentans* strains.[422]

Several investigators are currently examining the role of *M. fermentans* and other mycoplasmas as cofactors affecting progression of HIV-associated disease.[6,190] In vitro studies on HIV-infected CEM cells (a CD4-enriched T-lymphoblastoid tumor cell line) showed that treatment of the cells with tetracycline analogs or fluoroquinolones was able to inhibit the production of virus-induced cytopathic effects without inhibiting the replication and production of progeny virus.[266,363] Subsequent studies have indeed shown that *M. fermentans* acts synergistically with HIV-1 in lymphoblastoid and promonocytic cells to produce cell death; this ability has also been demonstrated to occur with other *Mycoplasma* species, including *M. penetrans*, *M. pirum*, and *M. arginini*.[267,292] Phillips and associates showed that mycoplasmal attachment to HIV-infected lymphocytes was associated with sites of viral budding, leading these investigators to surmise that attachment of mycoplasmas may trigger or enhance production of progeny virus by infected cells.[387] Several mechanisms have been proposed whereby mycoplasmas such as *M. fermentans* may act as cofactors or immunomodulators in the production of HIV-associated disease.[345] Lymphocyte

activation stimulates the replication of HIV, and *Mycoplasma* species, including *M. fermentans* and *M. penetrans*, have the capacity to behave as polyclonal activators of both B and T lymphocytes.[127,203,460] *M. fermentans* is able to induce the production of lymphokines (i.e., TNF-α, IL-1, and IL-6) in various cell types (i.e., monocytes, macrophages, astrocytes, and glial cells) in vitro.[133,350,351] Muhlradt and Frisch isolated and partially purified a substance, which they termed mycoplasma-derived high-molecular-weight material (MDHM), that was membrane associated, contained lipid, and existed in an aggregated form when purified.[349] The presence of MDHM in nanogram per milliliter amounts activates macrophages to release nitric oxide, IL-6, and TNF. Another group identified a 48-kDa, hydrophobic, membrane-associated *M. fermentans* antigen distinct from MDHM that also stimulates the secretion of both TNF and IL-1 from cultured monocytes.[250] *M. fermentans* may also produce superantigens that bind directly to the major histocompatibility complex (MHC) proteins, thereby stimulating T-lymphocyte activation. Another *M. fermentans*-derived lipoprotein called the macrophage-activating lipopeptide 2 (MALP-2) has also been shown to induce the release of pro-inflammatory cytokines, chemokines, and nitric oxide by mouse peritoneal macrophages.[232] *M. fermentans* also enhances concanavalin A-induced apoptosis of T cells.[441] Some investigators have proposed that the production of peroxides and free radicals by mycoplasmas or the production of enzymes that inactivate the normal intracellular catalase may induce HIV gene expression and activate viral replication. Peroxides and other reactive oxygen species induce HIV gene expression in vitro by transactivation of viral promoter regions and contribute to programmed cell death.[265] *M. fermentans* incognitus strain is able to fuse with both T cells and peripheral blood lymphocytes.[105] The delivery of mycoplasmal components into these cells may directly affect normal lymphocyte functions. In addition, the fusion of mycoplasmal and lymphocyte membranes may significantly change the structure or orientation of various receptors on the lymphocyte surface, thereby altering the binding, induction, or production of various lymphokines. These fundamental alterations in lymphocyte structure and function may influence attachment, integration, and gene expression of HIV. Both *M. fermentans* and *M. penetrans* also possess membrane-associated phospholipase C, which can induce an inflammatory response, cause membrane damage to host cells, and activate the arachidonic acid cascade.[440]

M. fermentans may also play a role in genital tract infections. In 1989, *M. fermentans* was detected in the placental tissues of two women with AIDS.[284] Blanchard and coworkers used culture and PCR methods to examine the presence of both *M. genitalium* and *M. fermentans* in the genital tracts of sexually active adults and in the amniotic fluids of women with intact fetal membrane who were undergoing cesarean deliveries.[38] *M. genitalium* was detected by PCR, but not by culture in 11%

of 94 men and 87 women with clinical symptoms of NGU or cervicitis, but was not detected by either method in any of 232 amniotic fluid specimens. Conversely, *M. fermentans* was not detected by either method in any of the urogenital specimens, but was detected by PCR in 4 of 232 amniotic fluid specimens, suggesting that the organism may be transmitted transplacentally. Interestingly, 2 of the 4 patients with positive PCR results for *M. fermentans* had histologic evidence of chorioamnionitis, implying that *M. fermentans* may also be a genital tract pathogen.[38]

M. fermentans has also been implicated in rheumatoid arthritis and other rheumatic disorders.[189,222] In a 1996 study conducted in London, *M. fermentans* was detected by PCR in synovial fluid specimens from 21% of 38 patients with rheumatoid arthritis, 20% of 10 patients with spondyloarthropathy, 20% of 5 patients with psoriatic arthritis, and 13% of 31 patients with unclassified arthritic condtions.[427] Genotypic characterization of isolates from synovia of patients with arthritis revealed no unique characteristics; some were related to the *M. fermentans* American Type Culture Collection (ATCC) type strain, while others were related to strains isolated from tissue cultures as contaminants.[426] *M. fermentans*, *M. salivarium*, or both were detected by PCR in synovial fluid specimens from aspirates from the temporomandibular joint of patients with destructive osteoarthritis of the jaw.[544] This condition is thought to result from the action of proteases and cytokines on the articular cartilage of this joint. Watanabe and coworkers suggested that mycoplasmas present in the temporomandibular joints damage the synovium and cartilage by their enzymatic activities plus the recognized ability of mycoplasmas to stimulate release of cytokines from immune effector cells.[544] In a 2000 study, *M. fermentans* was detected by PCR in the synovial fluid of 88% of 26 rheumatoid arthritis patients. Seven of 8 patients with a variety of other inflammatory arthritides were also positive for *M. fermentans*.[189] A study published in 2001 also found *M. fermentans* DNA in synovial fluids of 17% of 35 patients with rheumatoid arthritis, 25% of 44 patients with undifferentiated seronegative arthritis, and 17% of 24 patients with psoriatic arthritis.[142] More recently, *M. fermentans* was found by culture or PCR in 23% of blood samples taken from 87 patients with rheumatoid arthritis, and in the same study, there was a higher incidence of IgG and IgM antibodies to an antigen of *M. fermentans* in these arthritic patients than in healthy controls.[139]

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M. fermentans infection, like many other organisms, has been investigated for its relationship to chronic fatigue syndrome (CFS). Using PCR-based assays for *M. fermentans*, MH, and *M. penetrans*, Choppa and colleagues examined PBMCs from 100 patients with CFS.[70] *M. fermentans*, MH, and *M. penetrans* were detected in the

PBMCs of 32%, 9%, and 6% of the 100 patients with CFS, respectively, whereas they were detected in 8%, 3%, and 2% of healthy controls, respectively. A subsequent study by the same research group detected *M. fermentans* in PBMCs of 36% of 50 patients with typical CFS as defined by the CDC, 32% of 50 patients with atypical CFS, and in 8% of 50 healthy control subjects.[521] Nasralla, Haier, and Nicolson used PCR methods to detect *M. fermentans*, *M, pneumoniae*, *M. penetrans*, and MH in blood specimens of 91 patients with CFS and/or fibromyalgia syndromes.[355] Among the 91 subjects, 59%, 48%, 31%, and 20% were positive for *M. pneumoniae*, *M. fermentans*, MH, and *M. penetrans*, respectively. Of the 91 subjects, 53% had molecular evidence of bloodstream infection with multiple *Mycoplasma* species. In a survey of 261 patients with CFS and 36 healthy volunteers, bloodstream infection by at least one *Mycoplasma* species was detected by a forensic PCR test in 68.6% of the patients with CFS and in 5.6% of healthy volunteers.[361] MH was detected in 36.8%, followed by *M. fermentans* and *M. pneumoniae* in 25.7% and 25.7%, respectively. Infection with multiple *Mycoplasma* species was detected in 17% of the patients with CFS. Although these studies show that high rates of infection with *Mycoplasma* species are found in patients with CFS, there is no definitive evidence that these mycoplasmas are the causative agents of CFS. The development of sensitive assays and improvements in culture techniques for these organisms will hopefully enable natural history and treatment studies to be undertaken.

M. fermentans was also postulated to play a role in the illness observed among veterans of Operation Desert Storm. A serologic survey study was conducted among Persian Gulf War veterans and nondeployed servicemen using both prewar and postwar serum specimens. *M. fermentans* infections were documented to have occurred in this military population both before and after service in the war, but these infections were independent of deployment to the Persian Gulf, indicating that epidemiologic and serologic data did not support a role for *M. fermentans* in the illness seen in Gulf War vetereans.[153] A similar study conducted by the AFIP also concluded that there was no serologic evidence for an association between *M. fermentans* infection and the "Gulf War illness."[289]

Even with its relatively short but fascinating epidemiologic history and the intensity of investigations by several research groups, the role of *M. fermentans* in HIV pathogenesis, STDs, rheumatoid arthritis, CFS, and other conditions is not known. Although molecular methods facilitate the detection of specific gene sequences of the organisms, the lack of reliable culture techniques complicates studies that may include various treatment methods. *M. fermentans* has been recovered in culture from joint fluid specimens, but it is often not known whether molecular assays are detecting viable or nonviable organisms. Molecular studies have shown that *M. fermentans* is genotypically heterogeneous, as evidenced by the presence of insertion sequence-like elements at various locations in the DNA, resulting in interstrain and intrastrain

variation.[391] In studies in which the organism has been successfully grown in SP-4 medium, biochemical testing has delineated four phenotypic clusters based on the utilization of arginine, glucose, fructose, and *N*-acetyl-glucosamine.[373] Systematic phenotypic and genotypic analysis of *M. fermentans* isolates from a variety of clinical specimens and conditions may help to define the pathogenicity and pathogenesis of *M. fermentans* infections and the role of this organism in various clinical settings.

Mycoplasma penetrans

In 1991, Lo and colleagues at the AFIP in Washington, DC, reported the isolation of a new *Mycoplasma* species from the urogenital tracts of homosexual men with HIV infection.[287] This organism exhibited unique morphologic characteristics, including a flask-shaped cell body composed of two compartments: one containing loosely packed, coarse granules consistent with ribosomal structures and the other tapered, smaller compartment containing densely packed fine granules.[286] This organism fermented glucose and hydrolyzed arginine, but not urea, required cholesterol for growth, and could be cultivated in SP-4 medium. It was also able to adhere to and actively invade mammalian cells.[285] Intracellular organisms were observed to be inside membrane-bound vesicles, with subsequent cell disruption and necrosis. This new species was given the name *Mycoplasma penetrans*.[286,424] Two separate studies investigating the prevalence of genital mycoplasmas in Nigerian men and women have shown *M. penetrans* in low numbers as compared with other genital mycoplasmas. *M. penetrans* was cultured from three vaginal specimens. In this study, 81% of the isolated *Mycoplasma* spp. were MH and 13% *M. fermentans,* as compared with 6% *M. penetrans*. One isolate of *M. penetrans* was isolated from semen samples in men seeking help with infertility; in this study, 24% of the *Mycoplasma* spp. were MH, 10% *M. fermentans*, and 5% *M. penetrans*.[3,4]

Seroepidemiologic studies have documented a high frequency of antibodies against *M. penetrans* in HIV-infected individuals.[93,149,512,540] Wang and associates found that 35.4% of serum samples from 444 HIV-1-infected patients were positive for antibodies against *M. penetrans*.[540] Among 234 men with AIDS, 41.5% were seropositive; in comparison, 20.3% of 118 asymptomatic HIV-1-infected individuals were also seropositive. Only one of 384 HIV-negative blood donors had antibodies against *M. penetrans*. Interestingly, 40% of 85 serum samples archived from patients with "GRID" (gay-related immunodeficiency), a term used in the early 1980s to describe AIDS, were also positive for anti-*M. penetrans* antibodies. Among 336 serum specimens from STD clinics in southern California, Brooklyn, and Milwaukee, only three were positive for *M. penetrans* antibodies. None of the 178 serum specimens obtained from HIV-1-seronegative individuals with other diseases or immunologic

disorders (e.g., patients on dialysis, or patients with systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, leukemia, lymphoma, or other cancers) were positive for antibodies against *M. penetrans*.[540] Additional seroepidemiologic studies by the same research group found high rates of mycoplasmal antibodies in both symptomatic and asymptomatic HIV-1-positive homosexual men, but only 1% of 308

specimens from intravenous drug users and only 0.6% of 165 specimens from patients with hemophilia with or without HIV-1 infection, suggesting that *M. penetrans* may be sexually transmitted.[541] Grau and coauthors reported very similar findings on the presence of *M. penetrans* antibodies in a study conducted in France.[149] These workers found that positive *M. penetrans* serology was associated with both HIV-1 infection and with high-risk sexual practices among homosexual men; the seroprevalence in these patients increased with progression of AIDS-related disease. During the early 1990s, some researchers reported an epidemiologic relationship between the presence of *M. penetrans* antibodies and the development of KS. Lo and colleagues examined *M. penetrans* seroprevalence in a cohort of 33 HIV-seropositive and 31 HIV-seronegative homosexual men who were enrolled in an AIDS natural history study in New York City in 1984.[288] Testing of archived serum specimens from 1984 to 1985 revealed that 45.5% of the HIV-positive men and 22.5% of the HIV-negative men had antibodies against *M. penetrans*, suggesting that *M. penetrans* was circulating as a probable sexually transmissible agent early in the AIDS epidemic and was not necessarily associated with HIV infection. Over the subsequent 8 years, KS developed in nine men; seven of which had *M. penetrans* antibodies. Baseline seropositivity to *M. penetrans* was statistically associated with the subsequent development of KS, and among the HIV-positive men, KS was more likely to occur among men who had positive baseline serologies for *M. penetrans*. These workers concluded that *M. penetrans* infection may act as a cofactor with HIV in the subsequent development of KS.[81,288,541] However, others have found no association between *M. penetrans* infection and KS, which is now known to be associated with human herpesvirus type 8 infection.[149]

The use of sensitive molecular techniques for direct detection of *M. penetrans* has shown that this organism may be involved in chronic infections of cells and/or tissues other than blood mononuclear cells. Kovacic and colleagues used specific PCR assays for *M. pneumoniae*, *M. fermentans*, *M. genitalium*, *M. pirum*, *M. penetrans*, and MH to investigate the presence of these organisms in PBMCs from subjects infected or not infected with HIV-1.[254] Only *M. fermentans* was detected in 5.8% of 154 HIV-seropositive and 11.1% of HIV-seronegative patients. These investigators suggested

that the urogenital tract is the probable ecologic niche of this organism, since it was originally isolated from urine specimens and has been detected by PCR in urine specimens from patients who are seropositive for *M. penetrans* antibodies.[149,286,287] *M. penetrans* bacteremia has also been reported in non–HIV-infected patients with antiphospholipid syndrome.[558]

The exact role of *M. penetrans* in the pathogenesis of HIV infection is not known. Most work has centered on its putative role as a cofactor in HIV-associated disease progression. Sasaki and colleagues at the Institut Pasteur demonstrated that *M. penetrans* was able to activate human T lymphocytes to undergo blastogenesis followed by cell proliferation and expression of cell surface markers of activation.[421] These phenomena were observed with lymphocytes from healthy donors and from both symptomatic and asymptomatic HIV-infected patients. Characterization of the lymphocytes activated by exposure to *M. penetrans* showed that lymphocytes expressing either CD4 or CD8 underwent blastogenesis as a result of this expression. Activation activity was associated exclusively with cells of *M. penetrans* and not with culture supernatants. In cultured lymphoid cells, infection with *M. penetrans* stimulates the release of TNF-α and enhances replication of coinfecting HIV-1.[203] Longitudinal serologic studies have also demonstrated higher HIV viral loads and more rapid declines in CD4 cell counts in patients with persistently high or increasing anti-*M. penetrans* antibody titers, suggesting an association between active *M. penetrans* infection and progression of HIV disease.[150]

M. penetrans has been shown to bind human serum IgA and secretory IgA, but not IgG. The authors of this research suggested that this binding might afford a defense mechanism to *M. penetrans* in avoiding clearance by IgA antibodies as they establish infection.[348]

Mycoplasma pirum

Although originally described in 1985, *M. pirum* has attracted some attention recently because it is another of the AIDS-associated mycoplasmas, along with *M. fermentans* and *M. penetrans*. Before this association, *M. pirum* had been isolated only from cell cultures as a presumed contaminant, but the cell lines from which it was isolated were of human origin; it is now thought that these mycoplasmas may have been in the cultured tissues naturally.[102] *M. pirum* has been isolated from PBMCs of a patient with HIV infection and has been detected by PCR in blood mononuclear cells of HIV-seropositive individuals.[147] Similar to *M. fermentans*, HIV-associated cytopathic effects in vitro are also enhanced by the presence of *M. pirum*, leading to the suggestion that, similar to *M. fermentans*, *M. pirum* may act as a cofactor in the pathogenesis of HIV-related conditions.[345]

M. pirum is closely related to *M. penetrans* and *M. pneumoniae*, and, similar to these two other mycoplasmas, has a flask-shaped morphology and a "tip" by which the organism is able to attach to glass or plastic. The P1-like adhesin protein of *M. pirum* and the gene coding for it have been characterized.[494] Following attachment, *M. pirum* is able to actively invade cells, as are *M. fermentans* and *M. penetrans*.[475] Research is underway to further define the interaction of *M. pirum* and other mycoplasmas with human cells and to investigate the role of these organisms, if any, in the pathogenesis of HIV-associated disease.

Mycoplasma primatum

M. primatum is a common inhabitant of the oral and urogenital tracts of cercopithecine monkeys, and was first isolated from these animals in 1971.[101] The strains isolated from monkey tissues in 1971 were found to be similar to unclassified mycoplasmas that had been isolated from an infected umbilicus and from the vagina of a human patient in 1955.[411] Reexamination of the monkey isolates and the unclassified human isolates found them to be all the same. This mycoplasma used arginine, required serum or cholesterol for growth, and was negative for glucose fermentation, urease, hemolysis, hemadsorption activity, and tetrazolium reduction. These human/primate isolates were given the name *M. primatum*. To date, this mycoplasma has not been associated with infections or clinical disease.

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Mycoplasma salivarium

M. salivarium is most often isolated from the respiratory tract and resides in dental plaque and gingival sulci.[122] It has been most often associated with periodontitis, but has been isolated from joints and other sterile sites in patients with hypo- or agammaglobulinemia and has been found in a submasseteric abscess in an elderly woman.[154,452] Two patients with cerebral abscess in which *M. salivarium* was felt to have a causal role were reported from Denmark in 2011. Both were men, 53 and 39 years old, and presented with signs and symptoms of CNS disease; CT scans revealed an abscess in parietal-occipital and temporal lobe, respectively. The 53-year-old man had prior tuberculosis, poor dentition, and prior alcohol abuse. He presented with 3 weeks of dyspnea, headache, and impaired extremity coordination. The 39-year-old man had no known prior conditions. The aspirate from these abscesses disclosed the presence of *M. salivarium* by both culture and PCR; the abscesses also contained combination of oral aerobes and anaerobes. A combination of penicillin, metronidazole, and moxifloxacin was used in each case. The older patient fared better

than the younger, who had neurologic deficits and associated problems up to 6 months after treatment.[371]

Mycoplasma spermatophilum

M. spermatophilum is a newly described anaerobic *Mycoplasma* species that has been isolated from five sperm specimens and from a single endocervical specimen obtained from six patients who were attending an infertility clinic.[181] In culture, this obligate anaerobic species grows best at 35°C to 37°C and produces a hemolysin that lyses guinea pig, sheep, and human erythrocytes. Colonies have the typical fried-egg appearance on agar media. This species requires sterols for growth and does not use glucose or hydrolyze either arginine or urea; it is not yet recognized as a human pathogen.

Human Infections Due to Mycoplasmas *of Animal Origin*

M. arginini is one of the few mycoplasmas found in a number of animal hosts, including sheep and goats (respiratory and ocular tissues), cattle (respiratory tract, blood, udder, eyes, urogenital tract), and felines (respiratory tract). These animals may or may not exhibit pathologic symptoms, and the role of this organism in animal diseases is still unknown. Yechouran and colleagues reported a 64-year-old man with stage IVB non-Hodgkin's lymphoma who developed pneumonia complicated by fatal septicemia caused by *M. arginine.* This patient had hypogammaglobulinemia and had been receiving prednisone.[562] Before his admission to the hospital, he had worked in a slaughterhouse, where he slaughtered sheep, cows, and chickens. *M. arginini* grew from three blood cultures: a bronchial brush specimen, a bronchoalveolar lavage (BAL) specimen, and the tip of a Swan–Ganz catheter. Susceptibility testing of the isolate using a broth dilution procedure found that it was susceptible to tetracycline, doxycycline, and ciprofloxacin, but resistant to erythromycin and streptomycin. This susceptibility pattern has also been found in *M. arginini* isolates recovered from animals. The authors of this case report suggested that the patient acquired his infection by inhalation of contaminated aerosols in the slaughterhouse setting. *M. arginini* has also been isolated along with *U urealyticum* from an otherwise healthy 23-year-old Hungarian man with eosinophilic fasciitis presenting with generalized sclerodermiform skin lesions that progressed over 19 months. Blood was cultured for the *M. arginini*, and a species-specific PCR was positive from the skin biopsy. No comments about animal contact were given.[444]

Armstrong and colleagues reported a pulmonary infection caused by *M. canis* in a woman who was receiving antineoplastic therapy for metastatic carcinoma of the cervix.[17] The same organism was isolated from the respiratory tract of other family members and from the family dog, all of whom had upper respiratory tract infections

at the same time. Bonilla and colleagues also reported a case of septic arthritis of the left hip and right knee due to *M. canis* in a woman with common variable immunodeficiency.[45] This patient had a history of exposure to cats. Despite surgical debridement and treatment with doxycycline, widespread chronic osteomyelitis of the hip developed, necessitating hip arthroplasty.

Hemotrophic Mycoplasma *Species*

Haemobartonella and *Eperythrozoon* species are mycoplasma-like bacteria that adhere to and grow on the surfaces of red blood cells of several vertebrate species.[258] Until recently, these organisms were classified in the order *Rickettsiales*, since they resemble the rickettsiae with respect to their size, staining properties, and transmission from animal to animal by arthropod vectors. On the red blood cell surface, these bacteria appear as small, blue-to-purple, coccoid, rod- or ring-shaped structures when stained by the Wright–Giemsa method or by acridine orange.[41] Phenotypically *Eperythrozoon* species form ring-shaped structures on the surface of red cells and may often appear unattached to red cells and free in the plasma. *Haemobartonella* species, in contrast, do not form ring-shaped structures and are rarely seen in the free unattached state. The arbitrariness of the phenotypic differentiation of these two species has been recognized for some time and has led to their recent reclassification (see below). These bacteria are noncultivable using standard bacteriologic media and are maintained in research laboratories by serial passage in their animal hosts. Infections in animals caused by *Eperythrozoon* and *Haemobartonella* species are usually asymptomatic and may persist in a latent form for years; it is thought that the organisms are cleared from the circulation by the spleen.[311] Because of predisposing factors that are not completely understood (i.e., stress, etc.), large numbers of organisms may reappear in the bloodstream. In some animals, a subtle, chronic anemia develops or an overt, severe, and often fatal hemolytic anemia may develop.[336,337] For example, the well-studied feline hemotrophic mycoplasmas ordinarily do not cause disease in naturally infected animals, but may cause anemia in feline leukemia virus (FeLV)–infected cats and may accelerate the development of FeLV-induced myeloproliferative diseases.[464] Hemotrophic mycoplasmas are transmitted from animal to animal by various blood-feeding arthropods, including lice, ticks, flies, fleas, and mosquitoes.

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As they were discovered, the various species of *Haemobartonella* and *Eperythrozoon* were named after the host that they primarily infected, although the host ranges of the various species were really not known. As of the mid-1990s, the

recognized species of *Haemobartonella* and *Eperythrozoon* were *H. canis* (canines), *H. felis* (cats), *H. muris* (rodents), *E. coccoides* (mice), *E. ovis* (sheep), *E. suis* (swine), *E. parvum* (pigs), and *E. wenyonii* (cattle). In 1997, the 16S rRNA gene sequences of *H. felis*, *H. muris*, *E. suis*, and *E. wenyonii* were investigated. This demonstrated that these bacteria were not phylogenetically related to the rickettsiae, but were most closely related to members of the genus *Mycoplasma*.[359,403] In 2001, these four species organisms were removed from the order *Rickettsiales*, family *Anaplasmataceae*, and were given "candidatus" status in the genus *Mycoplasma* as "*Candidatus* Mycoplasma haemofelis," "*Candidatus* Mycoplasma haemomuris," "*Candidatus* Mycoplasma haemosuis," and "*Candidatus* Mycoplasma wenyonii."[221,357,469] "Candidatus" status is usually reserved for new, incompletely described taxa. Because of this, the Judicial Commission of the International Committee on the Systematics of the Prokaryotes ruled that, by changing validly published organism names to the taxonomically demoted "candidatus" status, the original names would lose standing because there would be no new or replacement names to add to the Approved List of Bacterial Names. As a result, all the species that had been renamed under "candidatus" status were revised by eliminating the "candidatus" status and renaming them as new species in the genus *Mycoplasma*.[358] At the same time this opinion was published, three additional "*Candidatus*" species —"*Candidatus* Mycoplasma haemodidelphidis," "*Candidatus* Mycoplasma haemolamae," and "*Candidatus* Mycoplasma haemominutum"—were described as hemotrophic bacteria from naturally infected opossums, alpacas, and cats, respectively.[338]As a result, the former *Eperythrozoon* and *Haemobartonella* species are now classified in the genus *Mycoplasma* as *M. haemofelis* (cats), *M. haemomuris* (mice), *M. haemosuis* (pigs), *M. wenyonii* (cattle), *M. ovis* (sheep and goats), *M. haemolamae* (lamas),*M. haemocanis* (canines), *M. haemodidelphidis* (opossums), and "*Candidatus M. haemominutum*."[356,357,470] Together, these hemophilic mycoplasmas have been given the trivial name "hemoplasmas."

Culture of Human Mycoplasmas From Clinical Specimens

General Considerations

Human mycoplasmas can be divided into three groups on the basis of the utilization of three substrates: glucose, arginine, and urea (Table 18-1). Depending on the species of *Mycoplasma* that is being sought, an enriched peptone basal medium, containing yeast extract and serum, is supplemented with one of these three substrates and a pH

indicator (usually phenol red) is added. *M. pneumoniae* metabolizes glucose to produce lactic acid, resulting in a shift to an acidic pH. MH metabolizes arginine with the production of ammonia and a shift in pH from neutral to alkaline. Similarly, UU produces urease enzymes that hydrolyze urea to ammonia, which also results in an alkaline pH shift. *M. fermentans* produces acid from glucose and also metabolizes arginine.

Specimens for the isolation of mycoplasmas, particularly the more rapidly growing genital mycoplasmas, are routinely inoculated onto both solid agar media and into some type of selective/differential broth enrichment media. Many broth media formulations used for isolation of mycoplasmas are diphasic, with medium containing agar in the butt of a tube overlaid with broth of similar composition. Media for isolation of mycoplasmas also contain antibiotics (e.g., ampicillin, penicillin, polymyxin B, and amphotericin) to inhibit contaminant bacteria and fungi.

Human mycoplasmas differ in their optimal pH for growth and in the atmospheric conditions that are required for successful recovery from clinical specimens (Table 18-1). Media for the isolation of *M. pneumoniae* are buffered at an initial pH of about 7.8, whereas the growth medium for MH is buffered initially at a neutral pH (7.0). UU grows optimally in an environment with a slightly acid pH, so a primary isolation medium for this species is buffered at about pH 6.0. The optimal temperature for mycoplasmal growth is 35°C to 37°C. *M. pneumoniae* and MH grow well in air or in an atmosphere of 95% nitrogen and 5% carbon dioxide. UU tends to be capnophilic, with optimal growth occurring in an atmosphere of 10% to 20% carbon dioxide and 80% to 90% nitrogen. An agar medium inoculated directly with specimens or subcultures from broths should be incubated under the appropriate conditions described in the foregoing; a broth medium can always be incubated under aerobic conditions.

Broth media are generally inoculated with 0.1 to 0.2 mL of the specimen contained in a transport fluid. Agar plates are inoculated with a similar amount, and the inoculum is spread over the surface of the agar with a sterile bent glass rod. Agar plates are sealed with air-permeable cellophane tape to prevent desiccation of the agar. Media for isolation of *M. pneumoniae* should be incubated for up to 4 weeks before a final culture report is made. Cultures for genital mycoplasmas should be incubated for 7 to 8 days; most positive broth cultures will be detected after 5 days of incubation. Diphasic media are compared with co-incubated tubes of the same media inoculated with sterile transport medium and with tubes inoculated with control mycoplasma strains to detect subtle differences in color or turbidity. Cultures should be inspected daily for subtle changes because the organisms die rapidly once growth occurs and the substrates are utilized. If a potentially positive culture is detected visually in diphasic medium, the broth is subcultured onto a solid agar medium, such as SP-4 agar or A7 differential agar (see discussion below).

Identification of the mycoplasmas requires the recognition of typical colonies on

solid media directly inoculated with the specimen or with a loopful of broth medium from a presumptively positive diphasic broth medium. Colonies on agar media can be directly examined under 30× to 100× magnification with peripherally incident light to ascertain morphology and growth characteristics. Supravital dyes, such as Dienes' stain, can be used to further characterize colonies and to differentiate them from artifacts. Various identification tests may be performed directly on solid medium, such as the hemadsorption test for presumptive identification of

M. pneumoniae, or the utilization of substrates such as arginine or urea, plus a phenol red indicator, may be incorporated into the agar to provide a direct assessment of substrate utilization and, therefore, a presumptive identification. Media for culture of respiratory and genital mycoplasmas and identification procedures for *M. pneumoniae* and UU are detailed in Charts 18-1 to 18-6.

Specimen Collection

M. pneumoniae may be recovered from both upper and lower respiratory tract specimens, including throat swabs, nasopharyngeal swabs, throat washings, sputum, tracheal and transtracheal aspirate, bronchoscopy, BAL, and lung tissue specimens. The organism may be recovered from these specimens throughout the course of the illness and for some time after symptomatic recovery. Mycoplasmas are very susceptible to heat and drying out, so culture media should be inoculated as soon after collection as possible or transport media should be used to preserve their viability if there are anticipated delays in processing. Culture media dispensed into small vials or purchased transport tubes may be used for transport of swab specimens; other specimens (e.g., sputum, tissue, washings) may be transported quickly to the laboratory in sterile screw-capped containers. Specimens should always be homogenized; however, Sputolysin or other chemical treatments for sputum liquefaction can be toxic to mycoplasmas and should be avoided.

Genital mycoplasmas may be isolated from a variety of specimens. These include urethral, vaginal, and cervical swab specimens; prostatic secretions; semen; urine; blood; miscellaneous body fluids (CSF, amniotic fluid, respiratory tract secretions, synovial fluid, pericardial fluid); and tissues (e.g., endometrial washings and biopsies, placental or amniotic tissues, fetal or abortus tissues, fallopian tube biopsies, uterine biopsies, wound biopsies, rectal tissues). Swab specimens should be obtained using Dacron, calcium alginate, or polyester swabs, with either plastic or aluminum shafts. Swabs with wooden shafts should not be used because the wood may be toxic. Contact of the swab surfaces with antiseptic solutions, creams, jellies, or lubricants should be avoided. Swab specimens should not be allowed to dry and should be

placed immediately into a transport or culture medium after collection. Other body fluids and tissue biopsy specimens should be submitted in sterile containers. Saline should not be used to moisten tissue specimens, for it may cause lysis of the organisms. In the laboratory, tissue specimens should be minced in sterile transport medium to produce a 10% (w/v) suspension and serially diluted 10- and 100-fold. This is necessary to prevent inhibition of mycoplasmal growth by organic materials such as hemoglobin, toxic phospholipids, antibodies, or complement that may be present in the tissue specimen. These dilutions are then used to inoculate growth media.

Blood specimens can be cultured for mycoplasmas by inoculation of growth media at a ratio of one part blood to nine parts broth (1:10 dilution). Optimally, at least 10 mL of blood from adult patients should be cultured. Ideally, the growth medium should not contain sodium polyanethol sulfonate (SPS), which inhibits the growth of *Mycoplasma* species, but should be a medium specifically formulated for mycoplasmas (see below). Inoculated broth media should be subcultured blindly, since the automated, continuously monitored blood culture instruments may not be able to detect the growth of mycoplasmas.[524,525]

Transport Media

Mycoplasmas are very susceptible to adverse environmental conditions, so specimens should be placed into appropriate transport or growth media as soon after collection as possible. A variety of transport media may be used for genital mycoplasma cultures. These include trypticase soy broth with 0.5% bovine serum albumin, 2SP broth (10% heat-inactivated fetal calf serum with 0.2 M sucrose in 0.02 M phosphate buffer, pH 7.2), or various types of mycoplasmal growth media (e.g., SP-4, Shepard's 10B broth). Antibiotics and antifungal agents (penicillin, 100,000 units/mL; polymyxin B, 5,000 mg/mL; amphotericin B, 2 mg/mL, final concentrations) are generally added to decrease contamination by other bacteria and fungi. Specimens should be transported to the laboratory immediately and may be held up to 24 hours at 4°C before inoculation of growth media. If culture is delayed beyond this time, the specimen should be frozen at –70°C. Prior to culture, these specimens should be thawed rapidly in a 37°C water bath. Urine specimens for culture should be centrifuged and the sediment diluted 1:1 with transport medium before freezing. The protein stabilizers in the transport medium prevent loss of organism viability that may occur if urine specimens are frozen without this protective measure.

Media for Culture of Mycoplasmas

Several types of broth and agar media have been described in the literature for the cultivation of *M. pneumoniae* and the genital mycoplasmas.[524] All have a source of

serum, as well as glucose, arginine, and/or urea, and a pH indicator is also included for media designed to detect genital mycoplasmas. SP-4 broth and Hayflick's broth or agar are excellent for growth of *M. pneumoniae*, MH, and other slow-growing strains of *Mycoplasma* spp.[508,524] Shephard's 10B broth and A8 agar are good for MH and *Ureaplasma* spp.[524] For *M. pneumoniae,* a biphasic medium is often recommended rather than broth or agar alone. One such medium recommended by the CDC for isolation of *M. pneumoniae* is methylene blue-glucose diphasic medium. This medium contains PPLO (pleuropneumonia-like organism) broth and agar, yeast extract, and serum supplements, as well as glucose, methylene blue, and phenol red. The methylene blue in the medium inhibits the growth of other human mycoplasmas that may be found in the respiratory tract, making the medium selective for *M. pneumoniae*. During growth of *M. pneumoniae*, the medium becomes more acidic and the phenol red turns color from salmon to yellow. At the same time, the organisms reduce the methylene blue and turn it from blue to colorless. Therefore, the color of the broth phase changes from purple to green or yellow-green, while the agar phase turns from a purple color to a yellow or yellow-orange. This medium can be used in conjunction with mycoplasmal glucose agar medium. Colonies recovered either directly on this medium or from subcultures of positive broths are then subjected to

inspection and identification procedures. The components and formulas for *M. pneumoniae* isolation medium are presented in Chart 18-4.

In a comparative study conducted by Tully and coworkers, diphasic SP-4 medium recovered *M. pneumoniae* from 69 of 200 specimens that were negative when cultured by other "standard" methods.[508] As with the methylene blue-glucose diphasic medium, growth is detected by the change of the phenol red indicator from red to yellow, indicating the production of acid from glucose. SP-4 agar and broth media can be used for isolation of MH, if arginine is added instead of glucose.

Because MH and UU metabolize different substrates and differ in the pH for optimal growth, many laboratories use two types of agar medium for the cultivation of mycoplasmas. In addition, diphasic broth and agar formulations of each medium type can be inoculated for optimal recovery of mycoplasmas. Media used to isolate MH should be buffered at a neutral pH (7.0). Some formulations may also contain arginine as the growth substrate, with both medium turbidity and a shift in the color of the phenol red indicator into the alkaline range being used to detect organism growth. Media used to isolate UU are buffered at a lower pH (5.5 to 6.0) and contain urea as the growth substrate. The latter media are also made more selective for UU by the inclusion of lincomycin because ureaplasmas are resistant to this drug and MH strains are susceptible. Medium formulations for isolation of genital mycoplasmas are found

in Chart 18-5.

Several other media have been described in the literature for the isolation and identification of genital mycoplasmas. Broth media include the U-9 urease medium described by Shepard and Lunceford, bromthymol blue broth, S-2 Boston broth, and the SP-4 medium used for recovery of the respiratory tract mycoplasmas that is supplemented with arginine and urea.[434,436,437,443,524,555] S-2 Boston broth is a single medium that contains an enriched base medium, horse serum, yeast extract, phenol red indicator, L-cysteine, urea, and penicillin. Growth of UU is detected by the appearance of a pink color in the medium, whereas growth of MH is detected by a pale salmon-pink to orange color or by the appearance of slight turbidity. Shepard's A7 differential agar medium and various modifications (e.g., A7B and A8) are particularly useful because both MH and UU grow well on these formulations and can be easily differentiated from one another by colony morphology and by the direct detection of urease formation by the latter species (see discussion below).[434,436,437] As described below, presumptive identification of MH and definitive identification of UU can usually be attained with these media, without the need for additional tests or reagents.

Isolation and Identification of Mycoplasma pneumoniae

In general, the growth of *M. pneumoniae* from clinical specimens is detected by the ability of these organisms to produce acid from glucose.[91] Methylene blue-glucose diphasic medium, inoculated with 0.2 mL of specimen, is incubated at 35°C. Tubes are inspected daily for color changes and turbidity for up to 4 weeks. The development of gross turbidity and an acid or alkaline shift of the indicator in <5 days is generally due to bacterial contamination. As soon as color changes in the medium are apparent, the broth must be subcultured to appropriate agar medium (e.g., SP-4 agar) and incubated in air for 5 to 7 days. Inspection of the agar surface under the low power of the microscope will reveal small colonies of the organisms. In the absence of obvious color change in diphasic media, a blind subculture to agar media should be performed after 1 and 3 weeks of incubation. A general scheme for the isolation of *M. pneumoniae* is shown in Figure 18-1.

Glycolytic, spherical colonies that grow on SP-4 agar, for example, from a respiratory tract specimen between 4 and 20 days can be presumptively considered to be *M. pneumoniae*. *M. genitalium* may resemble the colonies of *M. pneumoniae,* but would require more than 20 days for growth, and *Acholeplasma* spp. may mimic the glycolytic activity of *M. pneumoniae* on SP-4 agar, but will grow in less than 4 days and the colonies will have a "fried-egg" appearance, which is not typical of *M. pneumoniae*. To confirm colonies suspected to be *M. pneumoniae,* PCR sequencing or epifluorescence techniques can be performed, if these are available.[524] Colonies may also be identified as *M. pneumoniae* specifically using a hemadsorption assay

(Chart 18-2), tetrazolium reduction test (Chart 18-6), or by using specific antibodies to inhibit growth of the organism.[91,524] The hemadsorption assay is accomplished by flooding the SP-4 with 5% washed guinea pig erythrocytes, incubating 30 minutes, and washing with sterile saline 2 to 3 times; colonies on the agar surface are then examined microscopically for the adherence of the erythrocytes. If positive, *M. pneumoniae* is confirmed; *M. genitalium*, although also positive, is much more fastidious and would not be present in the 4 to 20 days in which *M. pneumoniae* colonies will appear.[524] The tetrazolium reduction test exploits the unique ability of *M. pneumoniae* to reduce the colorless compound triphenyl tetrazolium to the red compound formazan. To perform, the agar surface is flooded with triphenyl tetrazolium, incubated at 35°C for 1 hour during which time the colonies, if *M. pneumoniae*, will appear reddish; with longer incubation, colonies may turn purple to black. The tetrazolium test can be performed even after the colonies are tested with the hemadsorption procedure.[524] Methods for performing these tests can be found in references 91 and 524; use of these tests is usually limited to reference laboratories.

Although considered to be the reference procedure, the growth inhibition test for identification of *M. pneumoniae* requires specific antibodies, is the most time-consuming procedure, and hence is not often utilized as a confirmatory test. A broth culture of the organism presumed to be *M. pneumoniae* is diluted 1:50 and 1:500 and spread on the agar surfaces to which are then added strips of filter paper that have been impregnated with anti-*M. pneumoniae* antibodies. After incubation, inhibition of growth around the paper strips would indicate the presence of *M. pneumoniae*. Normal animal serum should be used as negative control.[524] All of these confirmatory tests can be useful, but since culture for *M. pneumoniae* is considered less sensitive than molecular techniques for detection, it is doubtful whether they would ever be employed widely in routine clinical laboratories.[433]

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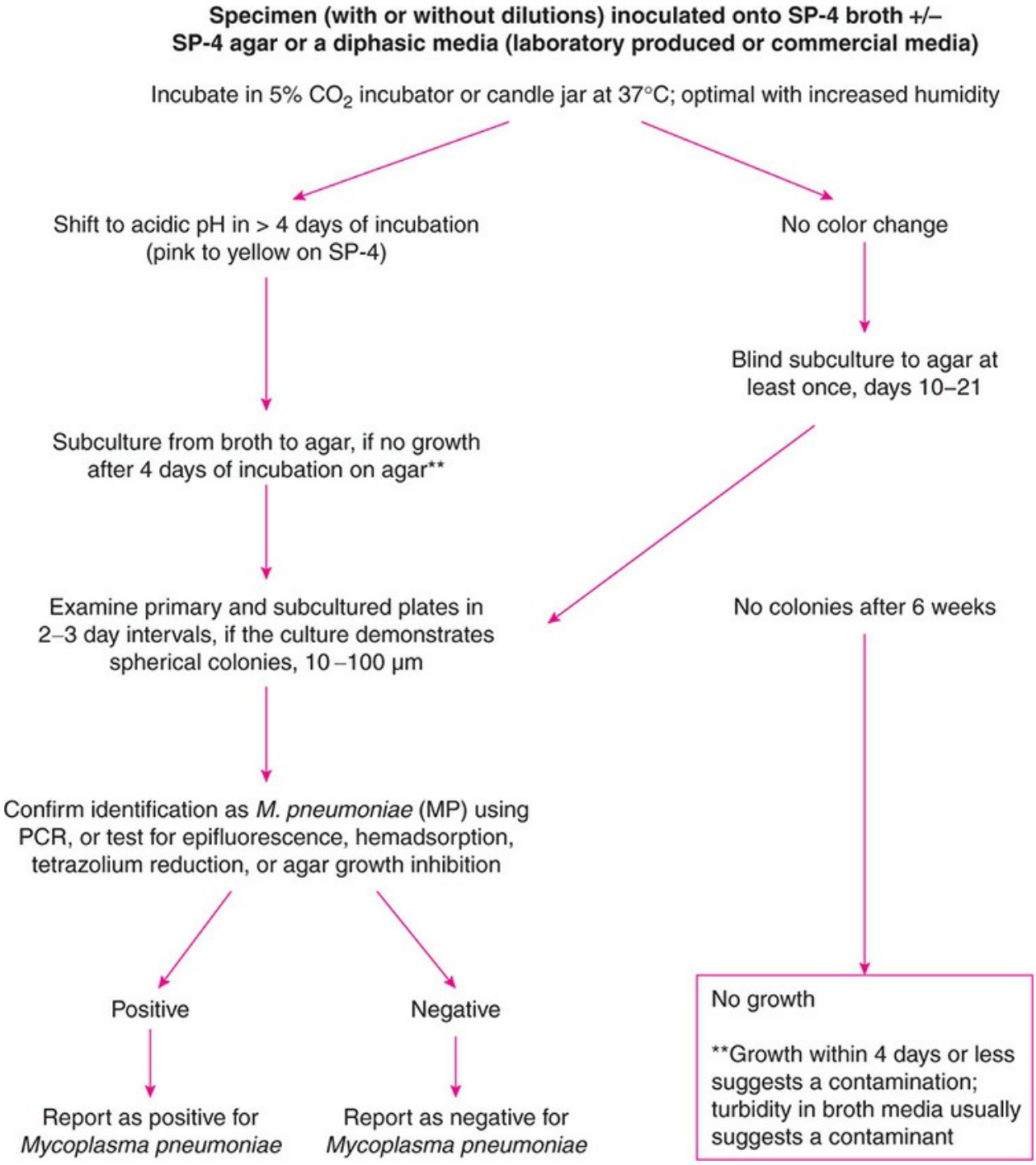

■ **FIGURE 18-1** Protocol for isolation of *Mycoplasma pneumoniae* from respiratory tract specimens.

Noncultural Detection of Mycoplasma *pneumoniae*

Because of the slow growth of *M. pneumoniae*, non–growth-dependent, direct detection methods continue to be developed. These have included antigen capture

assays, direct immunofluorescence, immunoblot analysis, and nucleic acid probe methods.[245,300,315,524] In general, most of these tests lack sufficient sensitivity to be clinically useful and have been largely replaced by nucleic acid amplification-based molecular techniques. They will not be further discussed in this chapter.

Prior to the development of amplification-based methods, a direct, radiolabeled DNA probe was developed for the diagnosis of mycoplasmal respiratory tract infections. The *M. pneumoniae* DNA probe (Gen-Probe, San Diego, CA) hybridized with the 16S rRNA of the organism and used a ^{125}I radioactive label to generate a detection signal. Tilton and coworkers compared the DNA probe with culture and found that the probe had a sensitivity of 100% and a specificity of 98% when compared with culture.[498] Dular and associates reported that the probe had both a sensitivity and a specificity of 89% compared with culture.[115] In both reports, the authors stressed the practical aspects of the probe as a rapid, sensitive, and timely approach to diagnosis, because probe results are available in about 2 hours, whereas culture requires several weeks. Despite these advantages, the Gen-Probe test was withdrawn from the market in 1992.

Several molecular methods, including nested and seminested PCR, multiplex PCR assays, nucleic acid sequence–based amplification (NASBA), Qb-replicase, and real-time PCR, have been developed since 1989 for the detection of *M. pneumoniae* in a variety of specimen types.[300,516] Specimen types include sputum, throat swabs, nasopharyngeal and tracheal aspirates, BALs, transthoracic needle aspirates, fixed lung tissues, and open lung biopsies. Major gene targets for these assays have included the P1 adhesin gene, species-specific 16S rRNA, the mycoplasmal ATPase operon genes, and the *tuf* gene, which codes for elongation factor 2, a factor

that functions in protein synthesis at the ribosome-mRNA level.[50,184,247,299–301,303,499,542,548,549] Early on, several investigators designed species-specific probes for *M. pneumoniae* based on *M. pneumoniae*-specific 16S rRNA sequences and found that amplification and subsequent probe detection of these sequences in clinical specimens was more sensitive than culture.[300,499,519] By using PCR technology, Narita and coworkers detected *M. pneumoniae*-specific DNA in 4 of 6 CSF specimens and in 3 of 4 serum specimens from patients with clinically and serologically confirmed *M. pneumoniae* CNS infection.[356a] A CDC study of community outbreaks of *M. pneumoniae* respiratory infections analyzed results of PCR and serologic assays and concluded that neither approach alone is sufficient, but they should both be used to enhance recovery of *M. pneumoniae* especially in absence of other pathogens.[496]

In addition to single assays for the detection of *M. pneumoniae*, multiplex assays

that can detect *M. pneumoniae, Chlamydophila pneumoniae*, and, in some, *Legionella pneumophila* are being developed to provide a comprehensive and sensitive approach to the diagnosis of the most common causes of atypical bacterial pneumonia.[77,297,548] Nilsson et al. compared a PCR for detection of *M. pneumoniae* in the oropharynx with serologic assays and found the PCR to be more sensitive, especially in the early stages of infection. DNA did remain in the specimens for quite a while after being detected as would be expected. In the same study, asymptomatic carriage of *M. pneumoniae* as detected by PCR was low even when testing was done during an outbreak.[362]Use of two PCR assays in the BAL of 50 hospitalized patients in a study in Italy was concordant for detection of *C. pneumoniae* 100% of the time and 98% for *M. pneumoniae*. The positive results agreed with clinical suspicion of community-acquired infection in many cases, and in acute infection, the molecular tests were in total agreement with the finding of IgM-specific antibodies for either or both pathogens.[389] Table 18-2 provides information on PCR assays that have been developed and corresponding evaluations. Review articles comparing methods for diagnosis of *M. pneumoniae* are also available.[298,299,522,524,535] In most instances, culture is less sensitive than PCR, but serologic methods (discussed below) still play a role in conjunction with molecular methods for optimal detection of *M. pneumoniae* infections.[108]

Isolation and Identification of the Genital Mycoplasmas

Mycoplasmas that may be recovered from the genital tract include MH, *M. genitalium*, *M. fermentans*, and UU. MH and UU are easily cultivated and usually grow within 1 to 5 days, whereas *M. genitalium* and *M. fermentans* grow much more slowly and are more difficult to detect in culture. Furthermore, culture systems that are in current use for the recovery of genital mycoplasmas may not support optimal growth of these species. Because of these considerations, genital mycoplasmas other than MH and UU are generally not cultured routinely in genital tract specimens.

M. genitalium is exceedingly difficult to recover using culture-based methods. Jensen and colleagues reported the successful isolation of *M. genitalium* from genital tract specimens.[216] From 11 specimens, 9 were successfully propagated in Vero cell cultures from PCR-positive urethral specimens from patients with urethritis. Growth in Vero cell culture was monitored by PCR. Of these nine isolates, six were adapted by serial passage to growth in Friis medium, a complex growth medium containing Hanks' balanced salts solution, brain–heart infusion broth, "homemade" yeast extract, and up to 20% (v/v) horse serum. These six isolates required from 1 to 19 serial passages in Vero cell cultures before they could be adapted to growth in broth, and from 2 to 6 passages in broth before visible colonies were produced on an agar medium similar in formulation to the broth. In this report, the authors note that, although the

development of a medium able to support the growth of *M. genitalium* represented a major breakthrough, PCR methods were essential for initial detection and for monitoring the cultures for growth. Molecular techniques that are highly sensitive and specific have been developed for detection of *M. genitalium* in genital tract specimens and first-pass urine specimens and should become more widely available in the future.[100,118–120,214,215,458,538,566–568]

For culture of MH and UU, appropriate specimens received in transport media are inoculated onto both broth and agar media. For the diagnosis of mycoplasmal infections of the lower urinary tract infections, urethral swabs may be optimal over urine or vaginal swabs.[197] In a study in women with symptoms of cervicitis, vaginal swabs were preferred over endocervical swabs for isolation of *M. genitalium*.[343] The correct specimen type is essential for optimal recovery. In general, about 0.2 mL of the specimen should be streaked onto agar media and placed in broth media. Broths may be incubated aerobically at 35°C, but plates should be incubated in a CO_2 incubator or a candle jar. UU and MH may also be recovered under anaerobic conditions. Plated media should be inspected daily under 40× magnification using oblique light to observe the "fried-egg" colonies of MH or the small, dense colonies of UU. In M broth, MH produces a slight turbidity in addition to a change in color. In U broth, UU will tend to produce a slight color change early in incubation with no distinct or obvious turbidity. In both cases, subcultures must be performed at these times to ensure recovery of viable bacteria because there is a rapid decrease in viability of the organisms once the pH has been elevated by substrate utilization and depletion. As with the primary culture plates, subcultures plates should be inspected daily. Most isolates of MH and UU will grow within 5 to 7 days. If growth is not detected on primary plates or broth subcultures after this time, the culture can be reported as negative for genital mycoplasmas. It is advisable to freeze aliquots of positive broths at –70°C in the event that problems are encountered with viability on direct agar cultures or subcultures. The general scheme for isolation of genital mycoplasmas is presented in Figure 18-2.

Identification of genital mycoplasmas is generally quite easy and straightforward. MH colonies grow in 1 to 5 days, have the typical fried-egg colonial morphology, and are usually 50 to 300 fm in diameter. Colonies may be stained with the Dienes' stain (Chart 18-1) to aid visualization (Image Plate 18-1B). Further identification procedures are not necessary because rapidly growing, arginine-positive organisms exhibiting the typical colony morphology are invariably MH. The small colonies of UU may be difficult to distinguish from various artifacts, such as mammalian cells and cellular debris or materials present in serum. Because of these problems, suspect UU colonies must be confirmed. This is done by exploiting the ability of UU to hydrolyze urea. If the U agar medium, which contains urea and phenol red, is used, then the suspect *Ureaplasma* colonies will be surrounded by a red halo.

TABLE 18-2 Molecular Methods for Detection of *M. pneumoniae*

Name of PCR or Type	Targeted Organisms	Method/Specimens	Results	Reference
Pro-Pneumo-1	*M. pneumoniae* *C. pneumoniae*	Prodesse real time-PCR	146 samples; 100% sensitive and 98% specific for *M. pneumoniae* vs. culture	180
Scorpion-based real time-PCR	*M. pneumoniae*	Real time-PCR and unimolecular probe	388 specimens; compared better than conventional PCR when both were compared with serology	104
Real time-PCR	*M. pneumoniae* *C. pneumoniae*	Molecular Beacons; throat and nasopharyngeal (NP) samples	100% sensitive and 98% specific for *M. pneumoniae* in 120 throat and nasopharyngeal specimens compared with conventional PCR	157
Comparison of simplex NASBA, multiplex NASBA, and conventional PCR	M. *pneumoniae* *C. pneumoniae* *L. pneumophila*	NASBA	251 respiratory specimens; for *M. pneumoniae*, the sensitivity was 78% for conventional PCR vs. 100% for simplex or multiplex NASBA	297
NucliSens EasyMag/EasyQ (NASBA, bioMérieux) vs. MagNA Pure/real time-PCR (Roche)	*M. pneumoniae* *C. pneumoniae*	NASBA vs. Roche real time-PCR	The sensitivity and specificity for *M. pneumonia* were equivalent, whereas the NASBA system demonstrated a higher sensitivity for *C. pneumonia*.	33
Microfluidic real time-PCR (Advanced Liquid Logic, NC)	*M. pneumoniae*	Hydrolysis (i.e., TaqMan) probes and primers; nasopharyngeal swabs	The microfluidic real time-PCR method was equivalent to conventional real time-PCR; considered less expensive and more convenient	553
PneumoBacter ACE Detection Assay (Seeplex Pneumo-Bacter; Seegene) vs. ProbeTec ET Atypical Pneumonia Assay (APA; Becton Dickinson)	*M. pneumoniae* *C. pneumoniae* *L. pneumophila*	Sputum and NP swabs compared	100% Agreement for CP and LP in PneumoBacter and APA Both assays demonstrated a 95% sensitivity and 100% specificity in sputum specimens, but only a 38% sensitivity and 94% specificity for nasopharyngeal specimens.	69
Colorimetric loop-mediated isothermal amplification assay (LAMP, China)	*M. pneumoniae*	Isolates and clinical samples	100% sensitivity and specificity in analytical studies; the LAMP assay performed well compared with the conventional PCR for the detection of *M. pneumonia* in clinical specimens.	570

Suspect UU colonies may also be confirmed with the manganous chloride–urea test (Chart 18-3). In this test, an agar plate containing colonial growth is flooded with an aqueous solution of urea (1%) and 0.8% (w/v) manganous chloride. Hydrolysis of urea by urease liberates hydroxyl groups from water, and these hydroxyl moieties oxidize manganous chloride to insoluble manganese oxide, which, in turn, causes the deposition of a golden brown precipitate in the colonies within a few minutes.

Shepard's A7 differential agar media and modifications thereof, A7B and A8 agars, have the urea and the urea-hydrolysis-detection reagent incorporated in the medium, so flooding the plate with manganous chloride is not required.[434,436,437] A7 agar contains manganous sulfate as the precipitating agent along with penicillin (1,000 U/mL) and amphotericin B (2.5 mg/mL). A7B differential agar is identical to A7 agar, except that the polyamine putrescine dihydrochloride (10 mM) is added to enhance the

Ureaplasma growth and development of the precipitate in the colonies. A8 differential agar incorporates calcium chloride (1 mM) as the divalent cation indicator for the detection of ammonia formation from urea, as well as colistin (7.5 mg/mL), ampicillin (1 mg/mL), and amphotericin B (2.5 mg/mL). On all three medium formulations (i.e., A7, A7B, and A8 differential agars), MH appears with the characteristic fried-egg morphology after 1 to 3 days. UU colonies appear within 1 to 3 days and are 15 to 50 mm in diameter (depending on crowding). UU colonies on A7 and A7B agars are dark brown due to the accumulation of manganese oxide in the colony, whereas colonies on A8 differential agar are gold to light brown (Image Plate 18-1B). Several comparative evaluations of A7, A7B, and A8 media indicate that they are probably the agar media of choice for culture of genital mycoplasmas and, when used with an appropriate broth medium (e.g., bromthymol blue broth, Boston broth), give the highest yield of positive cultures.[388,555] Because the growth characteristics of UU on these media are unique, further testing is not necessary. Both A7 and A8 differential agars are commercially available from Remel Laboratories (Thermoscientific).

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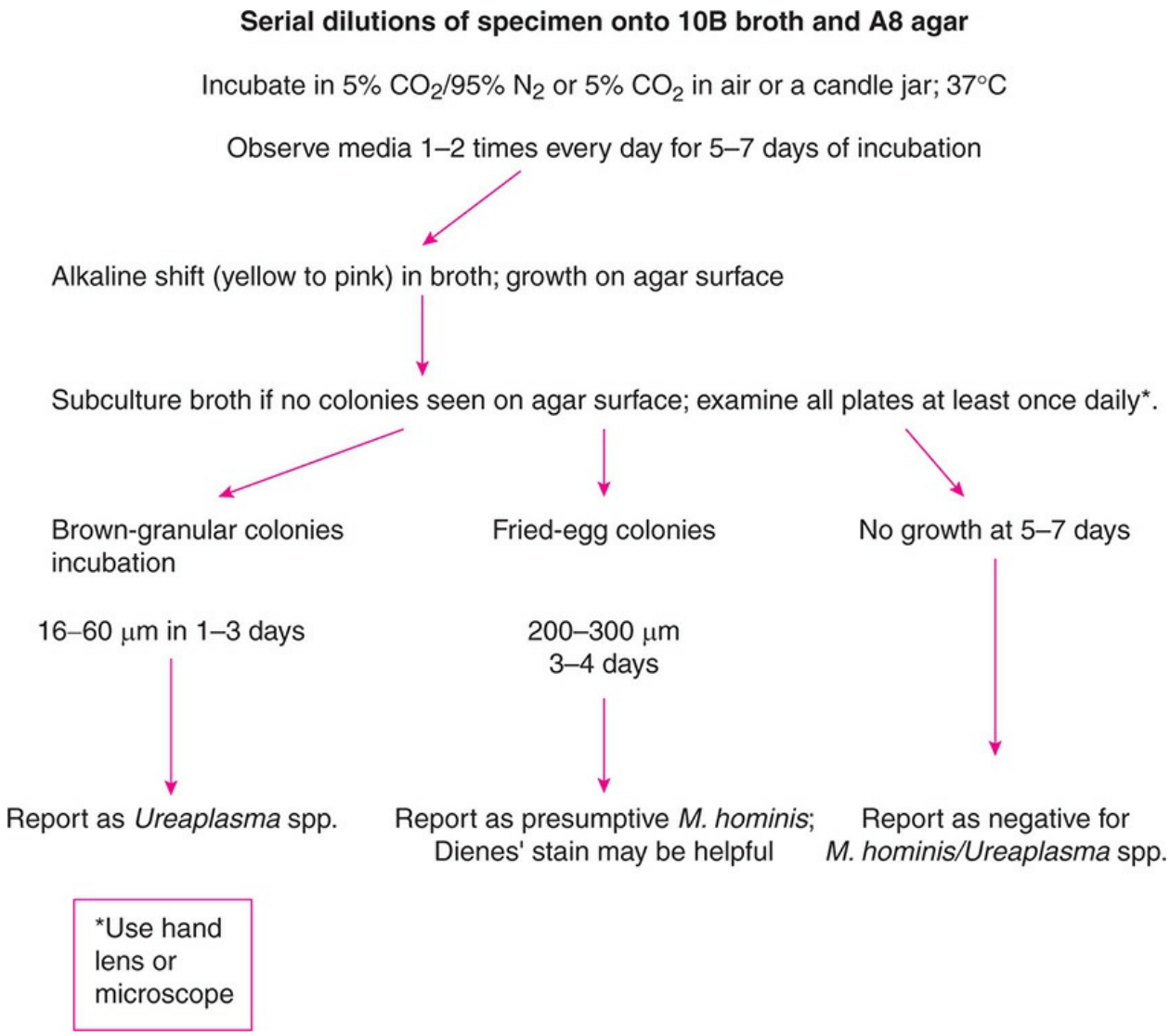

■ **FIGURE 18-2** Protocol for isolation of *Mycoplasma hominis* and *Ureaplasma urealyticum* from genital specimens.

Definitive identification of MH may be made by growth inhibition using specific antisera. An indirect immunoperoxidase method has also been published for the identification of mycoplasmas.[201] This method produces results comparable with those of the antiserum growth inhibition procedure. Similar procedures, using both immunofluorescence and immunoperoxidase techniques, have been developed for use in mycoplasmal studies in animals, where different *Mycoplasma* species that have similar tissue tropisms may be present in mixed culture.[30]

Noncultural Detection of the Genital Mycoplasmas

Nonculture methods have also been examined for their ability to detect genital

mycoplasmas. Hirai and colleagues developed an indirect fluorescence antibody (IFA) test for direct identification of MH in vaginal specimens.[183] The staining of Vero cells that were infected with other *Mycoplasma* species, including *M. orale*, *M. salivarium*, and *M. fermentans*, demonstrated that the IFA procedure was specific for MH. The IFA test was compared with culture on vaginal specimens collected from 193 healthy women. Among 22 culture-positive specimens, 17 were positive with the IFA test. Interestingly, 48 of 171 specimens that were negative by culture were positive with the IFA test, suggesting that the IFA method was more sensitive than culture. These workers also demonstrated that the location of granular aggregates that were observed on vaginal epithelial cells stained with the Papanicolaou stain frequently corresponded to the areas of the smears that stained with the MH-specific IFA reagents.[183]

Probe technology was initially exploited for detection of *M. genitalium* in urogenital specimens because this organism is very difficult to recover in culture. There are recognized antigenic cross-reactivity between *M. genitalium* and *M. pneumoniae* and similarities in certain nucleotide sequences between these organisms. Therefore, nucleic acid probes for *M. genitalium* had to be constructed to exclude nucleotide sequences that are shared with *M. pneumoniae* in order to prevent false-positive results.[564]Such carefully constructed DNA probes were used along with gene amplification techniques and PCR to detect *M. pneumoniae*- and *M. genitalium*-specific nucleotide sequences directly in clinical specimens.[219] In one study, 10 of 150 genital specimens recovered from eight patients (three men and five women) were positive for *M. genitalium*.[219] These results were verified by Southern blot hybridization tests because cultures were not performed.

Molecular assays have also been developed for detection and specific identification of MH and UU in genital tract specimens, amniotic fluids, and respiratory tract specimens from newborns.[302,458,568] PCR assays for MH have used MH-specific 16S rRNA sequences as the target for amplification

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and detection, whereas similar assays for UU have used organism-specific 16S rRNA sequences, the multiple banded (MB) antigen gene sequences or urease gene sequences.[39,207,491] Some of these tests are able to detect as few as one colony-forming unit (CFU) in specimens from the adult urogenital tract and amniotic fluids, and endotracheal aspirates from newborn infants. Teng and coworkers examined 50 clinical specimens (8 urethral swab specimens, 8 urine specimens, 12 endocervical swab specimens, 8 prostatic fluid specimens, and 14 semen specimens) by both culture and PCR.[490] Cultures were positive for five specimens, and an additional four specimens produced appropriate pH changes in broth but did not grow on subculture

to agar medium ("doubtful" positives). PCR was positive for all five culture-positive specimens, all four doubtful-positive specimens, plus three specimens (one prostatic fluid and two endocervical specimens) that were culture-negative. A broad-range PCR was used to detect MH in a patient with culture-negative, late-onset prosthetic valve endocarditis; targeted antimicrobials against MH were successful in curing the patient.[210] A PCR-based reverse line blot assay that can simultaneously detect 14 uropathogens from urine specimens has been reported to be sensitive, specific, user-friendly, and inexpensive. It includes targets for MH, UU, *U parvum*, and *M. genitalium*.[330] A genus-specific PCR for MH, *M. genitalium,* and UU was found more sensitive than culture in 210 patients with suspected genital mycoplasmal infections. Forty percent of patients were positive for one or more genital mycoplasmas in culture versus 57% via PCR. In addition, 11% of samples were found to be coinfected with *Mycoplasma* spp. and UU by PCR.[12] Scientists in France compared a quantitative real-time PCR targeting the *yidC* gene, encoding a membrane protein translocase, with quantitative culture of MH in 153 urogenital specimens. The clinical sensitivity of the PCR was higher than culture (45 positive by culture vs. 55 by PCR), the analytical specificity was 100%, and PCR limit of detection was 7 copies/ μL. The quantitation in those 45 samples that were positive in both culture and PCR correlated well.[129] A Bio-Rad PCR assay demonstrated excellent sensitivity and specificity for simultaneous detection of *C. trachomatis, N. gonorrhoeae,* and *M. genitalium* in urogenital specimens of 658 symptomatic and asymptomatic men and women as compared to a hydrolysis probe real-time PCR assay for detection of *C. trachomatis*, culture of *N. gonorrhoeae,* and a laboratory-developed PCR assay for the detection of *M. genitalium*.[270] A real-time PCR assay for detection of *M. genitalium* has also been described, which in addition to identification of macrolide resistance markers.[213] PCR methods have been used to detect a unique DNA sequence in *M. fermentans* that appears repetitively in the genome of the organism, whereas those for detection of MH, *M. penetrans*, and *M. pirum* detect unique nucleotide sequences within the 16S rRNA of these species.[147,148,539] Although serologic and genetic methods suggest that *M. fermentans* represents a homogeneous organism group, AIDS-associated strains, cell culture isolates, and other clinical isolates of *M. fermentans* can be differentiated from one another by pyrolysis mass spectrometry.[165] All isolates of *M. fermentans* have been detected by amplification of a conserved 206 base pair region of the IS1550 insertion sequence. Amplification of the macrophage-activating lipopeptide gene (*malp*) can also identify all strains of *M. fermentans* and provides another option for identification.[2]

Commercial Mycoplasma *Culture Systems*

The Mycotrim RS and Mycotrim Triphasic flask systems (Irvine Scientific, Irvine, CA) are commercially available systems that have been used extensively for the cultivation of *M. pneumoniae* and the genital mycoplasmas, respectively. The Mycotrim RS system consists of a bottle containing a layer of enriched glucose agar medium containing phenol red indicator on one side and a broth of similar composition. Thirty minutes before inoculation with the specimen, disks are saturated with antibiotics and thallous acetate, then these are added to the flasks to allow time for the antimicrobials to elute from the disk and diffuse into the agar. A pipette is used to add liquid specimens and to "streak" the agar surface. Swab specimens are rolled over the agar surface. The flask is incubated agar side up for 2 weeks at 35°C to 37°C. Reinoculation of the agar is performed after 3 days by manipulating the bottle so that the broth washes over part of the agar phase. Growth is first indicated by a change in the indicator from red to yellow-orange without noticeable turbidity. Definitive identification is made by placing the flask on the stage of a light microscope agar side up and inspecting the surface of the agar for mycoplasmal colonies. This may be done with or without the addition of a vital stain such as Dienes' stain.

Mycotrim GU broth contains arginine, urea, and phenol red indicator. This broth is inoculated with 0.1 mL of the specimen and is incubated aerobically at 35°C. The medium is observed for changes in the color of the indicator to a more alkaline pH; subculture to solid media, such as A7 or A8 differential agar; and subsequent incubation allows the recovery and identification of both MH and UU.

The Mycotrim Triphasic flask (formerly called the Mycotrim GU system) system is a diphasic system that contains modified A8 differential agar with calcium chloride in a tissue culture flask and a broth medium containing arginine, urea, and phenol red indicator. Before inoculation, antibiotic impregnated disks are added and antibiotics are allowed to elute from the disks and diffuse into the agar phase. The flask is inoculated with 0.10 mL of the specimen, and the broth–specimen mixture is washed over the agar phase. The flask is incubated at 35°C aerobically. Subculture of the agar is achieved by allowing the broth to wash over part of the agar phase after 24 hours of incubation. With the appearance of a color change in the phenol red indicator from yellow to orange or orange-red, the agar phase is examined under the microscope for the typical fried-egg colonies of MH or the small, brown calcium oxide–stained colonies of UU. Cultures are inspected daily and incubated for 7 days.

Early studies found good correlation of the Mycotrim GU system compared to conventional culture methods using arginine broth, urea broth, glucose agar, and A7B differential agar.[46,388,551] In laboratories doing a modest amount of mycoplasmal work, it appears that the Mycotrim GU broth combined with a differential agar medium and the Mycotrim Triphasic flask system are fairly comparable, although

the components for the "conventional" procedure may be less expensive. Another commercially available system for the recovery, identification, and susceptibility testing of MH and UU is MYCOFAST US (Wescor, Utah) and MYCOFAST EvolutioN 3 (Elitech Microbiology, Signes, France). This commercial kit has a special transport medium for specimens and plastic tray for inoculation of the specimens into media containing urea and arginine, which are metabolized if the genital mycoplasmas are present. There are enumeration wells for each organism and wells which contain antibiotics that are used for differentiation of the two organisms. Surveillance studies have used this media successfully in the laboratory for sensitive detection of MH and UU.[414]

Isolation of Mycoplasmas on Routine Culture Media

In general, *M. pneumoniae* and UU are more fastidious in their growth requirements than MH. This latter species is the one that is most frequently recovered from sites other than the genital tract, such as blood, wounds, and joint fluids. Therefore, MH may occasionally be isolated on routine bacteriologic culture media. The clinical microbiologist must be able to recognize these organisms under these less-than-optimal conditions. MH is able to grow on enriched media such as Columbia CNA agar and chocolate agar; the organism grows less well on tryptic soy–based blood agar.[377] The organisms will grow best under anaerobic conditions, with less rapid growth in an aerobic, CO_2-enriched environment. Mycoplasmal growth may not be apparent until after prolonged incubation (i.e., 72 to 96 hours), depending on the conditions of incubation and the numbers of organisms present. Colonies of MH are extremely small and may be detected only as a "film" or "speckling" of the agar surface when examined by strong, incident, reflected light. Examination of this type of growth is facilitated by using a dissecting microscope. The colonies are difficult to pick up with a loop and will not stain with the Gram's stain.

Suspected mycoplasmal colonies from routine agar medium can be subcultured by cutting out a block of the blood agar and rubbing the side with the growth across the surface of a mycoplasmal growth medium such as A7 differential agar. Another block should be aseptically removed and immersed in a broth medium such as H broth or Boston broth. Both broth and agar subcultures are then incubated and processed as described in the foregoing. Inspection of colony morphology, along with the growth rate and assessment of optimal incubation conditions for growth, will allow presumptive identification of the species.

MH may also be occasionally recovered from blood cultures, particularly in women with postpartum fever. If MH bacteremia is suspected, blood should optimally be inoculated at the bedside of the patient into an SPS-free medium. Incubation of the cultures should be prolonged, and blind subcultures to appropriate agar and broth mycoplasmal isolation medium, in addition to CNA agar, should be made at frequent

intervals. Blood for recovery of mycoplasmas should be inoculated directly into the mycoplasmal culture medium. The blood should be diluted 1:10 with growth medium; therefore, culture of a substantial blood volume may require multiple vials of culture media.

Serologic Tests for Diagnosis of *Mycoplasma pneumoniae* Infections

Because of the limited availability of culture, the technical difficulties inherent in the culture techniques, and the amount of time required for culture results to become available, the diagnosis of *M. pneumoniae* infection is often made by a combination of clinical, molecular, and serologic findings. Diagnosis on clinical grounds alone is difficult because many other pneumonic processes, particularly those caused by viruses, can present similarly. In addition, persistence of *M. pneumoniae* in the respiratory tract for variable periods after resolution of the disease process complicates the interpretation of positive growth-based and molecular detection methods. Because *M. pneumoniae* has lipid and protein antigens that promote the development of antibodies usually within 1 week of infection, serologic assays have been used for diagnosis.[522] In most instances, acute and convalescent titers are most beneficial, with single assay results not always providing accurate correlation to present infection. The most widely used serologic test for diagnosis of *M. pneumoniae* infection had been the CF test, but due to cross-reactivities with other organisms, including *M. genitalium*, and the inability to differentiate among antibody classes, it has been largely replaced by alternative methods.

Serologic test methods using indirect immunofluorescence, particle agglutination, and EIA approaches are currently available commercially. Table 18-3 lists a number of commercially available assays using these formats and, where available, references about their methodology and/or evaluation. The *M. pneumoniae* Antibody Test System (Zeus Scientific, Branchburg, NJ) uses indirect immunofluorescence for the separate detection of *M. pneumoniae*-specific IgG and IgM. In one evaluation of this assay, IFA for *M. pneumoniae*-specific IgM was compared with PCR, culture, and CF for diagnosis of mycoplasmal infection.[108] Based on the positivity of culture and CF as the criteria for a positive diagnosis of infection, the sensitivity of the IgM IFA test was 78% and the specificity was 92%. The predictive value of a positive IgM IFA in this study was only 57%. In another evaluation that used consensus results defining true positives as those yielding positive results in two or more EIA or CF assays in conjunction with clinical findings, the IgM IFA had a sensitivity and specificity of 89% and 99%, respectively.[10]

EIAs for the detection of *M. pneumoniae*-specific antibodies have become the most widely used in clinical and reference laboratories. There have been a number of evaluations performed, and readers are encouraged to review these when considering use of one of the assays.[28,468,493,524,534] Microtiter EIA kits that are available include the *Mycoplasma pneumoniae* IgG/IgM antibody test system (Remel Thermoscientific), ImmunoWELL *Mycoplasma pneumoniae* IgM EIA (GenBio, San Diego, CA), the Zeus *Mycoplasma* IgM EIA (Zeus Scientific, Inc.), the Platelia IgM and IgG *Mycoplasma* EIA (Bio-Rad), the ETI-*M. pneumoniae* IgM/IgG assay (Savyon Diagnostics, Israel), and others listed in Table 18-3.

Some assays detect IgM and IgG separately, whereas others detect these simultaneously. The EPI-MP products of Savyon Diagnostics offer assays for IgM, IgG, and IgA separately. Two assays, the Meridian ImmunoCard and Remel MP IgG/IgM antibody test system, offer a membrane-based format for EIA. The other EIA assays utilize a 96 well microtiter plate (e.g., GenBio ImmunoWELL) or breakaway strips (e.g., Savyon EPI-MP and Platelai). All of the EIA assays vary in the type of antigen used in the solid phase. Antigens used in these assays include solubilized and purified ultrasonicates from *M. pneumoniae* cultures (e.g., Platelia), purified *M. pneumoniae* glycolipids (e.g., ImmunoWELL assay), or purified membrane protein antigens that include the immunodominant *M. pneumoniae* cytadhesin P1 (e.g., the ETI assays) as the predominant antigen in the solid phase. The specificity of IgM testing by EIA is improved when an IgM capture method is used. In a comparative evaluation of several IgM assays, a specificity of 100% was noted only for the Platelia IgM EIA, which uses IgM capture (i.e., the solid phase is coated with anti-IgM antibodies), whereas assays that used IgG absorption or other IgG removal methods for IgM detection had specificities ranging from 90% (i.e., the ImmunoWELL IgM) to as low as 25% (ETI-*M. pneumoniae* IgM).[384] In general, the IgM assays are more sensitive and specific when testing sera from children, since adults may not generate a significant IgM response.[442,543] In addition, since adult populations tend to include individuals who have residual specific anti-*M. pneumoniae* IgG from past or subclinical infections, testing of paired sera collected 1 to 3 weeks apart is important for documenting a fourfold or greater rise in antibody titer and thereby determining acute infections in adults.[513] A recent study in Belgium evaluated 10 serologic assays that measured IgA, IgM, and/or IgG in 120 patients who were serologically positive for *M. pneumoniae* or positive for other agents of interstitial pneumonia. The IgG assays performed poorly; overall interassay performance was not good among the assays. The authors suggested that improvements were needed in these serologic assays, especially in regard to IgG determinations.[52]

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TABLE 18-3 Serologic Assays for Detection of *M. pneumoniae*

Name	Format	Immunoglobulin Detected	Antigen	Manufacturer	Reference
M. pneumoniae (MP) antibody test system	Indirect immunofluorescence assay (IFA)	IgM and IgG separately	Antigenic substrate or colonies on slide	Zeus Scientific, Inc. (Branchburg, NJ)	3, 524, 534
Serodia Myco II gelatin agglutination test	Particle agglutination	IgM and IgG simultaneously		Fujirebio & Fujirebio America; distributed in Japan and outside of Japan by Bayer (Europe, Canada, Australia)	20, 27, 524
SEROFAST	Particle agglutination	IgM and IgG simultaneously		International Microbiology	524
ETI-MP IgM or IgG	EIA, 96 well microtiter format	IgM, IgG, and IgA separately	MP P1 protein	Savyon Diagnostics Ltd., Israel	28, 468, 524
ImmunoCard	Membrane-based EIA	IgM only	MP antigen	Meridian Diagnostics, Cincinnati	10, 28, 318, 468, 493, 524, 534
M. pneumoniae IgG/IgM antibody test system	Membrane-based EIA	IgM and IgG simultaneously	MP cytadhesin protein	Remel Thermoscientific	468, 493, 524, 534
Mycoplasma IgG and IgM ELISA test system	Breakway strip EIA	IgM and IgG separately	Inactivated *M. pneumoniae*	Zeus Scientific, Inc. (Branchburg, NJ)	468, 524, 534
GenBio ImmunoWELL *M. pneumoniae* IgM and IgG	EIA, 96 well microtiter format	IgM and IgG separately	MP glycolipid antigen	Alexon-Trend, Inc. (Ramsey, MN)	28, 468, 493, 524, 534
Platelia IgG and IgM	EIA, breakaway strip	IgG and IgM separately	MP culture ultrasonicate	Bio-Rad	28, 524

These do not represent all commercial assays available for serologic testing of *M. pneumoniae* antibodies, but represent the ones most commonly mentioned in texts and for which there are evaluations available.

One of the advantages of the membrane-bound EIA assays is a quicker time to result, which are usually available in minutes versus hours that might be needed for the microtiter formats. Evaluations of the Remel *M. pneumoniae* IgG/IgM antibody test system, for example, disclosed a 5-minute EIA for simultaneous, qualitative detection of antibodies against *M. pneumoniae*. Thacker and Talkington evaluated this test with 50 paired serum samples and found that the Remel test detected antibodies in three specimens with CF titers of 32 and was positive for all but one specimen with CF titers of 64 or higher.[443] Another evaluation of this system reported a sensitivity of 91% and a specificity of 91%, with corresponding positive and negative predictive values of 87% and 93%, respectively.[126] The Meridian ImmunoCard *Mycoplasma*

IgM assay (Meridian Biosciences, Cincinnati, OH) is a 10-minute, qualitative, membrane-based EIA in which test serum is allowed to react with *M. pneumoniae* antigen extracts impregnated into filter paper. This assay does not require IgM separation or IgG removal from the serum prior to test performance. Evaluations of

this assay suggest that the ImmunoCard test is more sensitive than other serologic tests for detecting low levels of IgM antibodies.[10,117,318,468,493] In an evaluation of the assay using specimens from 40 pediatric patients, all acute-phase serum specimens were positive with the ImmunoCard, whereas the CF and particle agglutination tests were positive for 30% and 77.5% of the specimens, respectively.[318] In another comparative evaluation with 64 acute-phase sera from adults, the ImmunoCard detected specific IgM in 46% of the specimens, whereas the CF test, the Remel EIA, and the ImmunoWELL IgM EIA detected antibodies in 38%, 41%, and 23%, respectively.[493] A third evaluation using 145 acute-phase sera from children reported resolved sensitivity, specificity, and positive and negative predictive values of 85%, 97%, 93%, and 93%, respectively, when compared with both the Remel and Zeus IgM microtiter EIAs.[117] The evaluations of the ImmunoCard *Mycoplasma* IgM assay suggest that this rapid test is highly reliable for establishing a diagnosis of recent *M. pneumoniae* infection in both children and adults.[543] A newer immunoblotting method for serologic diagnosis of *M. pneumoniae* was developed in Germany using a new set of antigens, and the results of a study evaluating this assay demonstrated that 92% of PCR-positive patients were positive with this immunoblot method and the specificity was 93% to 100%.[116]

Microparticle agglutination assays have also been marketed for detection of *M. pneumoniae* antibodies. The Serodyne Color Vue IgG/IgM test (Serodyne, Indianapolis, IN) uses latex particles coated with *M. pneumoniae* lipid antigen, whereas the Serodia Myco II test (Fujirebio, Tokyo, Japan) uses similarly sensitized gelatin particles. Thacker and Talkington evaluated the Serodyne 40-minute passive agglutination test and found it less sensitive than the Remel EIA; only 68% of patients with mycoplasmal pneumonia could be serologically diagnosed with this kit, compared with 94% and 96% of patients who were diagnosed by the CF and Remel tests, respectively.[492] Lieberman and colleagues evaluated the Serodia Myco II gelatin agglutination test and found the sensitivity, specificity, and predictive values of positive and negative tests to be 48.1%, 86.9%, 49.3%, and 86.3%, respectively.[277] Inadequate specificity of the Serodia Myco II particle agglutination assay compared with an IgM-specific capture EIA and an IgM IFA has been noted by other researchers as well.[20]

The purification and sequencing of the P1 adhesin of *M. pneumoniae* and the recognition of immunodominant epitopes of P1 and other cell surface molecules may allow the manufacture of synthetic peptides that can be used as specific, chemically defined antigens in future serologic tests for *M. pneumoniae*.[206] Suni and colleagues evaluated a new EIA method (*M. pneumoniae* IgG, IgA, and IgM, Thermolabsystems, Helsinki, Finland) in which the solid-phase antigen is enriched for the P1 cytoadherence protein antigen of *M. pneumoniae*.[461] When compared with the

ImmunoWELL and Platelia EIAs, the sensitivity and specificity of this test were 100% and 96.5% to 100%, respectively. Another group investigated the performance of two novel EIAs in comparison with the CF test. In one assay, the solid phase contained recombinant P1 antigen; in the other assay, the solid phase contained recombinant p116 antigen.[109] Both of these antigens have demonstrable antigenicity and engender the formation of specific antibodies in infected patients.[113,114,196]One of these studies found that the recombinant antigen assays showed good agreement with the CF test, with the recombinant P1 EIA showing the best discrimination between positive and negative specimens.[109] The use of species-specific antigenic epitopes in serologic tests for *M. pneumoniae* infection may help to eliminate false-positive serologic tests resulting from *M. genitalium* respiratory or urogenital tract infection, since these organisms are known to possess cross-reacting antigens.[280]

Serodiagnosis by detection of anti-*M. pneumoniae* IgA levels has also been investigated as a possible method for diagnosis of *M. pneumoniae* infection. Watkins-Riedel and colleagues evaluated a 3-hour IgA EIA method (SeroMP-IgA kit; Savyon Diagnostics, Ashdod, Israel) and compared it with the microparticle agglutination assay (Serodia Myco II; Fujirebio), the CF test, an IgM-specific EIA (SeroMP-IgM kit; Savyon Diagnostics), and the rapid ImmunoCard IgM EIA (Meridian Diagnostics) on acute-phase serum samples from 23 patients with *M. pneumoniae* pneumonia.[545] The IgA assay performed with a sensitivity of 96% to 100% and a specificity of 91% to 100%. Sensitivity of the other assays ranged from 87% to 91%. During the course of this study, the investigators noted that younger patients tended to have higher levels of IgM than adults, whereas the adult patients tended to have higher levels of IgA antibodies. This assay appeared to address two of the recognized shortcomings of *Mycoplasma* serology: the comparatively lower sensitivity and specificity of the CF test and the inconsistent presence of *M. pneumoniae*-specific IgM in infected adult patients. A newer antigen test, utilizing an automated immunofluorescence multiplexed antigen array (InoDiag, France), was compared with standard culture, antigen, or PCR-based tests for diagnosis of six agents of atypical pneumonia including *M. pneumoniae.* Sensitivity was 100% and specificity 98% for IgM detection in the 19 sera from patients ≤30 years old who were positive for *M. pneumoniae* by the Meridian ELISA assay.[146]Other investigators have suggested that in young patients, a single elevated IgM titer could indicate acute infection, although seroconversion was definitely more specific.[384] This InoDiag assay does afford a rapid turnaround time (15 minutes), avoids the specificity issues associated with IFA, and can potentially be used in epidemiologic studies since various cutoff values can be used in populations with different prevalence and incidence.[146]

A nonspecific serologic test that is often used to support the diagnosis of *M. pneumoniae* infection is the production of cold agglutinins. These IgM antibodies react

in the cold with human erythrocyte I antigens. These antibodies are produced within 2 weeks after infection with *M. pneumoniae* and can persist for up to 5 months. The I antigen, which is similar in structure to some antigenic determinants in the glycolipid membrane of *M. pneumoniae*, is inactivated by α-glycosidase, and the binding of cold agglutinins to these cells is inhibited by galactose.[78,211,418,556] Furthermore, when erythrocytes containing the I antigen are subjected to

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chloroform–methanol extraction, an antigen is extracted that will fix complement in the presence of anti-*M. pneumoniae* antibodies.[556] As with other serologic tests, a fourfold rise in cold agglutinin titers in the presence of a compatible clinical illness is suggestive of *M. pneumoniae* infection. However, only about 50% to 60% of *M. pneumoniae* infected individuals will be positive for cold agglutinins, and several other conditions can cause increases in cold agglutinins, including infectious mononucleosis, mumps, influenza, rubella, respiratory syncytial virus infections, adenovirus infections, peripheral vascular disease, and psittacosis. Therefore, the specificity of this assay is not optimal.[418]

Serologic Tests for Genital Mycoplasmas

Serologic methods have also been investigated for the diagnosis of MH infections. Lipid-associated membrane proteins (LAMPs) constitute a major group of cell surface proteins that are highly antigenic and are major targets in the immune response against mycoplasmas.[263] LAMPs have been identified in several *Mycoplasma* species, including *M. salivarium*, *M. penetrans*, *M. pirum,* and *M. genitalium.* These antigens have been used in serologic assays for testing patients with various types of infections.[538,540,541] Antibodies to these LAMP antigens appear to be highly species-specific, with no apparent cross-reactions with those of other species. Antibodies against LAMP antigens have also been used to delineate serotypes of MH. In a recent study, the antigenicity and antibody profiles of 14 different MH strains were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot.[293] The LAMPs from these 14 strains showed similar SDS-PAGE profiles, and immunoblot analysis showed that these proteins were highly immunogenic when tested against sera from infected patients. These workers also demonstrated that sera from 28 of 31 HIV-positive patients with MH culture-positive urine specimens reacted with each of the 14 different MH isolates on Western immunoblot examination. These studies showed that LAMPs from any isolate of MH

could be used as a target antigen in an EIA test format for detection of antibodies against MH. This LAMP-derived EIA was used to demonstrate that the prevalence of MH-specific antibodies was significantly greater among patients attending STD clinics (67.8% prevalence) than among healthy blood donors (34.4% prevalence) and that MH infection occurred at a much younger age in women than in men. These EIAs for detection of serum antibodies against MH are research-based assays and are not widely available.

Serologic methods have also been evaluated as a way to establish the presence of UU infection. Serologic methods have included EIA techniques, with purified organism sonicates as the solid-phase antigen, or Western immunoblot techniques, wherein antigens were prepared by growing representative UU serovars in culture, lysing the organisms with SDS, separating the antigens by electrophoresis, and transblotting of the antigens onto nitrocellulose paper. One study examined the presence of the UU in amniotic fluids along with the serologic response as documented by EIA in women in their third trimester of pregnancy, who were undergoing amniocentesis for genetic indications, women admitted for preterm labor with intact fetal membranes, and women with preterm rupture of the fetal membranes.[192] Although the prevalence of UU infection was 2.9%, 4.3%, and 17.8%, respectively, in the three groups, the corresponding antibody prevalences were 50%, 86%, and 57%. These workers found that the incidence of adverse pregnancy outcomes—preterm birth, low birth weight, or fetal death—was significantly greater in women who had generated an immune response against UU.[192] Another study by the same group showed that 30% of women with postpartum endometritis had positive EIA serologies for UU, compared with 6% of the control group.[60] A serologic study of women and infants using immunoblots for UU-specific IgM and IgA found that 4.5% of infants born at less than 30 weeks of gestation, less than 1.7% of infants born between 30 and 34 weeks of gestation, and none of the infants born at 35 weeks or greater had detectable IgM- or IgA-specific antibodies; this suggested a possible role for the immune response in pregnancy outcome.[83] Serologic studies such as these may help to clarify the role of the specific immune response to UU in the pathogenesis of infection and to provide information for the development of sensitive and specific serologic tests to augment cultural and molecular diagnostic approaches in the clinical laboratory. As with MH, serologic tests for UU are not widely available and are mainly used in research settings.

Antimicrobial Susceptibility and Treatment of *Mycoplasma* Infections

Treatment of M. pneumoniae *and Genital Mycoplasmas*

Atypical pneumonia cause by *M. pneumoniae* is generally treated with tetracycline or a macrolide, including erythromycin, azithromycin, or clarithromycin. Although the organism may still be present in the upper respiratory tract during and after treatment, clinical manifestations of infection generally improve and infiltrates on chest radiography usually disappear during therapy. Standardized methods for performing in vitro susceptibility tests using agar and broth microdilution including quality control parameters have recently been described in the literature, and a Clinical Laboratory Standards Institute (CLSI) document is available that provides methods for and interpretation of results of susceptibility testing for *M. pneumoniae,* MH, and *Ureaplasma* spp.[74,530,534] Without these standards, testing has been limited to a few reference laboratories. If resistance to the usual drugs for treatment becomes more prevalent and molecular methods continue to increase the detection of mycoplasmal infections, then even more requests for susceptibility testing may be made. Now that susceptibility testing has been standardized, it will be necessary to know where such testing is available.[74,530] Box 18-3 provides an overview of the usual agents to which various mycoplasmas are susceptible and resistant. More specific information about specific treatment for each agent and the disease it may cause can be found in many of the references included in the table and in this chapter section.

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BOX 18-3

Usual Susceptibility Patterns of *Mycoplasma* spp. and *Ureaplasma* spp.

Organism	Usually Susceptible	Resistant or Usually Resistant	Comments
M. pneumoniae	Macrolides Fluoroquinolones Tetracyclines	Penicillins Cephalosporins Glycopeptides SXT Rifampin Nalidixic Polymyxins	High-level macrolide resistance has been in many countries[9,26,385,397,524,554,557]
M. hominis	Tetracyclines Fluoroquinolones Clindamycin Chloramphenicol Streptomycin Gentamicin	Penicillins Cephalosporins Glycopeptides Macrolides (intrinsic and induced resistance) SXT Rifampin Nalidixic Polymyxins	Occasional fluoroquinolone resistance with mutations in DNA gyrase/topoisomerases[524]
Ureaplasma spp.	Macrolides Fluoroquinolones Tetracyclines Chloramphenicol Streptomycin Gentamicin	Penicillins Cephalosporins Glycopeptides Clindamycin SXT Rifampin Nalidixic Polymyxins	High-level macrolide resistance rare, but has been detected.[524] Occasional fluoroquinolone resistance with mutations in DNA gyrase/topoisomerases.[112,524]
M. fermentans	Tetracyclines Clindamycin Chloramphenicol Fluoroquinolones	Penicillins Cephalosporins Glycopeptides Macrolides Aminoglycosides SXT Rifampin Nalidixic Polymyxins	There is some variability in the literature about in vitro activity of macrolides and aminoglycosides.
M. genitalium	Macrolides Tetracyclines (but therapeutic failures have occurred with tetracyclines) Clindamycin Fluoroquinolones	Penicillins Cephalosporins Glycopeptides SXT Rifampin Nalidixic Polymyxins	Macrolide resistance may be induced.[524] Occasional fluoroquinolone resistance with mutations in DNA gyrase/topoisomerases.[524]

SXT, Trimethoprim–sulfamethoxazole.
Most of this information unless specifically referenced was taken from references 524 and 534.

All mycoplasmas are innately resistant to β-lactam antibiotics (e.g., penicillins and cephalosporins), glycopeptides (e.g., vancomycin), as well as sulfonamides, trimethoprim, polymyxins, nalidixic acid, and rifampin.[474,524] *M. pneumoniae* strains are usually susceptible to a wide variety of antimicrobial agents, including the quinolones (i.e., ciprofloxacin, levofloxacin, ofloxacin, gemifloxacin, moxifloxacin), clindamycin, lincomycin, tetracycline, doxycycline, minocycline, the macrolides (i.e., erythromycin, azithromycin, and clarithromycin), and

streptomycin.[111,164,237,474,524,526,527]

Resistance to macrolides has been reported in many countries, including the United States.[9,26,397] Most of the resistance has been seen in children. In a study of 49 patients with *M. pneumoniae* pneumonia in 2007 to 2010, the incidence of macrolide resistance was 8.2%. Patients require treatment with tetracycline or fluoroquinolones when there is

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resistance to macrolides.[557] Simmons et al. have detected the presence of biofilms in certain strains of *M. pneumoniae* that might confer this macrolide resistance and impair host immune response, consequently preventing effective treatment of the infections.[445] A review article on the pathogenesis and detection of *M. pneumoniae* presents more information on antibiotic resistance in the United States.[523]

Most isolates of MH and UU are susceptible to tetracyclines. MH strains are usually also susceptible to clindamycin and lincomycin, but are innately resistant to erythromycin and other macrolides (i.e., azithromycin). Inducible resistance to macrolides like erythromycin can also occur in MH.[382] UU strains are usually susceptible to erythromycin and azithromycin, but not to clindamycin and lincomycin. The growth of MH and UU is also inhibited by chloramphenicol, streptomycin, and gentamicin, but not by cell wall active agents (e.g., cephalothin, ampicillin, and vancomycin). During the mid- to late 1970s and early 1980s, strains of both species were found that were resistant to tetracycline; these strains contained a genetic antimicrobial resistance determinant (transposon) called *tetM*.[404,524] Strains of UU that are resistant or intermediate in susceptibility to erythromycin and newer macrolides have also been described.[524] Global resistance to many antimicrobial agents has been shown in a recent study of 290 MH isolates and 179 UU isolates in Germany using either an agar dilution or Etest method from 1999 to 2008. Doxycycline remained the most active of the tetracycline agents tested. Although up to 13% of the MH strains were resistant to doxycycline, only 1% to 3% of UU isolates were resistant.[257] Macrolides remain the drug of choice for the treatment of UU non-CNS infections in neonates. Erythromycin is used much less frequently in older children and adults because of availability of newer macrolides, such as azithromycin and clarithromycin. Whether this will become the standard in neonates, replacing erythromycin, is yet to be determined.[533] CNS infections in neonates by these organisms are best treated with tetracycline.[533]Strains of UU are generally less susceptible to the quinolone antibiotics than either *M. pneumoniae* or MH.[239–241] Krausse and colleagues found that ofloxacin was effective against both species, but only 30% of the UU strains were susceptible to

ciprofloxacin as compared with 70% of the MH strains.[257] In general, the mycoplasmas are more susceptible to the newer fluoroquinolones in vitro as compared to older agents, such as ofloxacin.[524]

The treatment of *M. genitalium* infections has not yet been standardized, nor are methods available for susceptibility testing. Macrolides are recommended, especially single-dose azithromycin; tetracyclines are responsible for a large number of therapeutic failures even without any acquired resistance being demonstrated. The antimicrobial susceptibility profile of *M. genitalium* appears to be similar to that of *M. pneumoniae* in that isolates of this species are susceptible to tetracyclines and a variety of macrolides.[473,474] Renaudin and colleagues determined the susceptibilities of seven *M. genitalium* isolates using an agar dilution method and compared them with three *M. pneumoniae* strains.[401] Both species were susceptible to macrolides (i.e., erythromycin, spiramycin, roxithromycin, azithromycin, and clarithromycin), clindamycin, tetracycline, doxycycline, minocycline, and the fluoroquinolones (i.e., ofloxacin, ciprofloxacin, lomefloxacin, sparfloxacin).[98] Acquired resistance to macrolides and fluoroquinolones leading to therapeutic failure has, however, been described.[37,312,466] Despite in vitro susceptibility of *M. genitalium* to the tetracyclines, several studies have shown that tetracycline therapy is often associated with treatment failures. A study by Johannison and colleagues found that 62% of 13 men with *M. genitalium* urethritis who were treated with tetracycline for 10 days still had the organism in the urethra at follow-up.[220] In another study, all of seven men with *M. genitalium* urethritis who were treated with doxycycline still had urethritis on follow-up.[186] In a treatment study comparing tetracyclines and azithromycin, all six patients who had received azithromycin were negative for *M. genitalium* by PCR after treatment, while 63% of 16 patients treated with tetracycline were still PCR-positive for *M. genitalium* after treatment.[125] The presence of the organisms in the urogenital tract with or without attendant symptoms following treatment with these agents may be directly related to the dosage and duration of treatment. According to the treatment guidelines for STDs, azithromycin for treatment of urethritis is administered in a single 1-g dose.[59] Patients with *M. genitalium* urethritis who have been treated with azithromycin according to these recommendation have had partial clinical responses and remained positive for *M. genitalium* by PCR a week after completion of therapy.[134] In a 2012 North Carolina study of NGU in 293 homosexual men, persistence of *M. genitalium* after treatment was seen in 44% of those positive initially as compared to the 12% who persisted after 4 weeks of therapy for *C. trachomatis* infection. Persistence of the *M. genitalium* was found to be 66% in men treated with doxycycline versus 33% of those treated with azithromycin.[431]

With use of a modified broth dilution procedure with SP-4 medium, Hayes and

colleagues determined the antimicrobial susceptibilities of the *M. fermentans* incognitus strain along with reference strains of both *M. fermentans* (i.e., Strain PG18 [ATCC 19989]) and *M. pneumoniae* (ATCC 15531).[173,175] As expected, all three mycoplasmas were resistant to cell wall–active agents (e.g., penicillin, ampicillin). Both the PG18 and the incognitus *M. fermentans* incognitus strains were resistant to erythromycin (mean minimal inhibitory concentrations [MICs] of erythromycin, 31.2 mg/mL and 43.0 mg/mL, respectively), whereas *M. pneumoniae* was susceptible to erythromycin (mean MIC, 0.0073 mg/mL). The *M. fermentans* incognitus strain was susceptible to tetracycline, doxycycline, clindamycin, lincomycin, chloramphenicol, and ciprofloxacin. Both *M. fermentans* strains tested in this study were resistant to the aminoglycosides (i.e., gentamicin, kanamycin, streptomycin, and neomycin), whereas *M. pneumoniae* was susceptible. Aminoglycoside MICs for the incognitus strain of *M. fermentans* were higher than 1,000 mg/mL for all four of these drugs, while the MICs for gentamicin, kanamycin, streptomycin, and neomycin for the PG18 strain were 15.6, 20.8, 18.2, and 52.1 mg/mL, respectively. Subsequent antimicrobial susceptibility testing of 24 additional *M. fermentans* isolates also disclosed that this species is resistant to erythromycin and the aminoglycosides.[173] This subsequent study also documented that only ciprofloxacin and levofloxacin had a significant bactericidal effect.[173]

Using a modified dilution technique with Hayflick's broth, Hannon examined several *M. fermentans* strains isolated

from human infections and from tissue culture cells.[162] These strains were much more susceptible to azithromycin than to either erythromycin or clarithromycin. Clindamycin and several tetracycline congeners also showed good activity against the *M. fermentans* strains, although mycoplasmacidal concentrations were severalfold greater than inhibitory concentrations of antibiotic. This study also demonstrated that *M. fermentans* strains isolated during the 1960s in cell-free media were susceptible to the aminoglycosides, while recent human isolates and strains obtained from tissue culture cells were often resistant to a single or multiple aminoglycoside drugs, with MICs to these agents exceeding 500 mg/mL. Azithromycin was more active against *M. fermentans* strains than either erythromycin or clarithromycin.[162]

In a subsequent study, Hannon examined the aminoglycoside susceptibility of several *M. fermentans* strains, including three AIDS-associated isolates (including one incognitus strain), six recent respiratory tract isolates, one leukemia-associated isolate from 1962, and eight tissue culture isolates.[163] Two of the 3 AIDS-associated isolates and all of the non–AIDS-associated respiratory tract isolates were susceptible to the six

aminoglycoside drugs tested, while the *M. fermentans* incognitus strain and all the recent tissue culture isolates were highly resistant to all six agents, with MICs exceeding 250 mg/mL. The strain isolated from tissue culture of the bone marrow of a patient with leukemia in 1962 was highly resistant to streptomycin (MIC, >250 mg/mL), but susceptible to the other five aminoglycosides. These results were consistent with Hannon's earlier studies, which established that *M. fermentans* incognitus was the only human strain of the nine examined that demonstrated multiple aminoglycoside resistance.[162] Using a macrodilution, metabolic inhibition assay, Poulin and colleagues examined the antimicrobial susceptibilities of several AIDS-associated mycoplasmas, including *M. fermentans* (a stock strain, two clinical isolates from patients with AIDS, and the "incognitus" strain), *M. penetrans* (one strain), and *M. pirum* (one strain).[394] All isolates were susceptible to azithromycin, clarithromycin, clindamycin, doxycycline, ofloxacin, and tetracycline. *M. fermentans* strains and *M. pirum* were resistant to erythromycin, whereas *M. penetrans* was susceptible. Hayes and colleagues tested nine strains of *M. penetrans* by a broth dilution procedure.[174] All were susceptible to azithromycin, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, erythromycin, levofloxacin, lincomycin, and tetracycline, but were resistant to gentamicin and streptomycin.

Methods for Susceptibility Testing of Mycoplasmas

Agar disk diffusion is not an acceptable method for in vitro susceptibility testing of mycoplasmas. Both agar dilution and broth dilution methods for antimicrobial susceptibility testing of mycoplasmas have been described, as well as Etest methods.[74,524]SP-4 or Hayflick's media with pH of 7.3 to 7.4 are recommended for testing MH or *M. pneumoniae*. 10B broth, Bromthymol Blue Broth, or A8 agar can be used for testing *Ureaplasma spp.*; a pH of 6.0 to 6.5 is used for testing UU. For agar dilution, doubling dilutions of antimicrobial agents are incorporated into the agar medium and inoculation is performed using a Steer's replicator to deliver an inoculum of 30 to 300 CFUs per inoculum spot. This inoculum is determined by plating aliquots of 10-fold dilutions of a growing broth culture in triplicate, performing colony counts, and diluting the organism suspension appropriately so that the replicator delivers 30 to 300 CFUs per spot. Plates are incubated in air at 35°C to 37°C for 4 days (UU), 5 days (MH), or 14 days (*M. pneumoniae*). Growth may be enhanced for UU if media is incubated in increased CO_2.[74,524]

For broth microdilution, the antimicrobial agents are solubilized and diluted in microtiter trays with the proper broth medium containing a phenol red indicator to achieve a range of drug concentrations. The inoculum should be prepared from cultures that are in the exponential growth phase, where the numbers of organisms is generally about 10^7 CFU/mL for ureaplasmas and 10^8 to 10^9 CFU/mL for large-

colony mycoplasmas, which are also expressed as color-changing units (CCUs) per milliliter. An inoculum of 10^4 to 10^5 CFU (CCU) of organism is added to each of the microtiter wells containing the antimicrobial dilutions, and then the plates are sealed and incubated aerobically at 35°C to 37°C. The microtiter trays are examined daily until a color change due to a change in pH is detected in the drug-free growth control. The MIC is defined as the lowest concentration of antimicrobial agent at which the metabolism of the organisms is inhibited enough to prevent a change in the color of the medium (due to the pH indicator) at the time that the drug-free control first shows a color change. For UU, growth will usually be present within the control well after 24 hours, while growth of MH usually requires 48 hours. *M. pneumoniae* strains generally require 4 to 8 days of incubation before a change is noted in the control well. With both agar dilution and broth dilution procedures, strains with known susceptibilities and reproducible MICs must be included with the performance of testing, as external controls.[74,524] There are a few commercial products available for susceptibility testing of mycoplasmas, including *Mycoplasma* IST (bioMérieux), *Mycoplasma* SIR (Bio-Rad), MYCOFAST All IN (Unipath, UK), and MYCOKIT-ATB (PBS Orgenics), but these are available only in Europe presently.[524]

In all of these susceptibility procedures, problems that relate to the biologic characteristics of the organisms and to the method itself may be encountered. Biologic problems include the possibility of mixed cultures (e.g., both mycoplasmas and ureaplasmas) and the coexistence of susceptible and resistant populations in the same culture. The latter problem may be detected only in agar dilution-based systems, because colonies growing on antibiotic-containing media can be directly reidentified by urea hydrolysis or by serologic methods. Problems relating to methodology, including the lack of a standard medium for test performance, discrepancies related to the pH required for optimal mycoplasmal growth versus optimal antibiotic activity, the difficulty in standardization of inoculum size, the lack of standardized incubation times and conditions, and the lack of a standardized susceptibility breakpoints, have been greatly overcome with publications of documents such as Cumitech and CLSI 43-A.[74,238,524] Susceptibility testing on mycoplasmas will continue to be performed primarily in reference laboratories and larger institutions with special research interests in the therapy of genital mycoplasmal infections.

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Diagnosis and Treatment of Hemotrophic *Mycoplasma* Infections in Animals

Former members of the genera *Eperythrozoon* and *Haemobartonella* cannot be grown in culture, so diagnosis is based on the examination of blood smears stained by Romanowsky-type blood film stains, including Giemsa, May-Grünwald–Giemsa, Wright–Giemsa stains, or by the acridine orange stain.[336,337,469] Because the organisms display a cyclical parasitemia where they may be rapidly cleared from the blood, examination of multiple blood films collected over a 24-hour period increases the likelihood of a positive diagnosis in a clinically compatible host species. Blood films should be prepared immediately after collection using nonanticoagulated blood or blood collected in heparinized tubes; EDTA anticoagulant may cause detachment of the organisms from the red blood cell membrane, making microscopic diagnosis difficult.[336,469] The organisms appear as coccal forms that are 0.5 to 0.8 mm in diameter and adherent to the surfaces of the erythrocytes. They are usually found around the periphery of the erythrocyte and may appear singly or in pairs, clusters, and chains. With the acridine orange fluorescence stain, the organisms appear as bright orange to yellow-green. Molecular and serologic assays for the hemotrophic mycoplasmas have also been developed but are not widely available.[469] All species appear to be susceptible to tetracyclines and resistant to β-lactam antimicrobial agents. Treatment with erythromycin effectively controls acute infection, but treatment does not consistently clear animals of the infection.[336]

Vaccines and Prevention of *Mycoplasma* Infections

There have been attempts to develop inactivated vaccines for the prevention of *M. pneumoniae* infection. A systematic review and meta-analysis of the literature as of 2009 were published by Linchevski et al.[278] They found an efficacy against *M. pneumoniae* infection of 46% or 52% depending on the approach used with little adverse effects recognized. Overall, they reported that the vaccine could reduce rates of pneumonia and *M. pneumoniae* specifically by about 40% and suggested further development of such vaccines especially for high-risk populations.[278] A P1-C DNA vaccine fused with the B subunit of *Escherichia coli* heat-labile enterotoxin demonstrated increased efficacy in protecting mice against *M. pneumoniae* infection.[571]Strategies to create chimeric proteins derived from functional adhesin regions of *Mycoplasma pneumonia* for vaccine development have also shown efficacy in animal models.[171,429]

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CHAPTER 19

Mycobacteria

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Test methods and algorithms used for the growth, detection, identification, and susceptibility testing of mycobacteria have changed considerably over the past several decades. Several new techniques have been introduced that are directed toward the detection of *M. tuberculosis* complex (MTBC) directly in clinical specimens by nucleic acid amplification, the more effective recovery of mycobacteria from clinical specimens, the rapid identification of mycobacteria using molecular techniques, and the rapid determination of drug susceptibility profiles. Although the quest for pure research in molecular biology is an integral part of this evolution, the driving force for clinical laboratory personnel is to provide ever more rapid species identification and antimycobacterial drug susceptibility profiles. As molecular-based procedures evolve

from research laboratories into commercial products, some of which have received Food and Drug Administration (FDA) approval, the ability to diagnose tuberculosis and have a preliminary idea of drug susceptibility within days—or incredibly even hours—rather than weeks is now possible. These techniques are discussed in more detail later in this chapter.

Trends in Clinical Tuberculosis

Worldwide Incidence of Tuberculosis

According to the World Health Organization,[371] the global burden of tuberculosis (TB) has been slowly declining over the past several years, but it remains high. In 2012, there were an estimated 8.6 million incident cases of TB worldwide (range, 8.3 million to 9.0 million), of which 0.5 million occurred in children, 2.9 million in women, and 1.1 million in persons infected with HIV. Of those, 450,000 were multidrug-resistant TB (MDR-TB). Fifty-eight percent of the total TB cases occurred in South-East Asia and Western Pacific regions, 28% in the African region, and much smaller proportions elsewhere: 8% in the eastern Mediterranean region, 4% in the European region, and 3% in the region of the Americas. The five countries with the highest number of cases were India, China, South Africa, Indonesia, and Pakistan. The lowest rates were in the United States, Canada, Western Europe, Japan, Australia, and New Zealand. An estimated 1.3 million people died from TB in 2012, of which 320,000 were infected with HIV and 170,000 had MDR-TB.

In 2013, 9,588 new cases of TB were reported in the United States (incidence, 3.0 cases per 100,000 population), a 4.2% decrease from 2012.[43] The rate among foreign-born persons was about 13 times greater than the rate among US-born persons, and rates among non-Hispanic Asians were nearly 26 times greater than among non-Hispanic whites. Over half (51.3%) of the cases occurred in four states: California, Texas, New York, and Florida. Eighty-six cases of MDR-TB were reported in 2012 (the most recent year for which drug susceptibility test results are complete) and 88.4% of these occurred in foreign-born persons.

Persons at Risk for Mycobacterium tuberculosis *Infection and Active Tuberculosis*

Persons at increased risk for *M. tuberculosis* infection are as follows:

- Close contacts of those with known or suspected active TB
- Foreign-born from an area with a high incidence of active TB (e.g., Asia, Africa)
- Persons who visit an area with a high prevalence of active TB

- Residents and employees of congregate settings whose clients are at increased risk for active TB (e.g., correctional facilities, homeless shelters)
- Healthcare workers who care for patients at increased risk for active TB
- Persons who abuse drugs or alcohol

Factors that increase the risk for progression of infection to active TB are as follows:

- HIV infection, which is the strongest known risk factor for progressing to active TB among persons with latent *M. tuberculosis* infection
- Age <5 years
- Immunosuppressive therapy (e.g., tumor necrosis factor-α antagonists, systemic corticosteroids)
- Recent (within the past 2 years) infection with *M. tuberculosis*
- History of untreated or inadequately treated active TB
- Silicosis, diabetes mellitus, chronic renal failure, leukemia, lymphoma, cancer of the head, neck, or lung
- Gastrectomy or jejunoileal bypass
- Weight <90% of the ideal body weight
- Cigarette smoker or abuse of drugs or alcohol

Rapidly Progressive Disease

Recently acquired infections with *M. tuberculosis* do not invariably follow the classic, slowly progressive course of secondary disease. Primary progressive TB or the rapidly spreading miliary type of TB may occur in patients with profound immunosuppression, such as patients with AIDS. Progression of disease in such patients may be measured in terms of weeks, rather than months or years. The rapid replication of mycobacteria at sites of infection leads not only to high concentrations of organisms at these sites, but also to miliary spread and potential treatment failures secondary to the overwhelming burden of mycobacteria and possible emergence of multidrug-resistant strains.

Infection Control and Epidemiologic Measures

Measures should be instituted to ensure that patients with a high index of suspicion for TB are promptly placed in airborne (i.e., respiratory) precautions. Hospital personnel involved in the direct care of patients with TB are at high risk for infection and are required to follow isolation procedures and wear protective clothing and high-filtering masks. Laboratory personnel who handle clinical specimens from patients with TB are also at risk for infection, but may work in a safe manner with these specimens using universal precautions and processing specimens in laminar flow biologic safety hoods.

Ventilation systems in hospitals and in crowded settings, such as in prisons, nursing homes, and urban homeless shelters, where outbreaks of TB have been reported, are under intense scrutiny. Nardell and associates[221] found that

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poor air quality contributed to airborne infection in buildings fitted with air duct systems that delivered an inadequate mixture of outside air. Problems will remain in unsuspected settings, however, such as the outbreak of TB among patrons of a local bar, as reported by Kline and coauthors.[163] The index case in this report was a homeless person who was a regular patron of the bar, and who had a long asymptomatic interval before diagnosis. The possibility of heavy alcohol use among this population of patrons may also have contributed to the high rate of infectivity.

The Clinical Laboratory

Optimizing the Detection and Identification of Mycobacteria

The traditional practice of waiting for bacterial colonies to appear on solid agar, determining the species identification using only biochemical tests, and performing drug susceptibility tests only on solid media is too time-consuming. Decisions regarding the procedures and tests that will be performed in the on-site laboratory and which will be sent to a qualified reference laboratory must be determined within each practice care community. In many practice settings, activities of the on-site laboratory may be limited to rapid turnaround procedures, such as screening smears for acid-fast bacilli (AFB) or the inoculation of culture media and provisional assessment of isolates. Other procedures, again to be determined by local healthcare personnel, may be best performed in a reference laboratory. A team of healthcare workers, including clinicians, laboratory specialists, and support personnel, should be established to determine how diagnostic approaches are to be coordinated. Early diagnosis and prompt initiation of appropriate therapy are the major ingredients to ensure the cure of the infected patient, prevent the emergence of resistant strains, and, for TB, interrupt person-to-person transmission.

Laboratory Safety

The inappropriate handling of infectious biologic material such as clinical specimens places laboratory personnel at risk for infection. Any activity with the potential to generate aerosols must be done in a Class I or II biologic safety cabinet (BSC). Liquification and concentration of sputum specimens may be conducted safely on the

open bench if the specimens are first treated in a Class I or II safety cabinet with NaOH (as discussed later in this chapter). Biosafety Level 3 (BSL3) practices are required for laboratories that propagate and manipulate cultures for *M. tuberculosis* or *M. bovis*.

The BSL3 space, which includes the BSC and centrifuge, should have nonpermeable walls and work surfaces, directional airflow (with the lowest air pressure in the laboratory), and a double-door air lock to prevent the backflow of air. Air from the BSL3 space should be vented through high-efficiency particulate air (HEPA) filters directly to the outside. Laboratories and hoods should be equipped with gauges to monitor air pressure.

Because equipment and the worker's arms placed in a BSC may disrupt the laminar airflow and deflect contaminated air out of the cabinet, anyone working in the laminar flow BSC and in the BSL3 space should also wear personal protective equipment, including a respirator. Personnel must be tested each year to ensure that the respirator fits correctly. Disposable gowns should be worn over a scrub suit (street clothes should not be worn into the BSL3 space because they may become contaminated), along with gloves, caps, and shoe protection to complete the protective personal accessories, since accidental splashes may occur. Protective clothing should be removed and placed in a bag for autoclaving when work in the BSL3 is completed.

Each laboratory director must determine the extent to which renovations in the current laboratory space may be required. Renovations may cost many thousands of dollars and may not be cost-effective in certain settings. In such instances, a decision must be considered about whether cultures for mycobacteria would be more effectively performed at another facility, such as a reference laboratory that can comply with the recommended standards.

Specimen Collection

Mycobacteria can be recovered from a variety of clinical specimens, including respiratory specimens (sputum, bronchial washes, bronchoalveolar lavage fluid, and bronchial biopsies); urine, feces, blood, cerebrospinal fluid; tissue biopsies; and deep-needle aspirations of virtually any tissue or organ. Specimens that may contain normal bacterial flora should be processed as soon after collection as possible to minimize the degree of overgrowth with specimen contaminants.

Respiratory Specimens

Sputum samples collected by expectoration or by ultrasonic nebulization are best obtained shortly after the patient awakens in the morning, when mycobacteria are at their highest concentration. Twenty-four-hour collections are discouraged because the

sample containing the highest concentration of mycobacteria will be proportionately diluted by subsequent low-yield samples, and the chances for bacterial and fungal overgrowth during the prolonged collection process are significantly increased.

The irregular and intermittent release of mycobacteria into the bronchial lumen from mucosal ulcers or loculated cavities often results in a variable pattern of recovery from respiratory secretions. Cultures obtained from patients with pulmonary or renal TB, in particular, may be positive one day but negative the next; thus, a minimum of three early-morning sputum specimens should be collected on successive 24-hour periods to maximize the chance of recovery of mycobacteria. All specimens should be transported promptly to the laboratory and refrigerated if processing is delayed.

Blood Cultures

Several approaches may be used for the recovery of mycobacteria from blood cultures. Berlin and associates[21] report on the use of a biphasic system using modified 7H11 oleic acid albumin as the agar phase and brain–heart infusion as the broth phase. Positive cultures for *Mycobacterium avium* complex

(MAC) were obtained as early as 6 to 8 days. However, Agy and coworkers[3] report that only 43.8% of blood cultures from known blood culture–positive patients were recovered using a similar biphasic system. The use of the lysis-centrifugation blood culture system (ISOLATOR; Wampole, Alere, Waltham, MA) has been shown to increase the yield and shorten the time to recovery of mycobacteria from blood cultures.[97,191] The lysis-centrifuge tube contains an anticoagulant and a lysing agent to effect rupture of both erythrocytes and neutrophils. Thus, intracellular mycobacteria are released into the broth milieu, further enriched by the lysis of the red blood cells. Following centrifugation of the tube, the eluate is discarded and the sediment is transferred to appropriate culture media.

The first semiautomated system that could be used for culture of mycobacteria from blood was the BACTEC 460 (Becton Dickinson, Sparks, MD).[3,76,313,344,361] The detection of bottles positive for mycobacteria was based on measuring radioactive $^{14}CO_2$released in the blood culture vial from the metabolism of 1-[^{14}C]-palmitic acid that was included in the broth medium. This system is no longer supported by the manufacturer and, therefore, is not discussed further.

There are several newer commercially available options for the culture of blood for mycobacteria, some of which contain lytic agents to aid in the release of the mycobacteria from phagocytes. These include the VersaTrek (formerly ESP Culture System II, Trek Diagnostic), the BACTEC MYCO/F lytic (BD Diagnostic Systems),

and the MB/BacT bottle (bioMérieux), all of which are discussed further later in this chapter.

Whatever blood culture system is used, the policy of routinely obtaining at least two culture sets may not be cost-effective for patients in whom mycobacteremia is suspected.[311] From a total of 1,047 mycobacterial blood cultures obtained from 273 patients with disseminated MAC, Stone and colleagues[311] found that only 1 of the 2 bottles was positive in only 4 of 98 positive cultures (4%). In 85%, both bottles were negative, and in 11%, both bottles were positive. Thus, as a routine, they recommend obtaining only a single blood culture for the diagnosis of mycobacteremia and ordering a repeat culture only if there is strong clinical evidence of disseminated infection.

Other Specimens

In certain patients with AIDS, the concentration of mycobacteria, particularly MAC, may be sufficiently high in the lower intestinal tract to be recovered in culture. Kiehn and associates[153] have outlined a procedure for processing stool specimens for mycobacterial culture:

- Stool specimens are collected in a clean (not necessarily sterile) container with a tightly fitting lid, as for routine bacterial cultures.
- A direct smear is first prepared from a small quantity of the specimen and stained for AFB.
- If the smears are negative for AFB, the specimen is not processed further.
- If AFB are seen in the smear, 1 g of feces is suspended in 5 mL of Middlebrook 7H9 broth or equivalent and subjected to the same NaOH digestion–decontamination as used for sputum specimens (described below).

One might question the significance of detecting mycobacteria in fecal samples. Conlon and coworkers,[56] in a study of 89 patients with AIDS-related enteropathy in Lusaka, Zambia, found that chronic diarrhea did not correlate with the presence of mycobacteria in stool specimens. In contrast, Chin and colleagues[46] found that the recovery of MAC organisms from stool specimens was predictive of disseminated disease.

Specimens submitted for mycobacterial culture that are normally sterile, such as cerebrospinal fluid, synovial fluid, and other body fluids, need not be decontaminated before culture. Processing can commence with centrifugation, as described below, and aliquots of the sediment transferred to appropriate culture media. Low-volume fluid samples can be added directly to broth and incubated.

A first-morning clean-catch midstream urine sample, at least 40 mL, should be collected in a sterile container on 3 consecutive days. Twenty-four-hour pooled

specimens are not acceptable. Although these specimens can sometimes be processed without decontamination, the need for decontamination should be based on the experience of the individual laboratory. If the need to decontaminate is not clear, the specimen can be refrigerated until the results of routine bacterial cultures are available the following day.

Tissues and needle biopsy material should be placed in a small quantity of 7H9 or 7H11 broth as a holding medium. Depending on the size and nature of the material obtained, the specimen should be ground in a small amount of broth with a mortar and pestle and aliquots of the suspension transferred to appropriate culture media.

There is virtually no indication for obtaining material for mycobacterial culture with a swab because the hydrophobic nature of the lipid-containing cell wall of the bacteria inhibits transfer of the organisms from the swab to the aqueous culture medium. It is optimal to reject such specimens for mycobacterial culture. However, if a swab is received in the laboratory and material for culture cannot be recollected, the tip of the swab should be placed directly on the surface of the culture medium or into a tube containing approximately 5 mL of 7H9 broth and incubated for 6 to 8 weeks.

Laboratory Approach to the Recovery and Identification of Mycobacteria

The conventional laboratory approach to the diagnosis of mycobacterial infections involves the phenotypic characterization of colonies growing on a solid medium followed by a battery of biochemical tests. However, this process is too time-consuming and often does not provide an accurate identification. Currently, using a combination of phenotypic and molecular assays for the rapid identification of mycobacteria is recommended, particularly for the identification of MTBC.

Smears should be prepared from the material submitted and stained with a fluorescent dye (auramine O alone or in combination with rhodamine B). Some laboratories choose to confirm positive results with a carbol fuchsin-based stain (Ziehl–Neelsen or Kinyoun), but this is not required. However, some rapidly growing mycobacteria may not stain with a fluorochrome stain; therefore, if infection with a rapid grower is suspected, staining smears of the specimen with a carbol fuchsin stain should be considered.

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Sputum and other contaminated specimens are first treated with *N*-acetyl-L-cysteine–sodium hydroxide (NALC–NaOH), a decontamination–digestion procedure for concentrating any mycobacterial cells that may be present. It is currently

recommended that all sputum digests and other specimens be inoculated to broth culture media in addition to a solid medium (Löwenstein–Jensen [LJ] medium or Middlebrook agar). When mycobacterial growth is detected, a molecular test for identification of MTBC should be done as soon as the concentration of organisms exceeds the threshold of sensitivity for the assay.

Specimen Preparation

The high concentration of lipids in the cell wall of most mycobacteria makes them more resistant to killing by strong acid and alkaline solutions than other bacteria that may be present in the specimen. Consequently, specimens likely to contain a mixed bacterial microbiota are treated with a decontaminating agent to reduce undesirable bacterial overgrowth and to liquefy mucus. After treatment with the decontaminating agent for a carefully controlled period, the acid or alkali used is neutralized and the mixture is centrifuged at high speed to concentrate the mycobacteria.

Digestion and Decontamination

Some decontaminating solutions, such as 6% sodium hydroxide, are so strong that they may kill or seriously injure mycobacteria to the point that they will grow only very slowly, if at all. Decreasing the strength of the acid or alkaline decontamination solution has resulted in improved recovery of mycobacteria by culture, but frequently at the price of overgrowth of the culture with contaminants. Exposure of specimens to strong NaOH, 5% oxalic acid, or other agents must be carefully timed to prevent excessive chemical injury to the mycobacterial cells.

The use of mild decontaminating agents, such as trisodium phosphate (TSP) alone or combined with benzalkonium chloride (Zephiran; Winthrop Laboratories, New York, NY), is popular in some laboratories. Specimens containing a large quantity of *M. tuberculosis* can withstand the action of these agents for as long as overnight, and careful timing of exposure is not required. Specimens treated with TSP–Zephiran should be inoculated onto an egg-based culture medium to neutralize the growth inhibition of the Zephiran. If agar-based media are used, neutralization of the Zephiran can be accomplished by adding lecithin. The standard digestion–decontamination procedure developed by Kubica et al.[172] is shown in Box 19-1.

BOX 19-1

The Standard Digestion–Decontamination Procedure Developed by Kubica et al. at the Centers for Disease Control and Prevention[172]

1. Prepare the acetylcysteine-alkali digestant as follows: Combine 50 mL of 2.94%trisodium citrate-$3H_2O$ (0.1 M) with 50 mL of 4%NaOH. To this solution, add 0.5 g of powdered *N*-acetyl-L-cysteine (NALC) just before use. The NaOH (which becomes a 2%solution after mixing equal parts with the specimen) serves as a decontaminating agent. Occasionally, the concentration of NaOH must be increased to 3% (6%original solution) during warm weather or in treating specimens from patients with large pulmonary cavities associated with persistent bacterial contamination. The NALC is a mucolytic agent without bactericidal activity that liquefies mucus by splitting disulfide bonds. The mycobacteria are released when the mucus is liquefied, rendering them easy to concentrate by high-speed centrifugation.
2. Use 50-mL plastic centrifuge tubes with tightly fitting lids for the processing of all specimens. Add the NALC digestant mixture in a volume equal to that of the specimen. Typically, a volume of 10–15 mL of specimen is used. Tighten the screw cap.
3. Homogenize the mixture with a vortex mixer for 15–20 seconds or until well mixed and allow to stand at room temperature for 15–20 minutes, swirling the tubes periodically. Proper attention should be paid so that this digestion time does not exceed 20 minutes, because "overtreated" specimens result in fewer positive cultures.
4. After the digestion–decontamination step, add phosphate buffer, pH 6.8 (preferred over sterile water), up to the top ring in the tube. Mix well. The phosphate buffer makes strong shifts in pH less likely and also serves to "wash" the specimen, to dilute and neutralize toxic substances, and to reduce the specific gravity of the specimen so that centrifugation is more effective in the sedimentation of organisms.
5. Concentrate the specimen by centrifugation at 2,000 to 3,000 *g* for 15–20 minutes. A refrigerated centrifuge may be used at higher speeds to increase the yield of mycobacteria.
6. After centrifugation, decant all the supernatant carefully into a splash-proof receptacle containing a phenolic disinfectant. Wipe the lip of the tube with a cotton ball soaked with 5%phenol. Add a small quantity of phosphate buffer, pH 6.8 (1–2 mL), and resuspend the sediment with a Pasteur pipette.
7. Prepare smears on clean microscope slides for staining, preferably with a fluorochrome stain. Use either a sterile applicator stick or a flamed, 3-mm diameter bacteriologic loop and smear a portion of the sediment over an area 1 × 2 cm. If the quantity of sediment is very small, delay making smears until after the next step (i.e., the addition of albumin). Add 1 mL of sterile 0.2%bovine albumin.
8. Inoculate the concentrate to appropriate liquid and solid culture media.

Usually, concentrated HCl or concentrated NaOH is used to neutralize the decontaminating agents. Because of the strength of these solutions, a neutral end point is sometimes difficult to achieve. One advantage of the NALC procedure is that the addition of a large volume of phosphate buffer makes strong shifts in pH less likely. The addition of buffer serves to "wash" the specimen, dilutes toxic substances, and decreases the specific gravity of the specimen so that centrifugation is more effective in sedimentation of organisms.

Table 19-1 lists additional agents for decontaminating and concentrating specimens, along with comments about their use.[292,304] Each mycobacteriologist should select the agents to use in their laboratory on the basis of the number and types of specimens received and the time and technical staff available to process the specimens. As listed in Table 19-1, dithiothreitol is also an effective mucolytic agent when used with 2% NaOH. Cetylpyridium chloride has also been recommended for the decontamination of specimens, particularly those mailed from remote sites of collection.[304] The digestion–decontamination procedure is further described in Chart 19-1.

Ratnam and coauthors have described a simplified acetylcysteine–alkaline procedure that combines the decontamination and the concentration steps and the inoculation of the specimen to selective culture media (containing antibiotics, to be described later in this chapter).[262] Specimens are mixed with the NALC–NaOH solution on a vortex mixer and, without any waiting or addition of buffer or water to the digested specimens, the mixture is centrifuged at 3,000 *g* for 15 minutes. The supernatant is decanted, and the sediment is suspended in 1 to 2 mL of phosphate buffer (0.067 M, pH 5.3). The NaOH concentration in the solution may be reduced from the usual 2% to 1.5% if necessary or feasible. This method eliminates two steps: the 15- to 20-minute decontamination time following digestion of specimens and the addition of phosphate buffer or water. It also eliminates the potential for specimens to drip from the cap or run down the outer surface of the tube when diluent is added after vortex mixing and thus reduces the chance for cross-contamination. According to these authors,[262] the greatest killing of mycobacteria occurs during the first few minutes of exposure to the alkali digestion fluid, and as many as 10^4 mycobacteria may be lost, depending on the species. Eliminating the initial digestion step had no adverse effects on the recovery of mycobacteria. In fact, at times, the addition of phosphate buffer or water actually resulted in poorer recovery, owing to a greater dissolution of the particulate matter in the specimens. Alkali tolerance was not a consistent feature among the mycobacterial species and strains tested, with the rapid growers being the most susceptible to a high pH. By reducing or eliminating the standard treatment time of 15 to 20 minutes or by decreasing the concentration of NaOH in the digestant solution from the usual 2% to either 1.5% or 1%, the adverse effects can be easily

overcome.

Because of the emergence of drug-resistant strains of *M. tuberculosis*, rapid laboratory tests to determine drug susceptibility profiles are useful. Many of the new tests for identifying drug resistance profiles are based on nucleic acid amplification procedures. Consequently, it may be necessary to store specimens or digested concentrates for future batch testing or for shipping to a distant reference laboratory. It may be advisable to render such samples noninfectious at early stages of processing to minimize the biohazard to laboratory workers. Williams and associates[358] found that ethanol fixation of sputum sediments containing *M. tuberculosis* served to render the bacteria nonviable, while still preserving the integrity of genomic DNA in a state suitable for testing by polymerase chain reaction (PCR) techniques. In their studies, 0.25 mL of sputum sediments were diluted with 0.583 mL of 100% ethanol to bring the final ethanol concentration to 70%. They recommend storage of the ethanol-treated specimens at 4°C if PCR testing is to be delayed. Zwadyk and

coworkers[381] reviewed several methods for rendering specimens safe for further testing, and introduced the technique of heating the samples at 100°C for 30 minutes in a boiling water bath or a forced-air oven, which both killed and lysed the mycobacteria, releasing short DNA fragments suitable for amplification.

TABLE 19-1 Agents Commonly Used for Decontamination and Concentration of Specimens

Agent	Comments
N-Acetyl-L-cysteine plus 2% NaOH	Mild decontamination solution with mucolytic agent NALC to free mycobacteria entrapped in mucus. Limit exposure to NaOH to 15 minutes.
Dithiothreitol plus 2%NaOH[a]	Very effective mucolytic agent used with 2% NaOH. Trade name of dithiothreitol is Sputolysin. Reagent is more expensive than NALC. Limit exposure to NaOH to 15 minutes.
Trisodium phosphate, 13% plus benzalkonium chloride (Zephiran)	Preferred by laboratories that cannot carefully control time of exposure to decontamination solution. Specimens treated with TSP–Zephiran should be inoculated onto an egg-based culture medium to neutralize the growth inhibition of the Zephiran. If agar-based media are used, neutralization of the Zephiran can be accomplished by adding lecithin.

NaOH, 4%	Traditional decontamination and concentration solution. Time of exposure must be carefully controlled to no more than 15 minutes. NaOH, 4%, effects mucolytic action to promote concentration by centrifugation.
Trisodium phosphate, 13%	Can be used for decontamination of specimens when exposure time can be completely controlled. Not as effective as TSP–Zephiran mixture.
Oxalic acid, 5%	Most useful in processing specimens that contain *Pseudomonas aeruginosa* as a contaminant.
Cetylpyridium chloride, 1%, plus 2% NaCl[b]	Effective as a decontamination solution for sputum specimens mailed from outpatient clinics. Tubercle bacilli have survived 8-day transit without significant loss.

[a] See reference 292.
[b] See reference 304.

Centrifugation

Rickman and Moyer have called attention to the importance of carefully controlling centrifugal force in the recovery of mycobacteria from clinical specimens, particularly in the correlation of positive AFB smears with positive mycobacterial cultures.[266] The focus of their study was to understand the unique physical characteristics conferred on mycobacteria by the high lipid content of the cell wall (up to 30% dry weight). The lipid has the effect of making the specific gravity of the organism very low. If the organism is to be maximally sedimented during the centrifugation of the specimen, the specific gravity of the suspending fluid should be kept as low as possible, and the centrifugal force applied to the specimen should be as high as practical. Improved recovery of mycobacteria by culture occurred as the relative centrifugal force (RCF) was increased from 1,260 to 3,000 *g* (Table 19-2).[266,381] When the RCF was increased to 3,800 *g*, a twofold increase in the correlation of positive smears to positive cultures from 40% to 82% was realized, or more than a threefold increase from the correlation when the RCF was only 1,260 *g*.

Subsequent evaluation for recovery of mycobacteria from sputum samples seeded with known concentrations of organisms following centrifugation has confirmed that the recovery rate increases with centrifugation time and speed.[261] However, the recovery rates in these experimental samples were not significantly lower when using

an RCF of 2,074 *g* for 20 minutes (67% to 71%) compared with the recovery rates at RCFs of 3,005 *g* and 3,895 *g* for 15 minutes (76% to 80%). Acid-fast smear sensitivity for 25,000 specimens processed with an RCF of 3,800 *g* for 20 minutes was 71%. However, sensitivity was still 69%, as determined for an additional 30,000 specimens processed in a similar manner but with an RCF of 2,000 *g*. The authors conclude that the actual rates of recovery of viable mycobacteria from clinical specimens also depends on the method of treatment and individual species and strain differences of mycobacteria for alkali tolerance. Thus, although centrifugation at an RCF of 3,000 *g* for 15 minutes may be optimal for the recovery of mycobacteria from clinical specimens, the chances for recovery will not be substantially compromised in laboratories where lower cost centrifuges attaining only a maximum of 2,074 *g* are available, if the time is increased to 20 minutes. With the centrifugation forces produced at RCFs of 3,000 *g* or higher, glass or plastic centrifuge tubes may collapse and must be placed in sealed cups. Also, considerable heat is generated at high speeds, and refrigerated centrifuges may be required when RCFs exceed 3,000 *g*.

TABLE 19-2 Effect of Increasing Centrifugal Force on Results of Smears and Cultures for Mycobacteria

	Relative Centrifugal Force (g)		
	1260	3000	3800
Positive smears	1.8%	4.5%	9.6%
Positive cultures	7.1%	11.2%	11.6%
Correlation of positive smears/cultures	25%	40%	82%

Adapted from reference 266.

Centrifuges used for the processing of specimens potentially containing mycobacteria should be equipped with 50- or 250-mL centrifuge cups with aerosol-free tops that can be adapted to hold 50-mL centrifuge tubes. Such centrifuges should

not be vented to the outdoors because of the danger of reverse airflow through the vent during windstorms. Precautions should be taken to prevent the spontaneous rupture of fluid surface tension membranes when inoculating broth cultures or liquid specimens. If such a membrane in an inoculation loop breaks, for example, droplet aerosols may be created that can persist in the air for long periods. All such transfers must be performed in a biologic safety hood.

Bone Marrow, Tissue, and Body Fluid Specimens

Bone marrow and tissue biopsies are usually not contaminated with other microorganisms, so they can be homogenized and inoculated directly onto culture media without the use of a decontaminating solution. Draining sinuses or other cutaneous lesions suspected of harboring mycobacteria are best cultured by obtaining a small portion of infected tissue or drainage. Fine-needle biopsies are also being used with increased frequency to obtain material for tissue culture from a variety of subcutaneous and deep visceral lesions. In a study of 390 patients in which needle biopsy specimens were obtained to diagnose suspected tuberculous lymphadenitis, the overall rate of smear positivity was 23.6%, and culture positivity was 35%.[257] Caseating lesions were more likely to produce positive cultures (40%) than noncaseating lesions (9%).

Liquid specimens (e.g., cerebrospinal fluid, pleural fluid, peritoneal fluid, etc.) should be centrifuged, stained for AFB, and inoculated directly to broth and solid culture media. Pleural fluid specimens may be diluted with buffer to lower the specific gravity, thereby improving sedimentation of mycobacteria.

Staining of Acid-Fast Bacilli

The cell walls of mycobacteria, because of their high lipid content, have the unique capability of binding fuchsin dye so that it is not removed (destained) by acid–alcohol. This acid-fast staining reaction of mycobacteria, along with their characteristic size and shape, is a valuable aid in the early detection of infection and in the monitoring of therapy for mycobacterial disease. The presence of AFB in sputum, combined with a history of chronic cough and weight loss plus consistent chest radiographic findings (e.g., upper lobe

infiltrate with or without cavity formation), should be considered TB until proven otherwise.

It has been estimated that when using standard concentrating techniques, approximately 10,000 AFB per milliliter of sputum are required to be detected by

routine microscopy. Patients with extensive disease shed large numbers of mycobacteria, with a good correlation between a positive AFB smear and a positive mycobacterial culture. Many patients have minimal or less-advanced disease, and the correlation of positive smears to positive cultures in this group may be only 25% to 40%.

Smears stained for AFB are also useful in following response to treatment. After antimycobacterial drugs are started, cultures may become negative before the smears, suggesting that the organisms are not viable but still are capable of binding the stain. With continued treatment, more organisms are killed and fewer shed, so assessing the number of organisms in the sputum during treatment can provide an early objective measure of response. Should the number of organisms fail to decrease after therapy is started, the possibility of drug resistance must be considered, and additional specimens for culture and susceptibility studies should be obtained.

Two types of acid-fast stains are commonly used (Table 19-3):

1. Fluorochrome stain: auramine O, with or without a second fluorochrome, rhodamine
2. Carbolfuchsin stains: a mixture of fuchsin with phenol (carbolic acid)
   a. Ziehl–Neelsen (hot stain)
   b. Kinyoun (cold stain)

The carbolfuchsin and auramine O dyes used in these techniques each function by binding to mycolic acids in the mycobacterial cell wall. Smears stained with the carbolfuchsin technique must be scanned with an oil-immersion objective. This limits the total area of a slide that can be viewed in a given unit of time. In contrast to the carbolfuchsin stains, smears stained by the auramine procedure can be scanned with a 25× objective, thereby increasing the field of view and reducing the time needed to scan a given area of the slide. Fluorochrome-stained smears require a strong light source—either a 1200-W mercury vapor burner or a strong blue light with a fluorescein isothiocyanate (FITC) filter.

Fluorochrome-stained bacteria are bright yellow or orange-red against a dark background, allowing the slide to be scanned under lower magnification without losing sensitivity. The sharp contrast between the brightly colored mycobacteria and the dark background offers a distinct advantage in scanning the slide (Image Plate 19-1A). Modifications of the auramine fluorochrome stain include the addition of rhodamine, giving a golden appearance to the cells, or the use of acridine orange as a counterstain, resulting in a red to orange background. False-positive reactions may be due to

fluorescence of nonspecific tissue or cellular debris that can be mistaken for bacilli

with the 25× objective. The 40× objective should be used to confirm any suspicious forms. Dead mycobacterial cells will also stain with rhodamine and auramine, leading to a smear-positive, culture-negative situation. This feature is also important to remember when using acid-fast smears to assess treatment efficacy—the presence of AFB in fluorochrome-stained smears does not necessarily indicate treatment failure. Fluorochrome-stained smears can be stained subsequently with carbolfuchsin; the opposite situation does not apply.

TABLE 19-3 Acid-Fast-Staining Procedure

Ziehl–Neelsen Procedure	Kinyoun Cold Procedure	Auramine Fluorochrome Procedure
Carbolfuchsin: Dissolve 3 g of basic fuchsin in 10 mL of 90%–95%ethanol. Add 90 mL of 5% aqueous solution of phenol.	**Carbolfuchsin:** Dissolve 4 g of basic fuchsin in 20 mL of 90%–95% ethanol and then add 100 mL of a 9% aqueous solution of phenol (9 g of phenol dissolved in 100 mL of distilled water).	**Phenolic auramine:** Dissolve 0.1 g of auramine O in 10 mL of 90%–95% ethanol and then add to a solution of 3 g of phenol in 87 mL of distilled water. Store the stain in a brown bottle.
Acid–alcohol: Add 3 mL of concentrated HCl *slowly* to 97 mL of 90%–95% ethanol, in this order. Solution may get hot!	**Acid–alcohol:** Add 3 mL of concentrated HCl *slowly* to 97 mL of 90%–95% ethanol, in this order. Solution may get hot!	**Acid–alcohol:** Add 0.5 mL of concentrated HCl to 100 mL of 79%alcohol.
Methylene blue counterstain: Dissolve 0.3 g of methylene blue chloride in 100 mL of distilled water.	**Methylene blue counterstain:** Dissolve 0.3 g of methylene blue chloride in 100 mL of distilled water.	**Potassium permanganate:** Dissolve 0.5 g potassium permanganate in 100 mL of distilled water.
Procedure Cover a heat-fixed, dried smear with a small rectangle (2 × 3 cm) of filter paper. Apply 5–7 drops of		

carbolfuchsin stain to thoroughly moistened filter paper.
Heat the stain-covered slide to steaming, but do not allow to dry. Heating may be done by gas burner or over an electric staining rack.
Remove paper with forceps, rinse slide with water, and drain.
Decolorize with acid–alcohol until no more stain appears in the washing (2 minutes).
Counterstain with methylene blue (1–2 minutes).
Rinse, drain, and air dry (1–2 minutes).
Examine with 100× oil-immersion objective. Mycobacteria are stained red and the background is light blue.

Procedure
Cover a heat-fixed, dried smear with a small rectangle (2 × 3 cm) of filter paper.
Apply 5–7 drops of carbolfuchsin stain to thoroughly moisten filter paper. Allow to stand for 5 minutes. Add more stain if paper dries. Do not steam!
Remove paper with forceps, rinse slide with water, and drain.
Decolorize with acid–alcohol until no more stain appears in the washing (2 minutes).
Counterstain with methylene blue (1–2 minutes).
Rinse, drain, and air dry (1–2 minutes).
Examine with 100× oil-immersion objective. Mycobacteria are stained red and the background is light blue.

Procedure
Cover a heat-fixed, dried smear with carbol auramine and allow to stain for 15 minutes. Do not heat or cover with filter paper.
Rinse with water and drain.
Decolorize with acid–alcohol (2 minutes).
Rinse with water and drain.
Flood smear with potassium permanganate for at least 2 and not more than 4 minutes.
Rinse with tap water. Drain.
Examine with 25× objective using a mercury vapor burner and BG-12 filter or a strong blue light.
Mycobacteria are stained yellow-orange against a dark background.

With carbolfuchsin, the AFB stain bright red against either a blue or a green background, depending on the counterstain used (Image Plate 19-1B). Although the Ziehl–Neelsen and the Kinyoun techniques are theoretically the same, it has been the experience of some that the former is more sensitive in detecting lightly staining organisms, particularly some of the rapidly growing mycobacteria. The property of acid-fastness is due to the thick, waxy capsule that surrounds the mycobacterial cells. For aqueous carbolfuchsin to penetrate through the wax, the capsule must be "softened." This is done with heat in the Ziehl–Neelsen procedure, much like the melting of a paraffin film in the hot rays of the sun. Dye that penetrates the heat-softened cell wall binds to the mycolic acids; then, when the bacteria cells cool after the heat is removed, the wax again hardens, protecting the bound dye from the action of the acid–alcohol decolorizer ("acid-fast"). In the Kinyoun, or "cold" technique, a

surface-active agent is used to increase permeability of the dye through the waxy capsule; however, the re-formation of the waxy film may be incomplete, allowing most, if not all, of the bound dye to be extracted by the acid–alcohol decolorizer. Obviously, mycobacterial cells that are endowed with a thin waxy capsule will be more susceptible to decolorization, as may be the situation with many rapidly growing strains. The carbolfuchsin-based procedure is described in Chart 19-2.

One potential drawback of the fluorochrome stains is lower sensitivity for detection of rapid growers. Uribe-Botero et al.,[330] who used Truant's auramine–rhodamine staining of routinely processed bone marrow aspirates and biopsies, describe considerable success in the detection of mycobacteria with this method. In this study, 51 bone marrow specimens from 47 HIV-positive patients were studied and mycobacteria were detected in 72%. If the fluorescent stain was positive, the positive predictive value for culturing mycobacteria was 87%. McCarter and Robinson studied 782 primary sputum smears evaluated blindly by the rhodamine–auramine method both at room temperature and at 37°C, and found that the preparation stained at room temperature detected only 85.7% of the positive smears; 43.3% of the smears had more AFB in the 37°C stained smears, compared with only 13.3% more AFB in the room temperature–stained smears.[199] They concluded that staining at 37°C with this method enhances the detection of AFB. Regarding smears made from cytocentrifugation versus traditional NALC–NaOH concentration, Woods and coworkers[369] found no increase in the detection of AFB in 844 auramine O–stained cytocentrifuged sputum smears when compared with the traditional NALC–NaOH concentration method.

The fluorochrome stain offers the advantage of greater sensitivity compared with the carbolfuchsin method, since a significantly larger area of the smear can be scanned per unit of time with the fluorochrome stain. Some workers use the fluorochrome method for scanning purposes and then confirm their findings by reexamining the preparation after destaining and restaining with the carbolfuchsin method, but as previously stated, this is not required. The introduction of relatively low-cost blue light illuminators has made fluorescence microscopy available to clinical laboratories where the detection and recovery of mycobacteria is offered as a service.[250] In laboratories where expensive illuminators may not be affordable, use of the ultraviolet (UV) ParaLens adapter (Becton Dickinson, Sparks, MD), a lightweight, portable, inexpensive epi-illuminator, offers an alternative possibility. Patterson and associates[232] found a comparable sensitivity and specificity in the detection of fluorescing AFB using the UV ParaLens adapter when compared with Kinyoun-stained smears.

The recommendations of the American Lung Association for reporting mycobacteria seen on acid-fast-stained smears, listed in Table 19-4, are followed by many laboratories to

provide consistency of observations between technologists in a given laboratory and uniformity of reporting from one laboratory to another. A summary of the carbolfuchsin-based and fluorescent techniques for the staining of mycobacteria can be found in Charts 19-2 and 19-3.

TABLE 19-4 Method for Reporting Numbers of Acid-Fast Bacilli Observed in Stained Smears[a]

Number of Bacilli Observed	CDC Method	Report
0	Negative	–
1–2/300 fields	Number seen[b]	±
1–9/100 fields	Average no./100 fields	1+
1–9/10 fields	Average no./10 fields	2+
1–9/field	Average no./field	3+
More than 9/field	More than 9/field	4+

[a] Examination at 800× to 1,000× is assumed. Magnifications less than 800× should be clearly stated. If a microscopist uses a consistent procedure for smear examination, relative comparisons of multiple specimens should be easy for the clinician, regardless of the magnification used. To equate numbers of bacilli observed at less than 800× with those seen under an oil-immersion objective, adjust counts as follows: for magnifications about 650×, divide count by 2; near 450×, divide by 4; near 250×, divide by 10; e.g., if 8 bacilli per 10 fields were seen at 450×, the count at 1,000× would be equivalent to about 2/10 fields (8 ÷ 4).

[b] Counts less than 3/3,000 fields at 800× to 1,000× are not considered positive; another specimen (or repeat smear of same specimen) should be processed if available.

Adapted from the American Thoracic Society. Diagnostic standards and classification of tuberculosis and other mycobacterial diseases. *Am Rev Respir Dis* 1981;123:343–358.

Mycobacterial Culture of Specimens Using Solid

Media

The recovery of mycobacteria from agar culture media was poor when the first attempts were made late in the 19th century. Through experimentation it was found that a culture medium containing whole eggs, potato flour, glycerol, and salts solidified by heating to 85°C to 90°C for 30 to 45 minutes was effective in isolating *M. tuberculosis*. The process of solidifying protein-containing medium by heat is known as inspissation. An inspissated culture medium is more subject to liquefaction from the effects of proteolytic enzymes produced by contaminating bacteria than a medium solidified by the addition of agar. However, it was soon discovered that the use of aniline dyes, such as malachite green or crystal violet, in the inspissated medium helped control contaminating bacteria. The concentration of dye must be carefully controlled; if the concentration is too high, the growth of mycobacteria may also be inhibited along with the contaminating bacteria. Malachite green is the dye most commonly incorporated into nonselective culture media, in varying concentrations (Table 19-5).

Egg-Based Media

Egg-based culture media for the recovery of mycobacteria are listed in Table 19-5. LJ medium is commonly used in many clinical diagnostic laboratories; it is less inhibitory to the growth of mycobacteria than is Petragnani medium, which is used primarily to recover mycobacteria from specimens heavily contaminated with bacteria. Conversely, the American Thoracic Society (ATS) medium, which contains only 0.02 g/100 mL of malachite green, is less inhibitory to the growth of mycobacteria.

Agar-Based Media

During the 1950s, Cohen and Middlebrook developed a series of defined culture media for use in both research and clinical laboratories. These media were prepared from defined salts and organic chemicals; some contained agar, but all were found to require the addition of albumin for optimal growth of mycobacteria. The Middlebrook media that contain agar are transparent and in general allow earlier detection of mycobacterial growth compared with other media. This is partly due to the inclusion of biotin and catalase to stimulate revival of damaged bacilli in clinical specimens. Albumin is also incorporated to bind toxic amounts of oleate and other compounds that might be released from spontaneous hydrolysis of Tween 80. The albumin does not appear to be metabolized by the bacilli.

Middlebrook culture media used today include 7H9 broth and 7H10 and 7H11 agar media, which are used for isolation and susceptibility testing.[367] 7H11 differs from 7H10 only in containing 0.1% casein hydrolysate, an additive that improves the rate

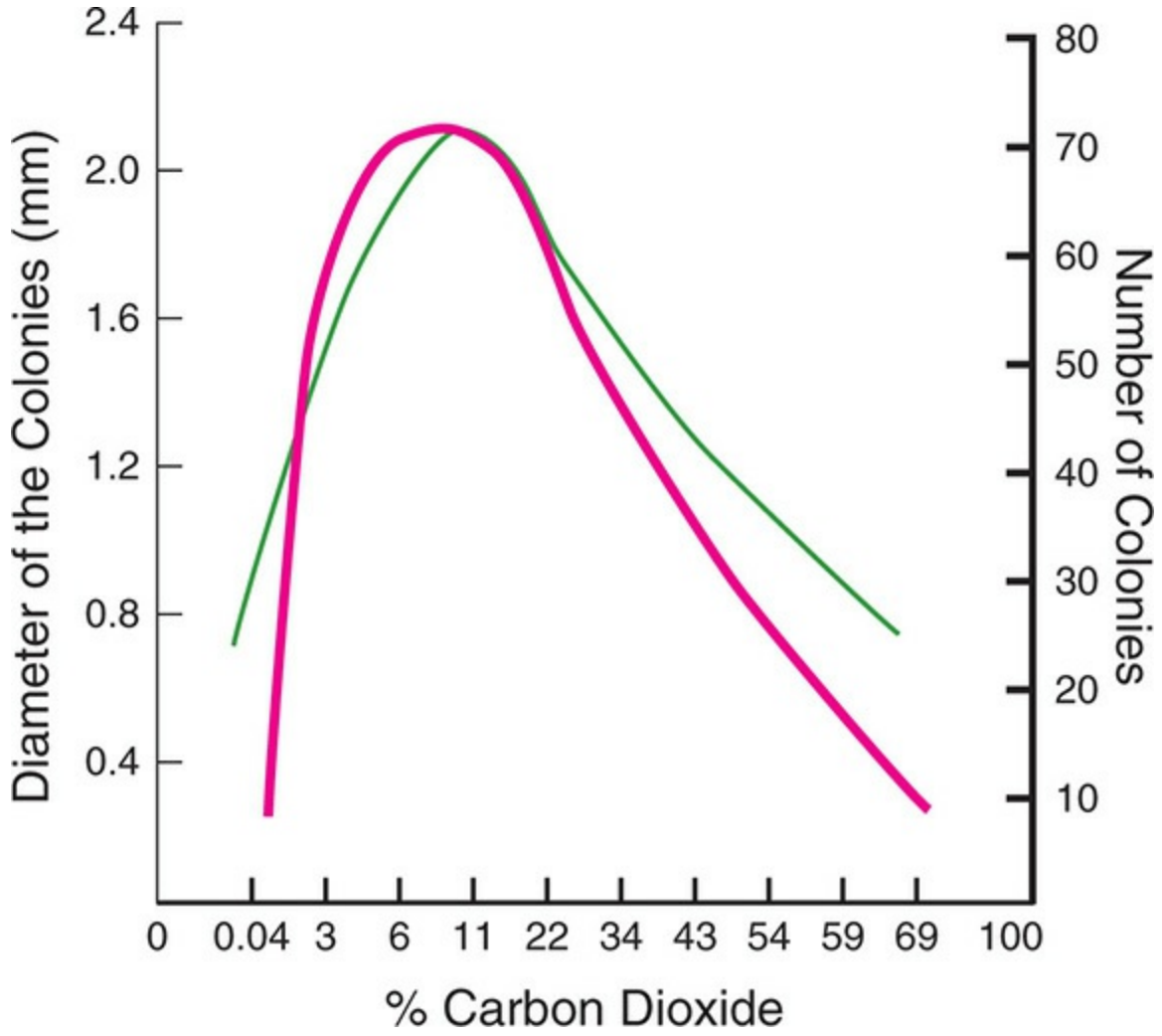
and amount of growth of mycobacteria resistant to isoniazid (INH).[54] Both 7H10 and 7H11 contain malachite green but at one-tenth the quantity usually used in egg-based media, explaining in part the higher incidence of contamination than on egg-based media.

One other advantage of the use of Middlebrook agar is that experienced mycobacteriologists can often make a presumptive identification of *M. tuberculosis* and other groups of mycobacteria within 10 days by examining early microcolonies on the agar and observing certain well-defined morphologic features.[277] Photomicrographs of representative microcolonies are illustrated in Image Plate 19-1C (*M. tuberculosis*) and Image Plate 19-2A and 2B (*M. avium* complex).

Although essentially all culture media yield more growth and larger colonies of mycobacteria when incubated in 5% to 10% CO_2, the Middlebrook media absolutely require capneic incubation for proper performance (Fig. 19-1). Exposure of 7H10 and 7H11 to strong light, or storage of the media at 4°C for more than 4 weeks may result in deterioration and release of formaldehyde, a chemical very inhibitory to mycobacteria.[211]

TABLE 19-5 Nonselective Mycobacterial Isolation Media

Medium	Components	Inhibitory Agent	Medium	Components	Inhibitory Agent
Löwenstein–Jensen	Coagulated whole eggs, defined salts, glycerol, potato flour	Malachite green, 0.0025 g/100 mL	Middlebrook 7H10	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, dextrose	Malachite green, 0.025 g/100 mL
Petragnani	Coagulated whole eggs, egg yolks, whole milk, potato, potato flour, glycerol	Malachite green, 0.052 g/100 mL	Middlebrook 7H11	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, 0.1% casein hydrolysate	Malachite green, 0.0025 g/100 mL
American Thoracic Society medium	Coagulated fresh egg yolks, potato flour, glycerol	Malachite green, 0.02 g/100 mL			



■ **FIGURE 19-1** Effect of CO_2 on the growth (colony size and number of colonies) of *M. tuberculosis* after primary isolation from sputum. (Redrawn from David HL. Bacteriology of the mycobacterioses. DHEW Publication No. [CDC] 76-8316, Atlanta, GA: Centers for Disease Control and Prevention, Mycobacteriology Branch, 1976.)

Selective Media

Culture media containing antimicrobial agents are used to suppress bacterial and fungal contamination, and their use can improve recovery of mycobacteria. Although certain antimicrobial agents are known to reduce contamination, they may also inhibit the growth of mycobacteria. Therefore, the times of exposure must be carefully controlled. Table 19-6 lists the names and components of several selective media.

The selective medium described by Gruft consists of LJ medium with penicillin, nalidixic acid, and RNA.[110] Petran subsequently described a selective medium containing cycloheximide, lincomycin and nalidixic acid to control fungal and bacterial

contaminants.[238] By varying the concentrations of these agents, the medium can be prepared with either LJ or 7H10 base.

Selective 7H11 is a modification of an oleic acid agar medium first described by Mitchison.[213] The medium was originally designed for use with sputum specimens without the use of a decontaminating agent. Mitchison's medium contains carbenicillin, polymyxin, trimethoprim lactate, and amphotericin B. McClatchy suggested reducing the concentration of carbenicillin from 100 mg/mL to 50 mg/mL and using 7H11 medium instead of oleic acid agar.[200] He called this modification selective 7H11, or S7H11. With the use of S7H11 medium with LJ and 7H11, recovery of mycobacteria is improved, particularly when the S7H11 medium is used with the NALC–1% NaOH decontamination procedure.

Incubation Temperature

Different species of mycobacteria show dependence on the temperature of incubation for optimal growth. Species having a propensity for infecting skin (such as the rapidly growing mycobacteria, *M. marinum*, *M. ulcerans,* and *M. haemophilum*) grow best at the temperature of the skin (30°C to 32°C). *M. tuberculosis* grows best at 37°C and poorly or not at all at 30°C or 42°C to 45°C (the body temperature of birds). *Mycobacterium xenopi*, a species not commonly found as a cause of infection in humans, grows best at 42°C and has been implicated as an environmental contaminant in the hot water system of a large hospital.[109]

Optimal recovery of different mycobacterial species depends on the incubation of at least part of the concentrated specimen at a temperature most likely to promote growth of that species. An incubator set at 30°C to 32°C should be used for all specimens from skin or subcutaneous lesions. If a 30°C to 32°C incubator is not available, the specimens can be held in a temperature-monitored, closed box placed in a sheltered area away from warm or cool drafts. The temperature in the box should be between 24°C and 25°C and recorded daily. An incubator maintained at 42°C can be helpful in recovering *M. xenopi*, although this organism will grow at 37°C.

Mycobacteria grow best in an atmosphere of 3% to 11% CO_2. Use of CO_2 is mandatory if Middlebrook 7H11 medium is used. However, if lack of incubator space to maintain cultures is a problem, cultures can be removed from the CO_2 atmosphere after 7 to 10 days, for organisms will be in a log

phase of growth and are less dependent on CO_2. For reasons that are not well understood, mycobacteria do not grow well in candle extinction jars. The CO_2 concentration in incubators should be monitored daily and a record kept of both the

incubator temperature and the CO_2 level.

TABLE 19-6 Selective Mycobacterial Isolation Media

Medium	Components	Inhibitory Agent	Medium	Components	Inhibitory Agent
Gruft modification of Löwenstein–Jensen	Coagulated whole eggs, defined salts, glycerol, potato flour, RNA, 5 mg/100 mL	Malachite green, 0.025 g/100 mL Penicillin, 50 U/mL Nalidixic acid, 35 mg/mL	Middlebrook 7H10	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose	Malachite green, 0.0025 g/100 mL Cycloheximide, 360 μg/mL Lincomycin, 2 μg/mL Nalidixic acid, 20 μg/mL
Löwenstein–Jensen	Coagulated whole eggs, defined salts, glycerol, potato flour	Malachite green, 0.025 g/100 mL Cycloheximide, 400 μg/mL Lincomycin, 2 μg/mL Nalidixic acid, 35 μg/mL	Selective 7H11 (Mitchison's medium)	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose, casein hydrolysate	Carbenicillin, 50 μg/mL Amphotericin B, 10 μg/mL Polymyxin B, 200 U/mL Trimethoprim lactate, 20 μg/mL

Rapid Methods for Establishing a Diagnosis

Smears for Acid-Fast Bacilli

One of the more practical and readily available methods to shorten the time to diagnosis of TB is to increase the RCF applied to clinical specimens to improve the sedimentation of the lipid-laden mycobacterial cells.[266] The improved correlation of positive smears to positive cultures has made the examination of a smear stained for AFB somewhat more reliable as an index of mycobacterial infection. Lipsky, in a review of factors affecting the clinical value of microscopy for AFB, concluded that when the results of all specimens from each patient are considered in total, the acid-fast smear has a high predictive value and remains one of the most rapidly performed tests in the detection of mycobacterial infections.[188]

Broth Culture Systems

For optimal recovery of mycobacteria, inoculation of both a broth system and a solid medium is recommended.[317] Broth systems are more sensitive than solid media, and they allow for more rapid growth. The first broth-based system for growth and detection of mycobacteria was the semiautomated radiometric BACTEC TB460. Many studies have shown that compared to conventional solid culture media, the BACTEC TB460 is more sensitive (i.e., results in recovery of more mycobacteria) and decreases time to detection of growth by several days.[13,35,65,66,82,216,268,290] The BACTEC TB460 also could differentiate MTBC from nontuberculous mycobacteria (NTM) using *p*-nitro-α-acetylamino-β-hydroxy-propiophenone, which inhibits growth

of MTBC organisms.[108] Disadvantages of this system are the use of needles, with the potential to cause cross-contamination,[58,219] and the need to dispose of radioactive material. Because the manufacturer no longer supports the BACTEC TB460, it is not discussed further. Virtually all laboratories that used the BACTEC TB460 have replaced it with one of the fully automated, nonradiometric broth systems described below.

Mycobacteria Growth Indicator Tube Systems. The Mycobacteria Growth Indicator Tube (MGIT) (BD Diagnostic) is a round-bottomed glass tube that contains modified Middlebrook 7H9 broth base. A fluorescent compound is embedded in silicone on the bottom of the tube. The fluorescent compound is sensitive to dissolved oxygen in the broth; that is, the presence of oxygen in the uninoculated medium serves to quench the emission of fluorescent light. As the actively growing mycobacteria consume the dissolved oxygen, the fluorescence is unmasked and can be detected manually by observing the tube under long-wave UV light (Wood's lamp). Growth may also be detected by observing a nonhomogeneous turbidity or small grains or flakes in the culture medium.

The MGIT system can be used to culture all specimen types except blood and urine. For optimal performance, OADC (oleic acid, albumin, dextrose, and catalase) growth supplement and an antibiotic mixture (PANTA: polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) are added to the broth. The antibiotic mixture inhibits the growth of contaminating bacteria. The OADC supplement provides oleic acid, an important metabolic stimulant for mycobacteria; albumin, to bind toxic free fatty acids; dextrose, as an energy source; and catalase, to destroy toxic peroxides that may be present in the medium. After adding the Supplement/PANTA mixture, the tube is inoculated with 0.5 mL of specimen or specimen concentrate; adding more than 0.5 mL of specimen may adversely affect the performance of the tube. The cap is replaced, and the ingredients are mixed by inverting the tube several times. For manual reading, tubes are placed in a 37°C incubator, and are read every other day starting on the second day after inoculation. Tubes are read with a Wood's lamp, placing the tube with the test mixture between a positive (sodium sulfite solution) and a negative (uninoculated MGIT tube) control. UV protective goggles are worn while looking for a bright orange color in the bottom of the positive tubes; an orange reflection is also seen at the meniscus. Positive tubes are stained for AFB, preferably using a carbol fuchsin method. Negative tubes are returned to the incubation rack and again observed at regular intervals for up to 6 weeks.

Hanna and coworkers[117] used the MGIT system to test 193 specimens (44 patients) from various body sites for the presence of *Mycobacterium* species. Sputum concentrations from 32 patients from whom at least one positive acid-fast smear was seen were studied. The MGIT cultures were positive in 31 (MTBC, 25; MAC, 4; and

M. haemophilum, 2) patients. MGIT tubes were positive for an additional three smear-negative patients; the one false-negative MGIT test occurred in a patient with very few AFB seen on smear. The mean time to detection was 10.4 days (range, 4 to 26). The two *M. haemophilum* isolates were recovered by adding an X (hemin) disk to the MGIT tube and incubating at 30°C.

Rather than read the tubes manually, tubes may be placed in an instrument, either the MGIT 960 or 320. Both instruments are nonradiometric, automated systems that use the MGIT tubes and sensors to detect the fluorescence that is visually interpreted in the manual version described above. The system holds 960 (or 320) plastic tubes, which are continuously monitored. The performance characteristics have been shown in several studies to be comparable to those of the BACTEC TB460 and/or other automated systems.[64,117,118,130,183,230,248,272,323,354,355]

MB/BacT ALERT Mycobacteria Detection System. The MB/BacT ALERT is an automated system to detect growth of mycobacteria, similar in design to the BacT/Alert blood culture system. The MB/BacT bottles contain enhanced Middlebrook 7H9 broth in an atmosphere of CO_2, nitrogen and oxygen under vacuum. This bottle therefore provides suitable nutritional and environmental conditions to recover the more commonly encountered *Mycobacterium* species from clinical specimens other than blood. An antibiotic

supplement, containing amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim and proprietary growth factors, is added to each bottle just before use to enhance the growth of *Mycobacterium* species and curtail the growth of contaminating bacteria that may survive the decontamination and concentration procedure. The bottom of each broth bottle is fitted with a gas-permeable sensor that changes from dark green to bright yellow when CO_2 is produced in the broth by metabolizing mycobacteria. Bottles are placed bottom down within individual cells in the incubation chamber, and reflected light is used to continuously monitor the production of microbial-generated CO_2.

As with the MGIT 960, the performance characteristics of the MB/BacT ALERT are superior to solid media alone[98] and comparable to the BACTEC TB460 and/or other fully automated growth and detection systems.[65,230,246]

VersaTREK (Formerly ESP Culture System II). The VersaTREK System (Thermo Scientific) is an adaptation of their blood culture system. Each culture bottle, when placed in a special drawer in the incubation module, is attached to a sensor, consisting of a plastic housing, a recessed needle, and a hydrophobic membrane. Thus, each

bottle is continuously monitored (every 24 minutes) for any change in gas pressure due to the metabolic activity of microorganisms in the culture bottle. Significant pressure change is signaled based on the rate of consumption of oxygen within the head space of the culture bottle by the metabolizing microorganisms.

The bottles contain a liquid culture medium (VersaTREK Myco Broth), a growth supplement, an antibiotic supplement and cellulose sponges. The sponges provide a growth platform for the mycobacteria, simulating the alveoli of the lungs.

Early studies showed that the ESP MYCO System was an acceptable option for growth and detection of mycobacteria from all specimen types, including blood.[319,368] Later, Williams-Bouyer et al.[359] compared the ESP Culture System II with the BACTEC MGIT 960 and Middlebrook 7H11 and 7H11 selective agar, examining a total of 3,151 specimens. The recovery rates for all mycobacteria were 71.2% for the ESP II, 63.9% for the MGIT 960, and 61.8% for the Middlebrook media. However, with the exclusion of *M. gordonae*, the recovery rates for the remaining mycobacteria were 70.2%, 72.6%, and 66.3%, respectively. These differences were not statistically significant.

For isolates recovered in both automated systems, the mean times to detection of all mycobacteria and members of the *M. tuberculosis* complex, respectively, were 15.8 and 17.4 days for ESP II and 12.5 and 11.9 days for BACTEC MGIT 960 ($P < 0.05$). False-positive signals occurred less frequently with the BACTEC MGIT 960 cultures (23 [0.7%]) compared with the ESP II cultures (84 [2.7%]) ($p < 0.01$).

BACTEC MYCO/F LYTIC. The BACTEC MYCO/F LYTIC blood culture bottle contains a lytic agent to release the mycobacteria that have been phagocytosed by white blood cells. It is incubated and monitored automatically in a manner similar to the other BACTEC blood culture bottles. In addition to growing mycobacteria, the BACTEC MYCO/F LYTIC is a good culture system for bacteria and fungi that may be present in the bloodstream.[93,335] In a direct comparison of many of the commercially available products, Crump et al.[65] evaluated the performance of the BACTEC 13A (BD Diagnostic), BACTEC MYCO/F LYTIC (BD Diagnostic), BacT/ALERT MB (bioMérieux), and ISOLATOR 10 lysis-centrifugation (Wampole Laboratories) systems for detection of mycobacteremia in adults. Of 600 patients tested, 85 (14%) yielded MAC and 9 (2%) yielded other species of mycobacteria. Of 26 complete (three bottles and one tube) and adequately filled (5 ± 1 mL) sets from which MAC was recovered, BACTEC 13A was positive for 19 (73%), BACTEC MYCO/F LYTIC was positive for 21 (81%), BacT/ALERT MB was positive for 22 (85%), and ISOLATOR 10 was positive for 21 (81%). Of the six possible two-way comparisons, the mean times to detection for the recovery of MAC from each bottle in adequately paired sets were 15.3 days for BACTEC 13A versus 12.8 days for MYCO/F LYTIC for 33 of 340 pairs, 14.1 days for BACTEC 13A versus 11.6 days

for BacT/ALERT MB for 38 of 380 pairs, 12.6 days for BACTEC 13A versus 20.0 days for ISOLATOR 10 for 26 of 261 pairs, 12.8 days for BACTEC MYCO/F LYTIC versus 11.0 days for BacT/ALERT MB for 33 of 340 pairs, 13.2 days for BACTEC MYCO/F LYTIC versus 20.4 days for ISOLATOR 10 for 24 of 230 pairs, and 9.9 days for BacT/ALERT MB versus 19.0 days for ISOLATOR 10 for 24 of 257 pairs. There were no significant differences in yields between the systems. However, there were differences in the mean times to detection among the systems. The time to detection was shortest for BacT/ALERT MB, followed by BACTEC MYCO/F LYTIC and BACTEC 13A and then ISOLATOR 10. They found the continuously monitored systems (BACTEC MYCO/F LYTIC and BacT/ALERT MB) to be as sensitive and, on balance, faster for the detection of MAC bacteremia than the standard manual ISOLATOR 10 and radiometric BACTEC 13A systems. Others have also found this method to be superior to traditional methods.[196]

Gas-Liquid and High-Performance Liquid Chromatography

Analysis of cellular long-chain fatty acids by gas-liquid chromatography (GLC) has been used to aid in the characterization of mycobacteria. The pyrolysis gas-liquid method of Reiner, in which long-chain mycolic acids are split into characteristic cleavage products, is largely restricted to research laboratories.[263] Tisdall has developed a GLC procedure for saponifying organisms in methanolic NaOH that permits the correct identification of most mycobacterial species based on chromatogram tracings and colony characteristics.[320] Guerrant and associates[111] have used acid methanolysis for isolating mycolic acid methyl esters from bacterial cells, a method that is not only less time-consuming than saponification techniques but is also more sensitive in detecting small numbers of cells. As little as a 1-mm loopful of organisms can be used, and the total time for analysis is less than 2 hours.

The FDA-cleared commercial system, the MIDI Sherlock Microbial Identification System (Microbial ID, Newark, DE), consisting of an 1100 HPLC and a computer is a rapid and reliable method for the culture confirmation of mycobacteria. The computer system includes a library of 40 cell wall lipid profiles for the most medically important *Mycobacterium*

species, derived from isolates acquired from the American Type Culture Collection (ATCC) and including numerous clinical isolates. The use of negative-ion mass spectrometry to detect tuberculostearic acid in clinical specimens also holds promise for the rapid detection of mycobacteria in such specimens for those equipped with the necessary instruments.[177]

High-performance liquid chromatography utilizing fluorescence detection (HPLC-FL) of mycolic acid 6,7-dimethoxycoumarin esters has been used for the rapid identification of *M. tuberculosis* and *M. avium* directly from fluorochrome-stained, smear-positive sputum specimens and BACTEC 12B cultures with early growth. In a study of 132 sputum specimens positive for *M. tuberculosis* and 48 positive for MAC, HPLC-FL made direct identifications with sensitivities of 56.8% and 33.3%, respectively.[147]When using HPLC-FL to test cultures grown in BACTEC 12B bottles, sensitivities of 99.0% and 94.3% were achieved. The specificity was 100% in both evaluations. Cage also successfully identified to the species level 117 of 126 (93%) mycobacteria grown in BACTEC 12B medium supplemented with oleic acid.[37] Glickman and colleagues[100] describe a computer-based file that contained the patterns of mycolic acids characteristic of 45 species of *Mycobacterium*. By using their system in the evaluation of 1,333 strains representing 24 *Mycobacterium* species, 97% were correctly identified (identification of *M. tuberculosis* was 99.85% accurate; of MAC, 98% accurate). Thus, for those possessing the equipment and using computer-based pattern recognition files, mycobacteria can be identified accurately and rapidly using HPLC-generated chromatographic data. These systems are in direct competition with nucleic acid-based methods for the rapid identification of mycobacteria.

Nucleic Acid Amplification

Nucleic acid amplification tests (NAATs) for direct detection of MTBC in clinical specimens became commercially available in the mid-to-late 1990s.[62] These assays, which are discussed in detail later in this chapter, have added considerable value to mycobacterial testing. Positive results allow earlier diagnosis and treatment of TB, a decreased period of infectiousness and better outcomes; negative results can eliminate unnecessary contact investigations. However, these assays have limitations, so results must be interpreted with caution. Their sensitivity is less than culture, so a negative result does not exclude the diagnosis of TB; false-positive results are possible; and they are an added laboratory cost.

The Centers for Disease Control and Prevention (CDC) has issued the following recommendations on the uses of NAATs for diagnosis of TB. All clinicians and TB control programs should have access to an NAAT for direct detection of MTBC, and these assays should become the standard of practice for patients with suspected TB. An NAAT should be performed on at least one respiratory specimen from each TB suspect, because a single positive result can support the diagnosis of TB, and all specimens with negative results should be tested for inhibitors. The time from specimen collection to NAAT report should be as brief as possible, preferably within 48 hours, and laboratories performing one of these assays should participate in a proficiency testing program.

Identification of Mycobacteria Using Conventional Methods

Although the applications of molecular techniques are currently the standard for the identification of cultured mycobacteria, conventional methods remain useful and are discussed briefly here. Charts 19-1 through 19-15 provide a detailed presentation of the principles, reagents, procedures, and interpretation of various laboratory techniques, including digestion–decontamination methods, and biochemical tests (included in Table 19-7). The use of molecular assays, including nucleic acid probe analysis, is covered separately.

Optimal Temperature for Isolation and Rates of Growth

Each *Mycobacterium* species has an optimal temperature for growth and a range of time for recovery in culture (Table 19-7). The time to recovery varies depending on the type of media used—the average time of recovery of mycobacteria on egg-based media is about 21 days, but ranges from as short as 3 to 5 days to as long as 60 days, depending on the species and the quantity of mycobacteria in the specimen. The time to recovery on solid media is generally shorter by several days when using 7H10 or 7H11 agar if the technique of microscopic observation for microcolonies is used (described below). The use of broth-based culture shortens the times of detection considerably.[66,160,268]

Any standard nonselective culture medium can be used when assessing the time of growth, either in tubed slants or in Petri plates. A well-isolated colony of the test organism is subcultured to 7H9 broth containing Tween 80 and incubated for several days or until the medium is slightly turbid. The broth is diluted 1:100, and isolation streaks are made to the test medium, either in tubed slants or on Petri plates, to obtain isolated colonies. To determine the growth rate accurately, it is necessary to use an inoculum sufficiently diluted to produce individual colonies. An inoculum of large numbers of slow-growing mycobacteria may form a visible colony within a few days and give an erroneous impression of the growth rate. *M. tuberculosis* stock cultures of known growth rates should be used as a control for slow-growing organisms; similarly, stock cultures of *M. fortuitum* can be used as a control comparison for rapidly growing organisms.

Petri plates of 7H10 agar or equivalent are preferred to the use of tubed media because the appearance of developing microcolonies can be studied with a dissecting or low-power microscope. With experience, an assessment of the morphology of these microcolonies can be useful in the exclusion of certain mycobacteria, and potentially the preliminary classification of others. Runyon published guidelines by which the microcolonies of various species of mycobacteria can be distinguished by those who

have gained experience with this approach.[277]

Koneman and associates in the clinical microbiology laboratory at the University of Illinois, Chicago, were able to detect the microcolonies of 29 smear-positive clinical sputum isolates of *Mycobacterium* species growing on 7H10 agar in an average of 6.1 days (range, 3 to 12) compared with average recovery times using standard visual observations of 32.4 days with LJ medium and 27.9 days with 7H11 agar

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(personal communication). The average detection time for these 29 isolates was 5.8 days by the BACTEC system, using radiometric 12B bottles. The results of this study indicate that mycobacteria can be detected by observing for microcolonies in the same shortened time period as that achieved with the BACTEC system. The microcolony method also allows an assessment of colony morphology, providing additional information not provided by the BACTEC system, which is helpful in making an early presumptive characterization of an unknown *Mycobacterium* species. If probes are used for identification, this morphologic data could be used to assist in probe selection. In a study that examined the time to detection, the average time to detection of microcolonies of *M. tuberculosis* was 11 days on Middlebrook 7H11 agar, whereas it was 16 days with the MB/BacT bottles and 19.5 days with the LJ medium.[98]

As noted above, some *Mycobacterium* species, such as *M. marinum* and most of the rapidly growing mycobacteria, grow better at temperatures that are slightly less than core body temperature. This may also be associated with the fact the disease produced by these organisms, particularly *M. marinum*, most commonly affects the extremities, which are slightly cooler than core body temperature. Therefore, laboratories should incubate mycobacterial cultures from specimens that may contain these organisms (e.g., skin biopsies from the extremities) at 30°C to 32°C.

Pigment Production

The determination of whether an unknown *Mycobacterium* species isolate is capable of producing colony pigmentation in the dark (scotochromogen) or only after exposure to light (photochromogen) is not very helpful in making a final species identification, but may be useful in narrowing the possibilities (Chart 19-5). *M. tuberculosis* fails to produce pigment, beyond a light buff color, even after exposure to light. The pigment-producing capabilities of other *Mycobacterium* species are listed in Table 19-7 (Image Plate 19-2C–E).

Niacin Accumulation

All mycobacteria produce niacin; however, only *M. tuberculosis*, *M. simiae,* and

occasional strains of *M. africanum*, *M. bovis*, *M. marinum,* and *M. chelonae* lack the enzyme necessary to further convert the niacin to niacin ribonucleotide. Thus, the determination of whether niacin has accumulated in the culture medium is a valuable differential test in identifying these species of mycobacteria, particularly *M. tuberculosis* (Chart 19-10). Reagent-impregnated filter paper strips have been developed that eliminate the need for using cyanogen bromide, a highly toxic substance, as required in the originally described method for performing this test. The development of a yellow color in the test medium incubated with a reagent strip is indicative of niacin accumulation and a positive test (Image Plate 19-1D). It is essential to have sufficient growth on the primary egg-based medium; otherwise, the risk of obtaining false-negative results is increased.

Reduction of Nitrates to Nitrites

Only a few species of mycobacteria, most notably *M. tuberculosis*, produce nitroreductase, which catalyzes the reduction of nitrate to nitrite. The development of a red color on addition of sulfanilic acid and *N*-naphthyl ethylenediamine to an extract of the isolate to be identified is indicative of the presence of nitrite and a positive test (Chart 19-11). The test must be carefully performed using three control cultures—one known to give a strong positive reaction, one a weak reaction, and the last a negative reaction. In addition to supporting the identification of *M. tuberculosis*, the nitrate reduction test is also a key test in the identification of *M. kansasii* and *M. szulgai* (Table 19-7 and Image Plate 19-1E).

Tween 80 Hydrolysis

Tween 80 is the trade name of a detergent that can be useful in identifying mycobacteria that possess a lipase that splits the compound into oleic acid and polyoxyethylated sorbitol. This test is helpful in identifying *M. kansasii*, which can produce a positive result in as quickly as 3 to 6 hours. Two scotochromogens with similar-appearing colonies, *M. gordonae* (positive) and *M. scrofulaceum* (negative), can be differentiated using the Tween 80 hydrolysis test. The Tween 80 hydrolysis reactions for other mycobacteria are listed in Table 19-7; the test procedure is described in detail in Chart 19-14, and a positive reaction is illustrated in Image Plate 19-2G.

Catalase Activity

Most of the mycobacteria produce catalase; however, not all species are capable of producing a positive reaction after heating the culture at 68°C for 20 minutes (heat-stable catalase). Most strains of *M. tuberculosis* and other members of the MTBC do not produce heat-stable catalase, except for certain INH-resistant strains, for which the

results of this test are of particular value as a possible surrogate marker. Catalase activity is assessed semi-quantitatively by measuring the height achieved by the column of bubbles produced when hydrogen peroxide is added to growing colonies in a tube culture (Chart 19-6). To perform this test, tubes of LJ medium must be poured in an upright position to produce a flat rather than a slanted surface. This surface is heavily inoculated with the test organism and incubated for 14 days before adding the hydrogen peroxide reagent. A column higher than 45 mm is considered a positive test (Image Plate 19-2H). A quick assessment of catalase activity can be determined by placing a few drops of hydrogen peroxide on colonies growing on the surface of Middlebrook 7H10 agar and observing for rapid effervescence of bubbles (Image Plate 19-2I).

Arylsulfatase Activity

Determination of the activity of the enzyme arylsulfatase in mycobacteria is helpful in differentiating the rapidly growing mycobacteria from group III nonphotochromogenic mycobacteria (Image Plate 19-2J). Small quantities of this enzyme can also be produced by *M. marinum*, *M. kansasii*, *M. szulgai,* and *M. xenopi*. These slower-growing species, however, do not produce sufficient enzyme to give a consistently positive reaction. The development of a red color in the test medium, indicating a release of free phenolphthalein, is a positive result (Chart 19-4).

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TABLE 19-7 Differential Characteristics of Mycobacteria

	Optimal Isolation Temperature (Time for Growth)	Pigmentation Growth in		Niacin Test	Nitrate Reduction	Tween 80 Hydrolysis—10 Days
		Light	Dark			
M. tuberculosis	37°C (12–25 days)	Buff	Buff	+	+	V
M. africanum	37°C (31–42 days)	Buff	Buff	V	V	–
M. bovis	37°C (24–40 days)	Buff	Buff	V	–	–
M. ulcerans	32°C (28–60 days)	Buff	Buff	–	–	–
M. kansasii	37°C (10–20 days)	Yellow	Buff	–	+	+
M. marinum	30°C (5–14 days)	Yellow	Buff	V	–	+
M. simiae	37°C (7–14 days)	Yellow	Buff	+	–	+/–
M. asiaticum	37°C (10–21 days)	Yellow	Buff	–	–	+
M. szulgai	37°C (12–25 days)	Yellow to orange	Yellow—37°C Buff—25°C	–	+	V
M. scrofulaceum	37°C (10 days)	Yellow	Yellow	–	–	–
M. gordonae	37°C (10 days)	Yellow to orange	Yellow	–	–	+
M. thermoresistible	45°C (7 days)	Yellow	Yellow	–	+	+
M. flavescens	37°C (7–10 days)	Yellow	Yellow	–	+	+
M. xenopi	42°C (14–28 days)	Yellow	Yellow	–	–	–
M. avium complex	37°C (10–21 days)	Buff to pale yellow	Buff to pale yellow	–	–	–
M. haemophilum	30°C (14–21 days)	Gray	Gray	–	–	–
M. malmoense	37°C (21–28 days)	Buff	Buff	–	–	+
M. shimoidei	37°C (14–28 days)	Buff	Buff	–	–	+
M. genavense	37°C (14–28 days)	Buff	Buff	–	–	+
M. celatum	37°C (14–28 days)	Buff	Buff to yellow	–	–	–
M. gasti	37°C (10–21 days)	Buff	Buff	–	–	+
M. terrae complex	37°C (10–21 days)	Buff	Buff	–	V	+
M. triviale	37°C (10–21 days)	Buff	Buff	–	+	+
M. fortuitum	32°C (3–5 days)	Buff	Buff	–	+	V
M. chelonae	28°C (3–5 days)	Buff	Buff	V	–	V
M. abscessus	32°C (3–5 days)	Buff	Buff	–	–	V
M. smegmatis	32°C (3–5 days)	yellow	yellow	–	+	+

+, positive; –, negative; V, variable; blank spaces, little or no data.

Urease Activity

Assessment of urease activity is an important test to differentiate *M. scrofulaceum* (positive) from *M. gordonae* (negative). It is also useful in differentiating *M. gastri* (positive) from other members of the group III nonchromogenic mycobacteria (Table 19-7). Determination of whether any given *Mycobacterium* species can produce urease can be performed either by inoculating the organism into distilled water containing a urea-base concentrate or by use of filter paper disks containing urea that are added to distilled water. Details of the urease test as applied to the identification of *Mycobacterium* species are presented in Chart 19-15 (Image Plate 19-2K).

Pyrazinamidase

The test for pyrazinamidase is especially useful in distinguishing weakly niacin-positive *M. bovis* from *M. tuberculosis*, although many other mycobacteria are positive (Table 19-7). Pyrazinamidase is an enzyme that deaminates pyrazinamide

Species	OX	CAT	Growth			Acid produced from:					Polysaccharide from sucrose	Reduction of:		DNase	Tributyrin hydrolysis	Habitat
			Selective media, 35°C	Chocolate agar, 22°C	Nutrient agar, 35°C	GLU	MAL	FRU	SUC	LAC		NO_3	NO_2[1]			
N. gonorrhoeae	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	Humans
N. meningitides	+	+	+	–	–	+	+	–	–	–	–	–	V	–	–	Humans
N. lactamica	+	+	+	–	–	+	+	–	–	+	–	–	V	–	–	Humans
N. cinerea	+	+	–[2]	–	–	–	–	–	–	–	–	–	V	–	–	Humans
N. polysaccharea	+	+	V	–	+	+	+	–	–	–	+	–	V	–	–	Humans
N. sicca	+	+	–	+	+	+	+	+	+	–	+	–	+	–	–	Humans
N. subflava																Humans
Bv. *subflava*	+	+	–	+	+	+	+	–	–	–	–	–	+	–	–	Humans
Bv. *flava*	+	+	–	+	+	+	+	+	–	–	–	–	+	–	–	Humans
Bv. *perflava*	+	+	–[3]	+	+	+	+	+	+	–	+	–	+	–	–	Humans
N. mucosa	+	+	–	+	+	+	+	+	+	–	+	+	+	–	–	Humans
N. flavescens	+	+	–	–	+	–	–	–	–	–	+	–	+	–	–	Humans
N. elongata																
Subsp. *elongata*	+	–	–	+	+	–	–	–	–	–	–	–	+	–	–	Humans
Subsp. *glycolytica*	+	+	–	+	+	+[w]	–	–	–	–	–	–	+	–	–	Humans
Subsp. *nitroreducens*	+	–	–	+	+	–/+[w]	–	–	–	–	–	+	+	–	–	Humans
N. bacilliformis	+	V	–	–	–	–	–	–	–	–	–	V	NA	NA	V	Humans
N. weaveri	+	+	–	+	+	–	–	–	–	–	–	–	+	–	–	Dogs
N. animaloris[4]	+	+	–	+	+	+	–	–	–	–	–	+	–	–	NA	Dogs

(PZA) to form pyrazinoic acid, which produces a red band in the culture medium.[345] Details of this test are presented in Chart 19-12, and the end reaction is illustrated in Image Plate 19-2L.

Iron Uptake

The rapidly growing mycobacteria have many similarities. The ability of *M. fortuitum* to take up soluble iron salts from the culture medium and to produce a rusty brown appearance on addition of an aqueous solution of 20% ferric ammonium citrate is a useful characteristic that differentiates this organism from species in the *M. chelonae/M. abscessus* group, which lack this property (Chart 19-9).

Growth Inhibition by Thiophene-2-Carboxylic Acid Hydrazide

Thiophene-2-carboxylic acid hydrazide (T2H) selectively inhibits the growth of *M. bovis*, whereas most other

mycobacteria, including *M. tuberculosis*, can grow in a medium containing this compound. This feature is particularly helpful for differentiating certain strains of *M. bovis* (Chart 19-8 and Image Plate 19-1F). For example, 30% of *M. bovis* BCG (bacille Calmette–Guérin) strains may be weakly niacin-positive, and others may be weak nitrate reducers, at times making differentiation of these strains from *M. tuberculosis* by these key tests somewhat difficult.

Growth in 5% Sodium Chloride

The ability to grow on an egg-based culture medium containing 5% NaCl when incubated at 28°C is a characteristic shared by *M. triviale,* some strains of *M. flavescens, M. fortuitum,* and *M. abscessus* (Table 19-7). Other mycobacteria do not tolerate this increased salt concentration. Slants of LJ medium containing 5% NaCl are commercially available. The test cannot be performed in an agar-based medium (Chart 19-13).[171]

Growth on MacConkey Agar

MacConkey agar, from which the crystal violet has been removed, will support the growth of the rapidly growing mycobacteria. However, most other *Mycobacterium* species cannot grow on this medium. The test procedure is detailed in Chart 19-7.

Classification of Mycobacteria

The recognition that an unknown microorganism is "acid-fast" is usually the first clue that the organism may be a mycobacterium. Acid-fast is a term that is used to describe bacteria that resist decolorization with acidified alcohol once they have been stained with carbolfuchsin. Certain bacterial species, notably *Nocardia* species and *Rhodococcus* species, retain carbolfuchsin only if a less stringent decolorizing agent is used, such as a low-concentration inorganic acid like 1% H_2SO_4 or 1% HCl. These bacteria are termed "partially acid-fast."

In the late 1950s, as species of mycobacteria other than *M. tuberculosis* were being encountered with increasing frequency in medical practices, Runyon proposed the grouping of these "atypical" organisms based on growth rate and pigment production (Box 19-2). The term "atypical," however, really is not appropriate because these organisms are simply different from MTBC. Preferred terms are nontuberculous mycobacteria (NTM) and mycobacteria other than MTBC (MOTT). Advances in our knowledge of the genetics, cell structure, and aberrant phenotypic properties of

previously known and newly discovered species of mycobacteria have advanced our knowledge beyond the neat packaging of species under the classic Runyon system of classification. In addition, certain strains of *M. kansasii*, for example, are either pigmented in the dark or are nonpigmented (qualifying for Runyon group II or group III, respectively). Therefore, a reliance on these phenotypic criteria may be misleading. Therefore, Woods and Washington have suggested a clinically oriented classification of mycobacteria (Box 19-3), refined from an earlier published proposal by Wolinski.[364,370]

BOX 19-2

Runyon's Scheme for Classification of Nontuberculous Mycobacteria

Group I: Photochromogens
Group II: Scotochromogens
Group III: Nonphotochromogens
Group IV: Rapid Growers

Nevertheless, for the traditionally trained mycobacteriologist, separating MTBC from NTM and the preliminary

placing of NTM into 1 of the 4 Runyon groups continues to provide a meaningful initial orientation. The fact that certain strains of the usually photochromogenic *M. kansasii* may be nonpigmented or even scotochromogenic, that some strains of the MAC may be lightly pigmented, and even that *M. szulgai* is scotochromogenic at 37°C but photochromogenic at 25°C (certainly disruptive to the Runyon scheme) does not suggest that past orientations should be totally discarded, but rather that the phenotypic variability of these organisms should be recognized and incorporated into conventional identification schemes.

BOX 19-3

Woods and Washington Classification Scheme, Updated

Species Potentially Pathogenic in Humans
M. avium complex
M. kansasii
Rapid growers: *M. fortuitum* group, *M. chelonae/M. abscessus* group
M. scrofulaceum
M. xenopi
M. szulgai
M. malmoense
M. simiae
M. genavense
M. marinum
M. ulcerans
M. haemophilum
M. celatum
Saprophytic Mycobacteria Rarely Causing Disease in Humans
M. gordonae
M. asiaticum
M. terrae
M. triviale
M. shimoidei
M. gastri
M. nonchromogenicum
M. paratuberculosis
Species With an Intermediate Growth Rate
M. flavescens
Other Rapidly Growing Species
M. thermoresistible
M. neoaurum
M. smegmatis group
M. mucogenicum group
M. mageritense
M. wolinskyi

Laboratory Identification of Mycobacteria and Related Clinical Syndromes

The identification of mycobacteria by traditional methods requires patience, familiarity with the end points of different identification characteristics, and a collection of control strains. Additionally, it is time-consuming and often does not provide an accurate identification to the species level. Not every laboratory needs the ability to identify all mycobacteria to the species level. The number of patients with TB in any given

hospital in the United States is not large, and it may be prudent to use the services of a reference laboratory to provide definitive identifications and susceptibility testing of clinically important isolates recovered in the primary care laboratory. Each laboratory director must determine the extent of services to be provided to meet the needs of the local clinical practice.

Review of Mycobacterium *Species: Laboratory Aspects and Clinical Correlations*

Although a presumptive diagnosis of pulmonary TB can be made based on clinical history, presenting symptoms, physical examination, radiographic evidence of disease and the presence of AFB in the sputum, the definitive diagnosis requires recovery of the causative organisms in culture or demonstration of these organisms by nucleic acid amplification methods. Several NTM are now recognized as important pathogens, each with a different potential for producing disease and sometimes with unique antimycobacterial drug susceptibility profiles that must be determined by laboratory tests. A wide variety of *Mycobacterium* species may be isolated from extrapulmonary sites. Laboratory culture characteristics and the clinical manifestations of select human mycobacterial diseases (Box 19-4 and Table 19-8) are discussed briefly in the following paragraphs and Clinical Correlation Boxes.

Mycobacterium tuberculosis *Complex.* Members of the MTBC are the only mycobacteria of public health importance and, therefore, the major focus of identification in clinical laboratories (Clinical Correlation Box 19-1).

Mycobacterium tuberculosis. Traditionally, it has been taught that classic strains of *M. tuberculosis* can be phenotypically differentiated from *M. bovis*, *M. microti,* and *M. africanum*. However, several parameters, including analysis of antigenic extracts, target epitopes for monoclonal antibodies, and antigenic and DNA-relatedness studies, suggest that *M. tuberculosis*, *M. bovis*, *M. microti,* and *M. africanum* represent a single species and currently are considered under the general term MTBC.

BOX 19-4

Mycobacterium species: Order of Presentation

Mycobacterium tuberculosis complex
Photochromogens
- *M. kansasii*
- *M. marinum*
- *M. simiae*
- *M. asiaticum*

Scotochromogens
- *M. scrofulaceum*
- *M. szulgai* (photochromogen at room temperature)
- *M. xenopi*
- *M. celatum*
- *M. gordonae*

Nonphotochromogens
- *M. avium* complex
- *M. paratuberculosis*
- *M. terrae, M. gastri,* and *M. triviale*
- *M. shimoidei*
- *M. malmoense*
- *M. haemophilum*

Rapid Growers
- *M. fortuitum* group
- *M. chelonae/M. abscessus* group
- Infrequent human pathogens

Other Mycobacteria
- *M. ulcerans*
- *M. genavense*

A few relatively simple tests are needed to identify most isolates of MTBC. The following are phenotypic characteristics by which an identification of *M. tuberculosis* can be made:

1. Formation of nonpigmented, rough, buff colonies after 14 to 28 days of incubation at 37°C on LJ or Middlebrook media (Image Plate 19-1G and H).
2. Appearance of microcolonies after 5 to 10 days incubation on Middlebrook 7H10 or 7H11 agars, with formation of serpentine cords owing to the production of "cording factor" (Image Plate 19-1C and H).
3. Accumulation of niacin (Image Plate 19-1D). *M. simiae*, certain strains of *M. bovis*, and occasional strains of *M. marinum* and *M. chelonae* may also be niacin-positive; therefore, this characteristic must be used in conjunction with the other findings.

TABLE 19-8 Nomenclature of Select Mycobacteria

Legitimate Species Name	Relative Pathogenicity for Humans	Equivalent Runyon Group	Acceptable Common Name	Comments
M. tuberculosis	+++		Human tubercle bacillus	Causes human tuberculosis—highly contagious.
M. bovis	+++		Bovine tubercle bacillus	Causes bovine and human tuberculosis; avirulent strains are used for bacille Calmette-Guérin (BCG) vaccines.
M. ulcerans	+++			Associated with skin infections in tropics.
M. africanum	+++			It is found in northern and central Africa.
M. kansasii	+++	I photochromogen		Rarely nonpigmented or scotochromogenic.
M. marinum	+++	I photochromogen		Causes skin infections, usually associated with water exposure.
M. simiae	++	I photochromogen		Photoreactivity may be unstable; niacin-positive.
M. genavense	++			Requires mycobactin J for growth on solid media. Propensity for causing disseminated disease in patients with AIDS.
M. asiaticum	++	I photochromogen		Similar to *M. simiae* but differs antigenically.
M. scrofulaceum	++	II scotochromogen		May cause cervical lymphadenitis.
M. szulgai	+++	I photochromogen at 25°C II scotochromogen at 37°C		Associated with chronic pulmonary and extrapulmonary disease; distinctive lipid composition of cell walls.
M. xenopi	++	II scotochromogen		Grows slowly; best at 42°C; may contaminate hot water systems.
M. celatum	+	Unclear (see text)		Propensity for causing respiratory infections in patients with AIDS. Closely resembles *M. xenopi*.
M. gordonae	Rare	II scotochromogen	Tap water bacillus	Rarely pathogenic for humans.
M. thermoresistible	0	II scotochromogen		Growth at 52°C. Not proven to be a human pathogen.
M. avium complex	+++	III nonphotochromogen[a]	Battey bacillus	May cause disseminated infection in patients with AIDS.
M. terrae	Rare	III nonphotochromogen	Radish bacillus	May be closely related to *M. triviale*.
M. shimoidei	+			Resembles *M. terrae* complex. Rare cases of pulmonary infections.
M. triviale	0/+	III nonphotochromogen	V bacillus	
M. malmoense	+++	III nonphotochromogen		Slowly growing mycobacterium usually causing pulmonary disease.
M. haemophilum	+++	III nonphotochromogen		Associated with skin lesions usually in immunosuppressed patients.
M. fortuitum group	++	IV rapid grower		Predominantly causes skin infections. It may cause pulmonary disease. May cause nosocomial infection.
M. chelonae	++	IV rapid grower		May cause skin infections, particularly in persons with compromised cell-mediated immunity.
M. abscessus	++	IV rapid grower		Predominantly causes skin infections. May cause pulmonary disease in patients with chronic respiratory disease or cystic fibrosis.

[a]Strains recovered from patients with AIDS may develop a pale yellow pigment with age.

CLINICAL CORRELATION BOX 19-1 *Mycobacterium tuberculosis*

Tuberculosis typically begins in the lungs, where diffuse, finely nodular, or patchy infiltrates may be seen, predominately in the apical portions of the upper lobes, but also spreading to other foci in miliary form or as progressive exudative tuberculosis (Image Plate 19-3A). Cavitary lesions, primarily in the apical portions of the upper lobes are common, and a solitary tuberculoma or "coin lesion" that may be part of an old Ghon complex may be seen (Image Plate 19-3B and C). Disseminated or miliary spread of infection occurs in certain persons, usually those with malnutrition, immunosuppression, or other chronic debilitating diseases. Jereb and coworkers,[143] using restriction-fragment-length polymorphism (RFLP) by PCR, were able to trace the source of an outbreak of *M. tuberculosis* among 10 kidney-transplant recipients in one hospital to a single patient who had posttransplantation exposure in another hospital before transfer. The mean incubation time for the onset of tuberculosis in the newly infected kidney-transplant recipients was 7.5 weeks. This situation illustrates how molecular techniques can be helpful in detecting point sources of tuberculosis so that early isolation can be implemented to prevent nosocomial transmission of disease.

Reactivation, or adult-type, tuberculosis is a slowly progressive inflammatory process in the lungs, characterized by granulomatous inflammation, which may erode into bronchi (Image Plate 19-3D). Large numbers of tubercle bacilli are spread to fresh foci within the lung when a cavity ruptures, and may be expectorated if the cavity breaks into a bronchus, potentially infecting others in close contact. Coughing, weight loss, low-grade fever, dyspnea, and chest pain are the usual clinical signs and symptoms of chronic progressive pulmonary tuberculosis. This is a description of what has been termed secondary- or reactivation-type tuberculosis. In patients with AIDS, the disease is more reminiscent of primary progressive tuberculosis, and is characterized by less focal fibrosis and caseation, and more rapid progression and miliary dissemination to involve virtually any organ in the body.

Tuberculous meningitis is relatively rare in the United States. Patients may present with headaches, change in mentation, or, uncommonly, progress to manifestations of severe meningitis. In most instances, at least 1 of 3 laboratory measurements of cerebrospinal fluid will be altered: an increase in cell count, usually a lymphocytosis; a decrease in glucose levels; or an increase in protein concentrations, although any one of these parameters may be normal. Acid-fast bacilli may be observed in centrifuged specimens in about 40% of patients, a yield that increases with the

number of spinal examinations performed. Pathologically, the meningeal involvement is most pronounced at the base of the brain, where visible changes range from a diffuse opacity to the presence of a thick gelatinous exudate, seen primarily in the areas overlying the pons and adjacent to the optic chiasm (Image Plate 19-3E).

Bacteremia with *M. tuberculosis* is traditionally considered to be rare; however, Bouza and associates,[28] in a study of 285 patients with culture-proved tuberculosis in whom blood cultures were obtained, found that 50 (14%) were bacteremic. Of these, 81% were infected with HIV. In 14 patients, blood was the first specimen from which organisms were recovered. Archibald et al.,[7] when studying 517 patients with possible community-acquired bloodstream infections in sub-Saharan Africa, found that of the 145 patients with a proven bloodstream infection, 81% of which were HIV-infected, the most frequently isolated pathogen was *M. tuberculosis*, at 39%.

The majority of *M. tuberculosis* isolates from untreated patients in the United States are susceptible to the primary antituberculosis drugs and respond to the recommended four-drug treatment. Susceptibility testing of mycobacteria is presented later in this chapter.

4. Reduction of nitrates to nitrites (Image Plate 19-1E).
5. Ability to grow in the presence of T2H (Image Plate 19-1F).
6. Lack of catalase activity.

The typical cell morphology of *M. tuberculosis* as seen in carbol fuschin-stained smears is a thin, slightly curved bacillus that measures 0.3 to 0.6 × 1 to 4 nm, deeply red staining (strongly acid-fast), with a distinct beaded appearance (Image Plate 19-1B). In the preparation of smears from cultures, the individual cells are often difficult to disperse, appearing as irregular aggregates, or lying in parallel strands. Distinctive cording may be seen in preparations from broth cultures, wherein aggregates of the AFB form long, serpentine cords. Mycobacteria may be seen in the Gram's stain as gram-positive, beaded bacilli. The beading represents nonuniform staining of the bacillus. The presence of a *Mycobacterium* species can also be suspected in Gram's stains if poorly or nonstaining bacilli are observed surrounded by a clear halo (Image Plate 19-1I). The crystal violet dye in the Gram's stain reagent does not penetrate the thick, waxy lipid cell wall of the organism, and they may appear as either beaded or almost as a negative image against the counterstained background.

Mycobacterium bovis. *Mycobacterium bovis* (Clinical Correlation Box 19-2) can be differentiated from classic strains of *M. tuberculosis* based on the following:

1. Most strains are niacin-negative.
2. Nitrates are not reduced to nitrites.
3. Pyrazinamidase is not produced.

4. Selective inhibition of growth by T2H; *M. bovis* will not grow in medium containing T2H.

The classic human strains have a very slow growth rate, producing "dysgonic"-appearing colonies on LJ medium. Growth of most strains is better on LJ medium than on Middlebrook 7H11 or equivalents. The medium most favorable for *M. bovis* contains 0.4% pyruvate without glycerol. Typical colonies are buff, low, and small and may appear either smooth or rough on egg-containing medium. On Middlebrook 7H11 agar, colonies are very thin and often show little or no stranding (referred to as "water-droplet-like"). These colonies may also simulate dysgonic forms of organisms in the MAC. The latter, however, are 68°C catalase-positive, do not produce urease, and are pyrazinamidase-positive. If pyruvate has been added to the medium, colonies may show serpentine cords similar to those of eugonic *M. tuberculosis*.

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CLINICAL CORRELATION BOX 19-2 *Mycobacterium bovis*

Mycobacterium bovis produces tuberculosis typically in cattle but may also infect other animals, including dogs, cats, swine, rabbits, elk, deer, and possibly, certain birds of prey. Fanning and Edwards traced 446 contacts of humans with domesticated elk in Alberta, Canada, 81 of whom were skin-test-positive for *M. bovis*.[84] Of these, 50 had been in contact with culture-positive animals, including one case of active *M. bovis* pulmonary infection diagnosed by a positive sputum specimen. With DNA fingerprinting assays with IS*6110* as the genetic marker, van Soolingen et al.[334] were able to determine that the strains of *M. bovis* causing human infection in Argentina were transmitted from cattle, whereas the strains causing human disease among persons living in the Netherlands were contracted from animals other than cattle (various wild and zoo animals). In some areas in Scotland and in eastern Europe, where cattle and dairy farming is still the chief livelihood, *M. bovis* still may constitute as many as 39% of all cases of tuberculosis.[357] Human bovine pulmonary tuberculosis closely resembles that caused by *M. tuberculosis*.

During the time that humans physically milked cows much more frequently than today, cutaneous infections of the fingers were common, often with underlying osteomyelitis and osteoarthritis of the digits (Image Plate 19-3F). A common site of recovery of *M. bovis* in clinical laboratories is from urine samples, as the bacille Calmette-Guérin (BCG) strain is used in bladder irrigations as an immunologic stimulant in the treatment of bladder carcinoma. This possibility should be considered when a slow-growing *Mycobacterium* is recovered from urine samples. In some

individuals, the BCG strain being used is not totally attenuated and may cause cystitis.

BCG strains of *M. bovis*, which have been used as a vaccine in highly endemic areas of the world, simulate *M. tuberculosis* by being "eugonic," or more rapidly growing (3 to 4 weeks on LJ medium), having a rough, buff appearance and, in some cases, accumulating niacin. However, these strains remain T2H-sensitive and can be differentiated on that basis. The microscopic morphology of *M. bovis* cells in acid-fast-stained smears is not distinctive.

Photochromogens

Mycobacterium kansasii. *M. kansasii* is an important cause of pulmonary disease that clinically, radiologically and histopathologically resembles TB (Clinical Correlation Box 19-3). Pulmonary disease due to *M. kansasii*, however, does not have the same public health implications as TB and is not transmitted from person to person. Originally known as the "yellow bacillus" following the first description by Buhler and Pollak in 1953, *M. kansasii* is a photochromogen classified within the Runyon group I. Although infections occur throughout the United States, most cases have been reported from the southern states (Texas, Louisiana, and Florida), Midwest (Illinois), and California. Males are infected more frequently than females, with a ratio of approximately 3:1. The disease is uncommon in children. Non-pulmonary disease, such as peritonitis in a patient undergoing continuous ambulatory peritoneal dialysis, is far less common than pulmonary disease, but has been reported.[95]

Laboratory aspects. Typical strains of *M. kansasii* grow at approximately the same rate as or slightly more rapidly than *M. tuberculosis* at 37°C. The distinctive feature is the dependence on light exposure for the production of a visible yellow pigment (Image Plate 19-2C) and the formation of reddish carotene crystals on prolonged incubation. The colonies are typically intermediate between fully rough and fully smooth; however, certain strains are totally one or the other. The microcolonies typically show elevated centers and curving strands of bacilli in the outer, thinner margins that may be confused with the serpentine cording of *M. tuberculosis*. Phenotypic properties, in addition to photochromogenicity, by which the identification of *M. kansasii* can be confirmed include:

1. Rapid hydrolysis of Tween within 3 days
2. Strong reduction of nitrate to nitrite
3. Rapid catalase reaction, including 68°C test
4. Strong pyrazinamidase activity

Less commonly, scotochromogenic or nonchromogenic strains may be encountered, including some strains with low catalase activity. The bacterial cells in acid-fast smear

preparations are characteristically long and broad and distinctly cross-banded or barred (Image Plate 19-2M), presumably from utilization of the lipid in the medium. Although experienced mycobacteriologists may make a presumptive identification based on acid-fast stain, additional testing is required before a definitive identification can be made.

Mycobacterium marinum. In 1926, while investigating infectious diseases in saltwater fish, Aronson discovered a new *Mycobacterium* species, later named *Mycobacterium marinum* ("of the sea").[8,346] The organism has also been called *M. platypoecilus* from infections observed in Mexican platyfish and also as *M. balnei* (a name referring to bath or spas). All are the same organism (Clinical Correlation Box 19-4). Exposure to water is often the clue in the clinical history to the etiologic agent of disease.[87]

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CLINICAL CORRELATION BOX 19-3 *Mycobacterium kansasii*

Chronic pulmonary disease simulating classic tuberculosis is the most common manifestation, classically involving the upper lobes. Cavitation with scarring is evident in most patients, and disease is slowly progressive. Extrapulmonary or disseminated infections are less common, although cases of scrofula-like lymphadenitis, sporotrichosis-like cutaneous infections, osteomyelitis, soft tissue infections, and tenosynovitis have been reported. Dillon et al.,[74] in particular, cite the progressive damage to the deep structures of the wrist and hand that can occur in cases of tenosynovitis. Disseminated disease may be seen in the presence of severe immunosuppression and has been reported in patients with AIDS. Jacobson and Isenberg described an AIDS-related infection caused by *M. kansasii* that presented as granulomatous interstitial pneumonitis, but report that only 0.2% of AIDS patients have superimposed *M. kansasii* infections.[138] However, Valainis and coworkers,[332] reporting from Louisiana, a region endemic for *M. kansasii*, in a 60-month review of patients attending two major referral centers in New Orleans, found that 31.9% of HIV-1-infected patients were coinfected with *M. kansasii*. In a retrospective study of 35 patients conducted in Kansas City, the incidence of *M. kansasii* infections in patients with AIDS was three times higher than *M. tuberculosis* infections.[12] Standard therapy for *M. kansasii* infections includes isoniazid, rifampin, and ethambutol, the most important drug of which is rifampin. An alternative, equally effective regimen is rifampin, ethambutol, and clarithromycin.

M. kansasii infections in patients with AIDS usually occur when CD4 lymphocyte counts are fewer than 50/μL, with serious and life-threatening complications in many instances. Levine and Chaisson reviewed 19 patients with *M. kansasii* and HIV

infection, 14 of whom had exclusive pulmonary infection, three had pulmonary and extrapulmonary disease, and two had extrapulmonary involvement exclusively.[184] All patients with pulmonary infection presented with fever and cough of at least 2 weeks' duration. Focal upper lobe infiltrates, diffuse interstitial infiltrates, or thin-walled cavitary lesions, or a combination thereof, were seen on chest radiographs. Other AIDS-related infections caused by *M. kansasii* have been reported.[124,294] In one patient, caseating granulomas were seen in the bowel wall and in the mesenteric lymph nodes, with accumulation of foamy histiocytes resembling Whipple's disease. Giladi and coauthors reported a catheter-associated *M. kansasii* peritonitis in a 62-year-old woman undergoing continuous ambulatory peritoneal dialysis, presumably the first such case report.[337] Tortoli and coworkers[328] found a cluster of *M. kansasii* strains that did not hybridize with the AccuProbe *M. kansasii* culture identification test (Gen-Probe, San Diego, CA) and that were distinctly associated with the HIV status. This suggests that additional tests for identification may be required for AccuProbe-negative strains.

Strains of *M. kansasii* resistant to rifampin have been reported. Wallace and coauthors reported on 36 patients from whom rifampin-resistant *M. kansasii* had been isolated, 90% of whom had previously received therapy with this agent.[340] The majority of patients, however, responded to a four-drug regimen, based on in vitro susceptibility studies, and sputum cultures that converted to negative in 90% of those treated in a mean of 11 weeks.

CLINICAL CORRELATION BOX 19-4 *Mycobacterium marinum*

Typical infections with *M. marinum* involve the skin, usually resulting when traumatized skin comes in contact with inadequately chlorinated freshwater or saltwater (e.g., swimming pools, tropical fish aquariums, water-cooling towers). Fisher has reported cutaneous infections in three lifeguards, citing this infection as a hazard of the trade.[50] Hoyt and associates[129] report several deep-tissue infections and destructive tenosynovitis among fishermen in the Chesapeake Bay area. Several other citations of deep cutaneous infections, usually of the hands, and commonly associated with aquatic activity, such as cleaning of fish, include a patient with tenosynovitis, arthritis and bursitis, and osteomyelitis.[16,50,131]

Sporotrichosis-like lesions, with central spread along the lymphatics emanating from an ulcerated area at the primary site of inoculation, may occur, but more typically, the lesions present as tender, red or blue-red subcutaneous nodules, usually involving the elbow, knee, toe, or finger ("swimming pool granuloma"). Such lesions have been mistaken for rheumatoid nodules.[9]

Antimicrobial therapy is discussed later in this chapter.

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Laboratory aspects. *M. marinum*, when recovered from clinical specimens, grows optimally at 30°C to 32°C, but it may grow, perhaps not as well, at 37°C. Subcultures generally grow better at 37°C than the initial culture of the specimen. Colonies appear in 8 to 14 days; those grown in the dark may appear nonpigmented. When exposed to light, a deep yellow pigment develops. Colonies vary between wrinkled and rough to smooth and hemispheric, particularly if grown on 7H10 or 7H11 media (which contains oleic acid and albumin). Microscopically, the cells are relatively long rods with frequent cross-barring.

Photochromogenicity and preference for growth at 30°C to 32°C are initial clues to the identification of *M. marinum*. The following are additional characteristics by which a definitive identification can be made:

1. Some strains may accumulate niacin.
2. Nitrates are not reduced to nitrites.
3. Tween is hydrolyzed.
4. Urease is positive.
5. Pyrazinamidase is produced.
6. Heat-stable catalase is not produced.

Mycobacterium simiae. Weiszfeiler and Karczag named *M. simiae* in 1969,[350] in recognition of the first recovery of the organism 4 years earlier by Karassova and colleagues,[349] from *Macaca* rhesus monkeys imported into Hungary from India (Clinical Correlation Box 19-5). This organism was subsequently found to be identical to a niacin-positive strain of a group III mycobacterium recovered in Cuba by Valdivia and coworkers from patients with pulmonary tuberculosis. They named the organism *M. habana*, which subsequently has been shown to be the same organism as *M. simiae*.

Laboratory aspects. Colonies of *M. simiae* develop within 2 to 3 weeks on egg-based media and are typically smooth. Most strains are photochromogenic. Prolonged exposure to light may be required for isolates that fail to produce pigment. The key identifying biochemical reactions are as follows:

1. Positive niacin accumulation
2. Hydrolysis of Tween (may be slow, requiring more than 10 days)
3. High thermostable catalase activity

Poor reproducibility of test reactions by some strains may make identification using conventional tests somewhat difficult.

Mycobacterium asiaticum. *Mycobacterium asiaticum* is in many ways phenotypically similar to *M. gordonae;* both have high catalase activity, hydrolyze Tween 80, and are negative for urease and nitrate reduction.[346] *M. asiaticum*, however, is a photochromogen, whereas *M. gordonae* is a scotochromogen. *M. asiaticum* is also biochemically similar to *M. simiae,* except it is niacin-negative. *M. asiaticum* has a distinct 16S rRNA profile, justifying a separate species status.[309]

Rare instances of human infection have appeared in the literature. The first indication that this organism could be pathogenic was in a report from Australia by Blacklock.[24] Two of the 5 patients had progressive cavitary pulmonary disease; three had no evidence of progressive pulmonary disease, and the sputum isolates were thought to represent secondary colonization. The first reported pulmonary infection caused by *M. asiaticum* in the United States occurred in a 62-year-old man living in Los Angeles.[316] Four isolates have also been reported from Florida.[102] Dawson and coworkers[69] have reported recovery of *M. asiaticum* from fluid aspirated from an olecranon bursa in a patient with a postsurgery infection. The infection cleared with drainage, regular dressing changes, and immobilization, without the need for antimycobacterial therapy.

Scotochromogens

Mycobacterium scrofulaceum. The species name *scrofulaceum* is derived from *scrofula* ("brood sow"). This mycobacterium was named in 1956 by Prissick and Mason in reference to the most common form of disease by this organism, cervical lymphadenitis in children[253] (Clinical Correlation Box 19-6). *M. scrofulaceum*, however, is not the only mycobacterium that causes cervical lymphadenitis. Gill et al.[96] reviewed the 16 children with this disease; 6 were caused by *M. scrofulaceum*, 4 by *M. tuberculosis*, and 4 by MAC.[96]

Kirschner and associates[162] recovered large quantities of *M. scrofulaceum* from swamp water in several locations in Georgia, West Virginia, and Virginia, indicating that environmental water sources are likely connected with the increased incidence of scrofula infections seen in these regions. High concentrations of organisms were found in warm water with low pH, low dissolved oxygen content, high soluble zinc levels, and high levels of humic and fulvic acids.

Laboratory aspects. Colonies of *M. scrofulaceum* grow slowly (4 to 6 weeks) at various temperatures (25°C, 31°C, and 37°C). They produce colonies that typically are smooth, buttery in consistency, and globoid, with pigmentation ranging from light yellow to deep orange (Image Plate 19-2D). Pigment production is not dependent on light exposure; thus, the organism is included in the scotochromogen group II of

Runyon.

CLINICAL CORRELATION BOX 19-5 *Mycobacterium simiae*

Reports of human infection with *M. simiae* are relatively few. Isolated reports of pulmonary infections have been described in France, Israel, Thailand, and the United States.[185] These authors report a 43-year-old man with AIDS in whom disseminated mycobacterial infection developed. Organisms were recovered from blood, jejunal fluid, and duodenal and rectal biopsies. Although a few other instances of *M. simiae* infections have been reported in patients with AIDS, the association is infrequent and does not serve as a marker.[236] The Israeli experience with *M. simiae* is interesting, in that 399 strains were isolated from 287 persons during the period 1975–1981, primarily among inhabitants of the coastal plain of Tel Aviv.[179] Most of the isolates were commensal organisms related to environmental water sources. A few pulmonary infections occurred among patients from whom multiple isolates were recovered, usually complicating preexisting chronic pulmonary conditions.

Disseminated disease, with renal involvement following pulmonary infection has also been reported.[275]

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CLINICAL CORRELATION BOX 19-6 *Mycobacterium scrofulaceum*

Lymphadenitis is the classic presenting symptom of infection with *M. scrofulaceum*, occurring most commonly in children between 18 months and 7 years of age, a time when a barrier break in the oral mucous membrane occurs during teething. The next higher incidence of disease occurs in young adults who are cutting molar teeth. The lymphadenitis is unilateral, involving nodes high in the neck adjacent to the mandible or posteriorly behind the ears (Image Plate 19-3G and H). The nodes often drain from the surface of the skin without complications, except for varying degrees of residual scarring at the sites of surface penetration of sinus tracts. The disease often affects healthy children; pain is usually minimal, and constitutional symptoms are absent. Colonization of the organism in the mouth and throat is presumed to be the site of origin. The high incidence of disease among young children is thought to be related to

disruption of the gums during tooth eruptions, during an age when the immune system is still relatively immature. The local disease is best treated with surgical incision and drainage of the involved nodes.

Key biochemical test reactions include the following:

1. Failure to hydrolyze Tween.
2. Nitrates are not reduced to nitrites.
3. 68°C catalase test is positive.
4. Urease is produced.

Mycobacterium szulgai. *M. szulgai*, officially reported as a species in 1972 by Marks and coworkers,[194] is named after the Polish microbiologist T. Szulg. The unique feature of this species is the temperature-dependent production of pigment. When grown at 37°C, the organism is scotochromogenic, whereas it is photochromogenic when grown at room temperature (25°C). Therefore, to assess the photochromogenicity of an unknown strain of *M. szulgai*, the light-exposed plates must be incubated at room temperature and not at 37°C. The scotogenic pigment produced at the higher temperature will mask any chance to visualize any photogenetic pigment that may be produced (Chart 19-5 and Clinical Correlation Box 19-7).

Laboratory aspects. Growth is relatively rapid, with either smooth or rough colonies developing within 2 weeks at 37°C. Orange pigment may be observed, intensifying with exposure to continuous light. In acid-fast smear preparations, the bacterial cells appear as moderately long bacilli, with some cross-barring, reminiscent of *M. kansasii*.

Key biochemical test reactions for *Mycobacterium szulgai* include the following:

1. Slow hydrolysis of Tween
2. Nitrates reduced to nitrites
3. Positive catalase activity
4. Intolerance to 5% NaCl

Mycobacterium xenopi. *M. xenopi* (*Xenopus*, a genus of frog), was first isolated from an African toad. Hot and cold water taps, including water storage tanks and hot water generators of hospitals, are potential sources for nosocomial infections (Clinical Correlation Box 19-8). Previously considered to be nonpathogenic, *M. xenopi* has been incriminated in several infections.[326] Wolinski reported *M. xenopi* infections of 50 patients, primarily in England, France, Denmark, Australia, and the United States.[364]Birds that frequent the coastal regions in Great Britain constitute an important reservoir.

Laboratory aspects. *Mycobacterium xenopi* colonies are slow-growing, small, erect, and produce characteristic yellow pigment, although occasional strains are nonpigmented. Growth is more rapid at 42°C than 37°C and is absent at 25°C. Although included with the nonphotochromogenic mycobacteria in the past, the brightly pigmented yellow colonies usually found on primary isolation suggest that the organism would be better considered with the scotochromogens. Colonies tend to be rough, and an aerial mycelium may be evident. Examination of young microcolonies on 7H10 agar reveals a distinctive "bird's nest" appearance, with sticklike projections. Branching and filamentous extensions appear in older colonies. Microscopically, acid-fast-stained smears reveal long, filamentous rods that are tapered at both ends, tending to arrange in palisades.

CLINICAL CORRELATION BOX 19-7 *Mycobacterium szulgai*

Maloney and associates[193] presented their experience with three patients with infections caused by *M. szulgai* and reviewed 24 cases previously reported in the literature. Lung infections were present in two-thirds of the cases, with chest radiographs revealing unilateral or bilateral apical disease, with cavitation, simulating *M. tuberculosis*. A patient with a persistent lung infection caused by *M. szulgai* has also been reported.[55] Fever, cough, hemoptysis, and weight loss were common symptoms. Extrapulmonary infections with *M. szulgai* cited in the foregoing review include olecranon bursitis, tenosynovitis, carpal tunnel syndrome, osteomyelitis, and localized cutaneous disease.

CLINICAL CORRELATION BOX 19-8 *Mycobacterium xenopi*

Most human infections caused by *M. xenopi* have been pulmonary, resembling those seen in patients with *M. tuberculosis*, *M. kansasii*, or MAC infections. Radiographic findings include multinodular densities, cavities, and fibrosis. Infections usually occur in patients with preexistent lung disease or predisposing conditions, such as alcoholism, malignancy, and diabetes mellitus. Contreras and coauthors, in reviewing 89 adult patients with pulmonary infections, report that *M. xenopi* was the second most frequent isolate (38% of patients).[57] Similarly, in a review of cultures positive

for nontuberculous mycobacteria, excluding MAC and *M. gordonae*, from 86 patients at the State University of New York Health Sciences Center at Brooklyn, it was demonstrated that *M. xenopi* was the species most commonly isolated (33 patients).[291] The majority of these isolates were recovered from respiratory specimens in patients with AIDS. The recovery of 28 isolates of *M. xenopi* from patients residing in the Ontario province of Canada has also been reported.[299] In 19 patients, the isolate was considered insignificant; 9 isolates were from middle-aged men with other chronic pulmonary diseases. In the province of Ontario, *M. xenopi* has been the second most common nontuberculous mycobacterial pathogen, after MAC.

Intrapulmonary spread usually occurs in patients with AIDS; in patients with disseminated disease, the organisms may also be recovered from bone marrow aspirates.[10] *M. xenopi* infections are uncommonly found in HIV-positive patients. In two instances of HIV-infected men with symptomatic pulmonary infection, night sweats, cough, and pleuritic pain were reported.[139] This organism grew in cultures of multiple respiratory specimens obtained from each patient. Both patients improved with multidrug therapy. A pulmonary infection in a 39-year-old kidney allograft recipient has been reported.[347] Isolated extrapulmonary infections involving bone, lymph node, epididymis, sinus tract, and a prosthetic temporomandibular joint have also been described.[364] Infections of the lumbar spine have been described; one patient was a 77-year-old immunocompetent woman who presented with a paravertebral abscess.[254,258] An outbreak of pulmonary infections in 13 residents of a housing project in Prague have been reported.[301] The point source was thought to be the local water supply, as organisms were recovered from water faucets in five of the flats. Tortoli and coauthors reviewed the microbiologic features of 64 isolates of *M. xenopi* recovered from patients living in Florence, Italy, during a 15-year period.[326] The homogeneity of the biochemical, cultural, and antimicrobial sensitivity patterns of these isolates suggested that there may be an endemic focus in the Florence area.

Key biochemical test reactions for *Mycobacterium xenopi* include the following:

1. Optimal growth at 42°C
2. Yellow scotochromogenic pigment
3. No niacin accumulation
4. Nitrate reduction-negative
5. Catalase produced at 68°C only
6. Arylsulfatase-positive
7. Pyrazinamidase-positive

Mycobacterium celatum. *M. celatum* is the name proposed for this

Mycobacterium species by Butler and coauthors.[36] The most appropriate Runyoun group for *M. celatum* is unclear. The initial isolate was described as nonchromogenic; however, other investigators found that isolates produced a weak yellow pigment in the dark. *M. celatum* most closely resembles *M. xenopi* phenotypically, differing only in its poor growth at 45°C, formation of large colonies on 7H10 agar, and the production of only trace amounts of the fatty acid 2-docanosol. It may cross-react with MTBC when using the acridinium ester–labeled DNA probe, AccuProbe (Gen-Probe, San Diego, CA), a problem that, although addressed, remains unresolved.[36] *M. celatum* has been recovered from respiratory specimens, and less commonly from the blood, stool, and cerebrospinal fluid, obtained from patients residing in diverse geographic locations, including the United States, Finland, and Somalia. It may cause serious disease (pulmonary and disseminated) in patients with AIDS.[247] In the original report by Butler and coauthors, approximately one-third of the isolates were recovered from patients with AIDS.[36] Haase and coworkers[114] report a case of scrofula-like unilateral cervical lymphadenitis in an immunocompetent child.

Mycobacterium gordonae. *Mycobacterium gordonae* rarely causes human infections, but is possibly the mycobacterium that is recovered in clinical laboratories with greatest frequency (Clinical Correlation Box 19-9). It is found particularly in aqueous environments, leading to the alternative designation of *M. aquae,* or the "tap water bacillus."

Laboratory aspects. *Mycobacterium gordonae* is a scotochromogen, and it is readily recognized by the smooth, deeply yellow-orange pigmented colonies that develop after 7 days of incubation at 37°C (Image Plate 19-2E). The organism hydrolyzes Tween 80 and produces heat-stable catalase; it is urease-negative and does not reduce nitrates to nitrites. An organism that may be phenotypically confused with *M. gordonae* is *M. flavescens*, which is a scotochromogen; however, *M. flavescens* possesses urease activity and reduces nitrates to nitrites, two characteristics not demonstrated by *M. gordonae*. *M. flavescens* is a commensal for humans and is not known to cause disease.

Nonphotochromogens

***Mycobacterium avium* Complex.** Strains of *M. avium* and *M. intracellulare*, often referred to as MAC, are widely distributed in water, soil, dust, mammals, and poultry. For humans, these organisms initially were considered of low pathogenicity and an uncommon cause of disease (Clinical Correlation Box 19-10). However, the AIDS epidemic has demonstrated the pathogenicity of these organisms in persons with depleted T-cell immunity.[102]

CLINICAL CORRELATION BOX 19-9 *Mycobacterium gordonae*

Mycobacterium gordonae most often is a contaminant. There should be a careful review of the clinical and histopathologic findings from the patient and, if possible, repeat isolation of the organism from the patient prior to ascribing disease to *M. gordonae*. There have been several isolated reports of infections caused by this organism, which include meningitis secondary to involvement of ventriculoatrial shunts, hepatoperitoneal disease, endocarditis in a prosthetic aortic valve, cutaneous lesions of the hand, and possibly patients with pulmonary involvement.[370] Disseminated disease, soft tissue infections, chronic urinary tract infection, and peritonitis in a patient undergoing peritoneal dialysis have also been described.[140,141,348,378] Wayne and Sramek, however, after reviewing many of the previously reported alleged infections caused by *M. gordonae,* caution that organism descriptions and convincing clinical correlations often are lacking in many of the papers, and that its true pathogenicity remains in question.[346] When recovered from specimens, each isolate must be accurately identified and a careful clinical correlation made to determine the clinical significance for what in most instances will be a contaminant.

CLINICAL CORRELATION BOX 19-10 *Mycobacterium avium* Complex

Conditions predisposing individuals to pulmonary infections with members of the *M. avium* complex (MAC) include chronic obstructive pulmonary disease by whatever primary cause, bronchiectasis, chronic aspiration or recurrent pneumonia, tuberculosis, pneumoconiosis, cystic fibrosis, and bronchogenic carcinoma.[107,149,156] Pulmonary disease also occurs in postmenopausal women without predisposing conditions, the so-called Lady Windermere's syndrome, after Oscar Wilde's Victorian character who had the peculiar habit of suppressing a cough. In immunocompetent patients, pulmonary manifestations of MAC disease are similar to those of *M. tuberculosis*: cough, fatigue, weight loss, low-grade fever, and night sweats. Radiographic findings include solitary nodules or more diffuse infiltrates, but cavitary disease may occur, albeit less frequently than in tuberculosis.

The greatest upsurge of MAC disease during the past decades has been in patients with AIDS. In a review of MAC disease in HIV-positive patients before the widespread use of highly active antiretroviral therapy (HAART), it was found that the disease was

usually dissemination and occurred late in the course of HIV infection.[190] The risk of MAC disease in patients with AIDS with CD4 counts lower than 50 is high (45% within 1 year after diagnosis).[46] When organisms were found in the sputum or gastrointestinal tract, 60% of patients had disseminated disease. Disseminated disease is characterized by intermittent fever, sweats, weakness, anorexia, and weight loss that is relatively rapidly progressive. Abdominal pain or diarrhea with malabsorption was seen in some patients. Significant pulmonary involvement was infrequent, despite recovery of MAC organisms in sputum specimens. Bacteremia occurs in over 90% of patients with disseminated disease, with organisms found within the circulating monocytes; therefore, mycobacterial blood culture is the recommended specimen for culture in these patients. Colony counts as high as 10^6 colony-forming units (CFUs)/mL have been reported, although counts in the range of $^{10^1}$to 10^2 are more common.[365]

In a study of mycobacterial infections in 94 patients with AIDS, significant pulmonary disease occurred in about 25% of patients infected with MAC, in contrast to 83% of patients with *M. tuberculosis* infections who had pulmonary disease.[215] Also, classic tuberculosis preceded the diagnosis of HIV infection in two-thirds of the patients, in contrast with MAC disease, which was a secondary complication of the patient reaching the AIDS stage of disease in all patients studied. Members of the MAC have also been recovered from patients with AIDS and tuberculous meningitis, reiterating the importance of considering a number of coincident pathogens in patients which reach that stage of immunosuppression.[137] The recovery of organisms from the spinal fluid indicated disseminated disease, and prognosis in this group of patients was poor. Disseminated MAC disease, however, has become much less common since the introduction of HAART for HIV infection.

The typical histologic findings in hematoxylin-and-eosin-stained sections of tissue from patients with AIDS who have disseminated MAC disease are aggregates of foamy macrophages filled with acid-fast bacilli, simulating the lepra cells seen in *M. leprae* infections (Image Plates 19-2P and 19-3I and J) or Whipple's disease when there is involvement of the gastrointestinal tract. Patients with histologic evidence of gastrointestinal (and pulmonary) involvement invariably have disseminated disease.[46] Ingestion of contaminated water or food could well be the major mode of transmission. The histology of localized MAC disease in patients without AIDS includes necrotizing inflammation, and/or granulomas with or without caseation necrosis.

MAC organisms also cause a scrofula-like cervical lymphadenitis in children.[159,175] Woods and Washington cite several other MAC infections, including granulomatous synovitis, genitourinary tract disease, cutaneous lesions, osteomyelitis, meningitis, and colonic ulsers.[370]

Members of the MAC can be recovered from many environmental sources, including water estuaries, pools, soil, house dust, plants, and bedding materials. Natural sources of water, including potable water, pose some risk for acquiring human infections.[337] Waters that have moderate salinity (1% to 2% salt) have relatively high acidity (pH 4.5 to 6.5) and are located at lower altitudes are ideal for supporting the propagation of MAC organisms.[31] Human MAC infections may occur from ingestion of contaminated water and food (the intestinal tract is thought to be the primary route of infection in HIV-infected patients) or by inhalation of organisms contained within aqueous aerosols. Wendt and coworkers[352] found MAC organisms in droplet sizes of 0.7 to 3.3 μm above freshwater surfaces, sufficiently small to reach the alveolar spaces, and organisms may become highly concentrated in jet streams emanating from air–seawater interfaces. Although poultry, swine, and other species of birds and animals become infected and excrete organisms in the feces that can remain viable for long periods in soil, animal-to-human transmission is a rare event, and human-to-human transmission has not been documented.[136]

MAC has a worldwide distribution; however, endemic areas have been found in temperate geographic areas, including the United States, Canada, Great Britain, Europe, the Netherlands, and Japan.[136] Members of MAC are the NTM most frequently associated with human disease. When HIV infection was first recognized, the overall increase in the incidence of infections due to MAC paralleled the incidence of AIDS cases. For example, of the 161,073 patients with AIDS reported to the CDC by December 1990, more than 12,000 cases of NTM infections were also reported; in 96% of these, the causative agent was a member of the MAC.[128] HIV infection is the primary risk factor for disseminated infections with this organism[224]; however, the incidence of disseminated MAC infections in HIV-infected patients has diminished dramatically since the introduction of highly active antiretroviral therapy. MAC is also an important cause of chronic pulmonary disease, typically involving three groups of patients: those who have underlying lung damage with apical fibrocavitary lesions, postmenopausal women with bronchiectasis and nodular opacities, and patients with cystic fibrosis.

Laboratory aspects. The members of the MAC may appear as 1 of 3 colony variants: (1) Smooth, opaque, and dome-shaped; (2) Smooth, transparent, and flat; and (3) Rough (Image Plate 19-2N and O). The isolates recovered from patients with AIDS commonly produce the smooth, transparent, flat colony variant, as seen in microcolony observations (Image Plate 19-2A and B). These flat, translucent colony types are thought by some to be more virulent than strains that produce other colony types.[63,280,286,312] Although *M. avium* was originally classified in Runyon group III

(nonchromogens), in the experience of many, some strains recovered from patients with AIDS have varying degrees of yellow pigmentation, intensifying as the colony ages. Doern and associates,[76]however, found that most of their strains were nonpigmented. Stormer and Falkingham have determined that the strains recovered from HIV-positive patients were nonpigmented and were more likely to be resistant to antimicrobial agents compared with pigmented strains.[312]

Microscopic examination of acid-fast smears reveals cells that are typically short and coccobacillary. Early in culture and under certain conditions, long, thin bacilli may be seen. Staining is usually uniform, without beading or banding. Phenotypically, MAC strains are best characterized by a battery of negative reactions (Table 19-7). The organism does produce heat-stable catalase and has the ability to grow on T2H-containing media; otherwise, the biochemical reactions are inert. With the availability of a nucleic acid probe for the culture confirmation of members of the MAC, the delay in obtaining a species identification by conventional methods is no longer warranted. Although it is common to perform the combination probe for MAC, it may occasionally be important to determine whether the causative agent is *M. avium* or *M. intracellulare*, since some suggest that disease with *M. avium* appears to be more severe than that caused by *M. intracellulare*.[375]

Most strains of MAC are resistant to the commonly used antituberculosis drugs. The underlying mechanism of resistance is based on the impermeability of the cell wall.[260] The synthesis of aminoglycoside- and peptide-inactivating enzymes has not been demonstrated, although some strains do produce β-lactamases.[214] The surfactant effect of Tween 80 on the cell wall may potentiate the effect of certain antimicrobial agents; ethambutol also has an effect on cell wall permeability, reflecting how this agent may work synergistically to potentiate the effect of certain other antituberculous drugs.[126] The addition of macrolides to treatment regimens has dramatically improved outcomes for patients with MAC disease.

***Mycobacterium paratuberculosis* and Crohn's Disease.** Hermon-Taylor and coauthors[123] cite the work of a Glasgow surgeon, T.K. Dalziel, who published a detailed description of chronic enteritis in humans and proposed that the disease was caused by the same microorganisms as those responsible for Johne's disease, an ulcerative intestinal condition associated with chronic diarrhea in cattle. Johne's disease had been known since 1895 and was thought to be associated with AFB, which could be seen in the tissues of infected animals but, in these early days, could not be cultured. Not long afterward, however, a slow-growing mycobacterium was finally recovered from the intestinal mucosa of infected animals, initially known simply as Johne's bacillus but later identified as *M. paratuberculosis*.

In 1984, Chiodini and associates,[48] working with Hermon-Taylor and colleagues at St. George's Hospital in London, reported the recovery of an unclassified, extremely

fastidious *Mycobacterium* species from three patients with Crohn's disease. After considerable effort, these workers identified the organism as belonging to the Runyon group III, closely related to *M. avium* and *M. paratuberculosis* complexes. Several additional cases linking *M. paratuberculosis* and Crohn's disease have been cited from the United States, the Netherlands, Australia, and France.[47] Further evidence that the Crohn's disease mycobacterium and *M. paratuberculosis* are the same was provided in yet another publication from the St. George's hospital group, who demonstrated identical DNA restriction digests between these groups of organisms.[201]

Gitnick and coauthors report the recovery of fastidious mycobacteria, including *M. paratuberculosis*, from 5 of 82

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surgically resected intestinal specimens from patients, 27 of whom had Crohn's disease.[99] These isolates required between 4 and 8 months for cultivation, which may be one reason why this organism has not been recovered in clinical laboratories. Prantera and coworkers[251] provided indirect evidence of the association of Crohn's disease with *M. paratuberculosis* by demonstrating high serum levels of antimycobacterial antibody that was demonstrated in patients following successful dapsone therapy, a phenomenon similar to that observed after treatment of classic cases of TB. With PCR and a portion of the *M. paratuberculosis* insertion sequence IS*900* as a probe, Sanderson and associates[282] were able to identify *M. paratuberculosis* DNA in 65% of rectal biopsy specimens in patients with Crohn's disease, in comparison with only 4.3% and 12.5% of patients with ulcerative colitis and healthy persons, respectively. Similarly, Fidler and colleagues[86] found that the material obtained from granulomata of Crohn's disease tissues (31 biopsy samples in the study) were much more likely to amplify *M. paratuberculosis*-specific DNA on PCR than non–Crohn's disease tissues (10 biopsies of ulcerative colitis as negative controls). If indeed mycobacteria are the cause of intestinal diseases, new methods for treatment of these conditions, notably Crohn's disease, may be in order. The association has not been proved by Koch's postulates (see Cocito and coauthors for a commentary on paratuberculosis[53]).

Mycobacterium terrae, M. gastri,* and *M. triviale. These mycobacteria are also slow-growing nonphotochromogenic bacteria that, in the past, have not generally been known as pathogens but are rarely encountered in the clinical laboratory (Clinical Correlation Box 19-11). They can be differentiated by the characteristics listed in Table 19-7. *M. triviale* colonies may resemble those of MAC or in some instances may be so rough that they are confused with tubercle bacilli; however, these strains are niacin-negative and can grow in media containing 5% NaCl. *M. triviale* also

hydrolyzes Tween 80 within 5 days, whereas MAC organisms are Tween-negative. *M. gastri* and *M. terrae* are other nonphotochromogenic species that may require biochemical differentiation (Table 19-7). *M. terrae* is also known as the "radish bacillus," since it was initially recovered from radish washings. Colonies of *M. terrae* tend to be smoother than the rough colonies of *M. triviale*.

Mycobacterium shimoidei. In 1988, Imaeda and coworkers,[135] based on DNA homology studies, officially established *M. shimoidei* as a distinct species. Additional clinical infections caused by *M. shimoidei* have been reported since the report of the index case. This organism has been reported as the cause of pulmonary infections in patients living in Australia and in Germany, the latter patient had this infection as a complicating condition of long-standing silicosis.[346] *M. shimoidei* differs phenotypically from members of the *M. terrae* complex by negative catalase and positive β-galactosidase reactions, and from *M. malmoense* by a positive acid phosphatase reaction. However, because β-galactosidase and acid phosphatase reactions are rarely performed in clinical laboratories, species identification based on phenotypic properties is difficult.

Mycobacterium malmoense. In 1977, Schroder and Juhlin recovered a new *Mycobacterium* species from four patients with pulmonary disease.[288] They called the organism *Mycobacterium malmoense*, after the Swedish city Malmo, in which these patients lived. The disease has also been noted to occur in Scotland.[90] The organism has been recovered from patients in the United States: 12 strains by Good and associates in 1980,[103] and from four patients with chronic lung disease by Albers and coworkers, in two of whom progressive disease developed[6] (Clinical Correlation Box 19-12).

Laboratory aspects. This organism typically grows slowly. Some strains are seen as soon as 2 to 3 weeks of incubation at 37°C; however, some strains may require as long as 12 weeks before colonies become visible. This need for prolonged incubation, beyond a period used in most clinical laboratories, may lead to under-detection. Typical colonies of *M. malmoense* are grayish white, smooth, glistening, opaque, and domed. They are colorless, and exposure to light does not produce pigment. On acid-fast smears, the organisms appear coccoid or as short rods without cross-bands.

CLINICAL CORRELATION BOX 19-11 *Mycobacterium terrae* and *Mycobacterium triviale*

M. terrae and *M. triviale*, although previously considered together in a complex, may also be considered separately. These mycobacteria have been incriminated in several

human infections, as cited by Woods and Washington: septic arthritis caused by *M. triviale* in an infant, synovitis and osteomyelitis caused by *M. terrae* in a young man with Fanconi's pancytopenia, and possible disseminated *M. terrae* infection in a young woman with previous miliary tuberculosis.[370] Several instances of pulmonary infection with *M. terrae* have been reported.[173,321] Krishner and coauthors report the presentation of one patient and cite six previously reported cases of respiratory infection.[168] Peters and Morice report the *M. terrae* pulmonary infection in a 64-year-old woman with ovarian carcinoma, which presented as miliary infiltrates on chest radiographs and accompanied by a rash on the extremities.[235] The lung and skin lesions resolved after 6 weeks of therapy with isoniazid, rifampin, and pyrazinamide, despite apparent "resistance" of these drugs, as determined by in vitro susceptibility testing. Petrini and coworkers[240] report a patient with *M. terrae* tenosynovitis of the hand. Both the tendon and tendon sheaths were swollen and chronic inflammation, granuloma formation, and necrosis were seen histologically. Tenosynovitis caused by this organism was also reported in the finger of a middle-aged fisherman who incurred puncture wounds while handling Crappie fins.[167]

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CLINICAL CORRELATION BOX 19-12 *Mycobacterium malmoense*

In the past few decades, *M. malmoense* has been reported with increasing frequency as a pulmonary pathogen. Infection typically occurs in patients with underlying lung diseases, often middle-aged men with pneumoconiosis.[379]Radiographic findings generally are indistinguishable from tuberculosis. Jenkins and Tsukamora[142] describe two cases of cervical adenitis, whereas Warren and associates[343] present a patient in the United States with chronic pulmonary disease. Albers and coworkers[6] believe that *M. malmoense* infections may be underreported, as some strains require 8–12 weeks or more of incubation before visible growth occurs, a period longer than cultures are held in most laboratories. A broth culture medium should be used in parallel, which has become the standard for mycobacterial culture, to improve the yield of particularly slow-growing strains.[125] Additional reports include lymphadenitis in a 5-year-old girl, septic cutaneous infection in a patient with hairy-cell leukemia, and several reported infections in HIV-positive patients.[39,51,60,244,360] In one patient, the infection was successfully treated with ethanbutol, cycloserine, and isoniazid, despite

multiresistance in in vitro susceptibility tests.[39] The lack of a clear correlation between in vitro susceptibility tests and clinical response was reported over a decade ago.[13] In the experience of these authors, omission of ethanbutol from the therapeutic regimen, even if it showed resistance in in vitro studies, has led to an unsatisfactory response.

Key biochemical test reactions for *Mycobacterium malmoense* include the following:

1. No accumulation of niacin.
2. Nitrate is not reduced to nitrite.
3. Tween 80 is hydrolyzed.
4. Catalase is produced at 68°C.
5. Pyrazinamidase is positive.

Mycobacterium haemophilum. Sompolinsky and associates[306] first recovered *M. haemophilum* in 1978 from a subcutaneous lesion of an Israeli patient with Hodgkin's disease (Clinical Correlation Box 19-13). As the species name would indicate, *M. haemophilum* requires hemoglobin or hemin for growth. Chocolate agar, 5% sheep blood Colombia agar, Mueller–Hinton agar with Fildes supplement, or LJ medium containing 2% ferric ammonium citrate is suitable for recovery of this organism. McBride and associates[198] report success in the recovery of *M. haemophilum* using a medium containing Casman base, 5% heated sheep blood, and crystal violet. Because of the remote chance for recovery of this organism in most laboratories in the United States, use of an X-factor strip in the area of inoculation on 7H10 agar, as suggested by Vadney and Hawkins, offers a suitable alternative in suspected cases.[331]

Most infections involve the skin and subcutaneous tissue, possibly reflecting the propensity of the organism to grow at lower temperature.[169] Gupta and coworkers[113] reported a patient with AIDS who had osteomyelitis and skin infection, which was successfully treated with minocycline. Kiehn and associates reported four cases in immunocompromised patients, two had AIDS and two were allogeneic bone marrow transplant recipients.[154] Because of the unique requirements for hemin and lower temperature for optimal growth, these authors suggest that *M. haemophilum* should be considered when specimens from immunocompromised patients with unexplained illness fail to grow mycobacteria under routine culture conditions or when AFB are seen on smears.[314]

Laboratory aspects. Optimal growth occurs at 28°C to 32°C; some strains grow at 20°C; little or no growth occurs at 37°C. Growth is stimulated by an incubation

atmosphere of 10% CO_2. Typical colonies may be rough or smooth after 2 to 4 weeks of incubation at 32°C on egg medium or 7H10 agar (supplemented with hemin or on the surface of which an "X-strip" has been placed, as discussed earlier). Pigment does not develop, even after exposure to light. Under the microscope, the cells are short, curved, and strongly acid-fast, without banding or beading. This organism is also

biochemically inert, with the production of pyrazinamidase the only positive reaction among the conventional tests commonly used to identify mycobacteria.

CLINICAL CORRELATION BOX 19-13 *Mycobacterium haemophilum*

Painful subcutaneous nodules, swellings, and/or ulcers that can progress into abscesses and draining fistulas are common clinical presentations of *M. haemophilum*. Rogers and coworkers[271] report instances of disseminated disease in patients with AIDS, in whom the skin lesions were multiple, involving the upper arm, hands, and feet. In a study of 13 patients with *M. haemophilum* infections culled from 7 metropolitan hospitals in New York, clinical manifestations included disseminated cutaneous lesions, bacteremia, and involvement of the bones and joints, lymphatics, and lungs.[89] The authors of this study stress that improper culture techniques may delay or result in a falsely negative culture, resulting in a missed laboratory diagnosis. It is possible that infections may be more common than realized due to these failures to culture the organism.

M. haemophilum has also been isolated from the wrist and ankles at sites of severe tenosynovitis, and from a cutaneous lesion, lymph node, and eye of a man who was the first reported patient with an *M. haemophilum* infection in Canada; the second patient was a 3-year-old Canadian girl with lymphadenitis, who was also included in this report.[192,318] Patients with lymphopenia are at particularly high risk for infections; renal dialysis and corticosteroid therapy have been shown to be predisposing conditions of *M. haemophilum* infection.[105,217]

Rapidly Growing Mycobacteria

***Mycobacterium fortuitum* Group and *M. chelonae/M. abscessus* Group.** The rapidly growing mycobacteria are ubiquitous land and aquatic organisms that

contaminate water supplies, including reagents and wash solutions used in hospitals. Clinical manifestations of infections that these mycobacteria cause are described in Clinical Correlation Box 19-14. The species of rapidly growing mycobacteria that most commonly cause human disease are *M. fortuitum* group (predominantly *M. fortuitum*) and *M. chelonae*/*M. abscessus* group (predominantly *M. chelonae* and *M. abscessus* subsp. *abscessus* [formerly *M. abscessus*]). Species within the *M. fortuitum* group are *M. fortuitum, M. peregrinum, M. senegalense, M. setense, M. septicum, M. porcinum, M. houstonense, M. boenickei, M. brisbanense,* and *M. neworleansense.* Identification to the species level requires molecular techniques, but in most instances has little clinical relevance; it may, however, be useful for epidemiologic investigations. Species in the *M. chelonae/M. abscessus* group are *M. chelonae, M. immunogenum, M. abscessus* subsp. *abscessus, M. abscessus* subsp. *massiliense, M. abscessus* subsp. *bolletii* (formerly *M. massiliense* and *M. bolletii),* and *M. salmoniphilum*. In the past, *M. chelonae/M. abscessus* complex was reported. However, differentiating *M. chelonae* from *M. abscessus* subsp. *abscessus* is recommended because the latter is more resistant to antimicrobial agents.[33,68,283,373]

CLINICAL CORRELATION BOX 19-14 Rapidly Growing Mycobacteria

M. fortuitum group and *M. chelonae/M. abscessus* group are the most clinically important of the rapidly growing mycobacteria (RGM). A wide variety of infections have been associated with these mycobacteria, including infections of the skin and subcutaneous tissue (which are the most common), bone, lung, central nervous system, prosthetic heart valve, and disseminated disease.[34,89,370,297] Wallace and Brown reviewed 100 isolates of *M. chelonae* from skin, soft tissue, and bone over a 10-year period.[339] Cutaneous infection (53%), localized cellulitis or osteomyelitis (35%), and catheter-associated infections (12%) were most commonly found. Underlying risk factors for infections included organ transplantation, rheumatoid arthritis and other autoimmune disorders, and trauma, including invasive medical procedures. Previously, Wallace and coworkers[341] had reviewed 125 human infections caused by RGM. Fifty-nine percent of these cases involved the skin and soft tissues (e.g., postsurgical wound infections, accidental trauma, and needle injections).

Nosocomial outbreaks of *M. fortuitum* skin infections have been associated with exposure to a contaminated ice machine and to contamination of an automated bronchoscope disinfection machine.[91,178] A clinically distinctive cutaneous syndrome associated with infection caused by the then *M. chelonae* complex (*M. abscessus* was not routinely distinguished from *M. chelonae* at that time) in kidney-transplant

recipients, consisting of indolent, tender, nodular lesions, on the extremities has been reported.[59] Sporotrichoid spread of these infections may occur in immunosuppressed patients, and, rarely, cutaneous infection with RGM may represent the extension of disseminated disease.[77,218] Cutaneous infections due to RGM also have been associated with tattoo ink.[252]

Several other clinical entities caused by RGM have been reported. Keratitis has been reported in wearers of both soft and hard contact lenses.[30,151] Most of these infections followed trauma, and topical antibiotic therapy often is not successful; therefore, keratoplasty may be required to effect a cure. It has been suggested that acid-fast smears of corneal scraping be performed on any patient with chronic corneal ulcers.[265] Patients receiving continuous ambulatory peritoneal dialysis are also at risk of acquiring peritonitis caused by RGM.[49,81,164,204,307] Therefore, it is reasonable to perform mycobacterial cultures on patients with peritonitis associated with continuous ambulatory peritoneal dialysis when routine cultures fail to reveal organisms. RGM should be considered when Gram's stain smears prepared from isolates recovered from peritoneal or dialysis fluids show what appears to be poorly characterized diphtheroids or beaded, gram-positive bacilli. Removal of the catheter, drainage of fluid collection, and appropriate use of antimicrobial agents usually result in rapid cure.

RGM may cause pneumonia, with pulmonary infections caused by *M. abscessus* being particularly serious.[341] RGM are ubiquitous in many water sources and may colonize the respiratory tract of patients who have compromised local defense mechanisms, who are debilitated or immunocompromised, or who have long-standing chronic obstructive pulmonary disease. Burns and associates[35] studied an outbreak of positive *M. fortuitum* sputum cultures among 16 patients being treated on an alcoholism rehabilitation ward. Pulsed-field electrophoresis of large genomic DNA restriction enzyme fragments disclosed that the 16 isolates were identical. The point source was found to be a tap connected to the water line supplying the showers being used by these patients; no further cases occurred after the showers were disconnected and decontaminated.

Several reports of catheter-associated infections caused by RGM have been made. Raad and coworkers[255] reviewed 15 infected patients with cancer, 9 were infected with *M. fortuitum*, whereas 6 had contracted *M. chelonae*. Four patients with bacteremia and associated catheter infections recovered after removal of the catheter and prompt institution of antibiotic therapy. Bacteremia recurred in seven patients in whom the catheter was

left in place and who were treated with antibiotics alone. Patients with infections of the catheter tunnel required surgery for cure. Patients with mycobacteremia are at

risk to develop infective endocarditis, particularly if they have a prosthetic valve or valvular abnormalities.

Mycobacterium chelonae infections have also been reported in patients with febrile neutropenia, which is a risk factor for disease.[202]

Other infections caused by the RGM include *M. chelonae* otitis media due to transfer of the organism between patients from contaminated instruments, aortitis following aortic valve replacement, sternal wound infection, endocarditis, cardiac bypass-related infections, hepatitis, synovitis, retroperitoneal abscess complicating a gunshot wound to the flank, and disseminated *M. fortuitum* infection in a patient with AIDS.[133,189,270,287,300,351,370] *Mycobacterium smegmatis* posttraumatic soft tissue infections were reported in a 21-year-old man and a 29-year-old woman who were involved in separate motor vehicle accidents.[223] The former patient presented with a draining left leg lesion and inguinal adenopathy and the latter with a chronically draining subcutaneous area of cellulitis of the posterolateral thigh. The authors also cite 12 cases of *M. smegmatis* infections reported in the literature, dispelling doubt that this organism is nonpathogenic. Finally, Newton and Weiss subsequently reported *M. smegmatis* as a cause of aspiration pneumonia.[222]

The RGM vary in their in vitro susceptibility to antimicrobial agents. For example, most isolates of *M. fortuitum* group are susceptible to trimethoprim/sulfamethoxazole, whereas virtually all *M. chelonae/M. abscessus* group are resistant. Most of the *M. fortuitum* group, *M. smegmatis* group, and *M. mucogenicum* are susceptible to imipenem, most *M. chelonae* are susceptible to tobramycin, and most *M. abscessus* are susceptible to amikacin. Antimicrobial susceptibility testing should be performed on all clinically significant isolates of RGM, following Clinical and Laboratory Standards Institute guidelines.[367]

Laboratory aspects. Mycobacteria are categorized as rapid growers, by definition, if they grow on a solid medium within 7 days after subculture. The young colonies appear smooth and hemispherical, usually with a butyrous or waxy consistency. Colonies of *M. fortuitum* group and *M. chelonae/M. abscessus* group are typically nonpigmented but may appear off-white or faintly cream-colored (Image Plate 19-2Q). *M. fortuitum* may produce branching, filamentous extensions from 1- to 2-day-old colonies on cornmeal–glycerol or Middlebrook 7H11 agar. Some strains produce rougher colonies with short aerial hyphae, best observed under a stereomicroscope. *M. chelonae* lacks these filamentous extensions.

The above mycobacteria often have the capability of growing on MacConkey agar without crystal violet. They also appear as smooth, dome-shaped colonies that may have a slight pink pigmentation (Image Plate 19-2R). Additionally, they may grow on routine 5% sheep blood agar, initially appearing as tiny pinpoint colonies (Image Plate 19-2S). Microbiologists must be alert to this possibility and perform acid-fast stains in

addition to Gram's stains if the correct identification is to be made.

Microscopically, in acid-fast-stained preparations, the bacterial cells are generally pleomorphic, ranging from long, filamentous forms to short, thick rods. Branching typically is absent or rudimentary; at times the cells may appear beaded or swollen, with nonstaining ovoid bodies present at one end. Some strains of *M. fortuitum* may grow within 48 hours on routine 5% sheep blood agar. The bacterial cells appear as short, slender, filamentous, faintly staining gram-positive bacilli (Image Plate 19-2T).

Silcox and associates[297] have identified the following characteristics for an isolate to belong to the rapidly growing mycobacteria:

1. Acid-fast
2. Lack of pigment production
3. Growth on solid media in less than 7 days at its optimal temperature
4. Evidence of arylsulfatase activity at 3 days
5. Growth at 28°C on special MacConkey agar (devoid of crystal violet)

Accurate identification of rapidly growing mycobacteria, however, requires molecular methods (described later in this chapter).

Infrequent Human Pathogens. Rapidly growing mycobacteria that infrequently or rarely cause human disease are *M. mucogenicum* group (*M. mucogenicum, M. auagnense,* and *M. phocaicum*), *M. mageritense, M. wolinskyi*, the early-pigmented rapidly growing mycobacteria (*M. neoaurum, M. canariasense,* and *M. cosmeticum*), and the pigmented rapidly growing mycobacteria *M. smegmatis, M. goodie,* and *M. thermoresistible,* the latter of which has the unique ability to grow at 52°C. The infrequency of reports describing disease caused by *M. thermoresistible* in humans indicates that exposure is either minimal or the organism is of very low virulence. Weitzman and coauthors reported an infection by *M. thermoresistible* in an immunocompromised woman with fever, cough, and weight loss.[351] Cavitary pulmonary disease was seen on radiographs. The organisms were recovered from sputum and a bronchoscopy specimen. Histologic examination of a lung biopsy revealed numerous microabscesses and granulomas with Langhans giant cells. Wolfe and Moore reported a breast infection in a woman following augmentation mammoplasty.[363]

Other Mycobacteria

Mycobacterium ulcerans. Most cases of *M. ulcerans* infections have been reported from Central and West Africa, Malaysia, New Guinea, Guyana, Mexico, and Australia (Clinical Correlation Box 19-15). The name "Bairnsdale ulcer" has been used for the cutaneous lesions of *M. ulcerans* infections, named after the Australian town where the organism was first recognized by Alsop and Searls in the 1930s.[256] Most human

infections occur in the tropical regions following rain forest disturbance.[120] It is postulated that the mycobacteria are carried from the soil into draining lacustrine systems, where they multiply over a period of months or years. Humans become infected by contacting organisms from these contaminated estuaries.

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CLINICAL CORRELATION BOX 19-15 *Mycobacterium ulcerans*

Infection with *M. ulcerans,* termed Buruli ulcer, typically presents as a "boil" or lump under the skin on the lower extremities, which usually develops at the site of previous trauma. This, then develops into a shallow, nonhealing ulcer, with a necrotic base within a few weeks. Lesions are generally painless, unless they become secondarily infected with bacteria. Some lesions may be quite severe, with avascular coagulation necrosis extending deep into the subcutaneous fat.[134] Satellite nodules that ulcerate may also develop.

Disease has been reported from at least 33 countries. These are located in Africa, South America, and the Western Pacific. Although Delaporte et al.[72] reported one of the first *M. ulcerans* infections in a patient with AIDS, it is not a marker for that disease. Buruli ulcer is one of the 17 neglected tropical diseases. This infection is progressive and causes significant morbidity, disability, and disfigurement. Up to 80% of patients are thought to be curable, if treated with the appropriate antimicrobial agents. Up-to-date information about Buruli ulcer is available from the World Health Organization at http://www.who.int/topics/mycobacterium_ulcerans/en/.

Laboratory aspects. *M. ulcerans* grows optimally at 33°C and not at all at 37°C. Rough, lightly buff or nonpigmented, convex to flat colonies, with irregular outline, often simulating those of *M. tuberculosis*, develop after 6 to 12 weeks. Microscopically, the acid-fast cells are moderately long and rod-shaped, without banding or beading. *M. ulcerans* is biochemically inert, showing only heat-stable catalase activity among the several tests usually used to identify mycobacteria.

Mycobacterium genavense. *M. genavense* is an uncommonly recovered, slow-growing, nonpigmented, NTM that causes infection in patients with AIDS. The organism was first recognized by Boettger and associates[27] by finding a unique pattern using DNA extracted from mycobacteria growing in BACTEC 13A blood culture media. Amplified gene fragments were sequenced directly, and electrophoretic profiles were determined in 0.8% agarose gel stained with ethidium bromide. The authors

studied 16 isolates that had a unique DNA sequence profile and officially designated the organism *M. genavense* after the 28-year-old patient with AIDS, who was living in Geneva, from whom the first isolate was recovered. Subsequently, Coyle and associates,[61] using assays for sequencing of the gene for 16S rRNA that had been extracted from unidentified mycobacterial isolates of 15 blood cultures obtained from seven patients with AIDS, also confirmed the identity of *M. genavense* as a separate species. The sequence pattern was most closely related to *M. simiae*; however, the clinical presentation (fever, weight loss, diarrhea, hepatosplenomegaly, and anemia), response to therapy, and autopsy findings more closely resemble infections with MAC.[22] Histopathologically, *M. genavense* in HIV-positive patients produces lesions characterized by masses of foamy histiocytes and ill-defined granulomas, the development of which depends on the immunologic reactivity of the host.[197] In one autopsy series, the small intestine, spleen, liver, and lymph nodes were most commonly involved; the lungs, myocardium, and kidney were spared, a distribution similar to that seen in patients with disseminated disease caused by a member of the MAC.

Although the initial isolates were identified in specimens obtained from patients with AIDS in Switzerland, disseminated infections caused by *M. genavense* have been described from elsewhere in Europe, North America, and Australia.[27,234] This geographic distribution may reflect more the awareness by health workers of this new species rather than select areas of endemicity. *M. genavense* may be underrecognized because it grows only in broth culture media or on a solid medium to which mycobactin J (which is not available in most clinical laboratories) has been added, and it requires prolonged incubation.

The Detection and Identification of Mycobacteria by Molecular Methods

In the past several years, most clinical mycobacteriology laboratories have discontinued the time-consuming and tedious task of performing biochemical tests to make a species identification of mycobacteria recovered in culture. More and more often, nonculture methods based on molecular biology are being used in clinical laboratories for the identification of *Mycobacterium* species isolates recovered in culture. For example, the use of nucleic acid probe assays has virtually replaced conventional biochemical testing in the identification of MTBC, MAC, *M. kansasii,* and *M. gordonae*.[82,78,152,180,220,206,264,284,327]

There are four major applications for molecular techniques that are available for use in clinical laboratories:

1. Culture confirmation of isolates recovered from clinical specimens using DNA probes.
2. The identification of mycobacteria through DNA sequencing or other molecular techniques.
3. The direct detection of MTBC in respiratory and extrapulmonary specimens using nucleic acid amplification assays.
4. DNA fingerprinting and strain typing of *Mycobacterium* species.

The medical literature relative to molecular diagnostics as applied to the identification of the mycobacteria is vast. Although the molecular tools used in different

mycobacteriology laboratories vary, virtually all modern mycobacteriology laboratories use molecular diagnostics to some extent. Study of these techniques also provides a fundamental understanding of the basic biology of mycobacteria and helps to determine the best role for newer techniques when they are introduced into the clinical laboratory. The detection and characterization of mycobacteria by molecular methods may be separated into nonamplification- and amplification-based methods. After amplification, the product(s) of amplification may be analyzed by DNA sequencing to acquire identification information or by other techniques or typing. The salient features of these applications are described below.

Signal Amplification Methods

Nucleic Acid Probes. Nucleic acid probes were the first nucleic acid-based technology to be used routinely in the clinical microbiology laboratory for the identification of mycobacteria in positive cultures. These nonisotopic probes were pioneered and brought to market by Gen-Probe (now Hologic, San Diego, CA). The accuracy, sensitivity, and specificity of these probes are very high when used to identify mycobacteria in culture.[78,104,152,180,206,220,284] Nucleic acid probes are available for the identification of MTBC, MAC, *M. kansasii,* and *M. gordonae*. Separate probes are also available for *M. avium* and *M. intracellulare,* if the differentiation of these mycobacteria is desired.

In brief, this technology uses acridine ester-labeled single-stranded DNA probes that hybridize with the ribosomal RNA (rRNA), which is released from the test bacterium through the action of a lysing agent, heat, and sonification. Ribosomal RNA is a useful genetic target for the identification of microorganisms, since it often contains signature sequences and is present in the cells and culture in high quantities secondary to the growth of the organism (i.e., biologic amplification). The DNA probe and rRNA hybridize according to traditional Watson and Crick base-pairing for a stable DNA–

RNA complex. After the inactivation of the unhybridized probe, a signal-generating step is performed and light is produced and recorded by an instrument. The light produced is proportional to the amount of probe present, and a predetermined threshold is used to determine positivity. The 2 hours required for the probe-based determination of the species for which genetic probes are available represents a significant advance compared with identification by traditional methods.[206,284] The commercial kits have an extended shelf-life, which also extends the usefulness to a large number of laboratories.

One advantage recognized early was the use of these probes on positive broth cultures, which recover mycobacteria more rapidly than solid media in most instances.[82,152,237,264] In an effort to decrease the time of detection even further, Forbes and coworkers[88] applied PCR amplification to detect *M. tuberculosis* recovered in BACTEC 12B broth cultures. By using PCR, positive BACTEC 12B vials could be assayed when the growth index (GI) reached 10, shortening the time of incubation required for the GI to reach 100 or more. The use of PCR in this study resulted in a mean time to detection of *M. tuberculosis* of 9 days, compared with 14 days using nucleic acid probes from growth of BACTEC 12B subcultures on solid media. The observation of cord formation in acid-fast stains performed on broth from signal-positive cultures can be used as a guide to immediately select the MTBC probe assay.[148]

Nucleic Acid Amplification Methods

Commercially Available Applications. The burden of organisms in rare instances is large enough in the clinical specimen to achieve detection with a chemiluminescent genetic probe. However, in most instances, this method is too insensitive for direct use on clinical specimens. A nucleic acid amplification–based assay is recommended if identification of mycobacteria is to be attempted directly from the clinical specimens. In the late 1990s, two commercially available nucleic acid amplification assays for the detection of MTBC received approval by the FDA for use on respiratory specimens. These tests, the Amplicor *M. tuberculosis* PCR assay (Roche Diagnostics, Indianapolis, IN) and the Amplified *Mycobacterium tuberculosis* Direct test (AMTD), which uses transcription-mediated amplification, had both been approved for use with smear-positive specimens, whereas only the latter was been approved for use on smear-negative respiratory specimens. This represented the culmination of several developmental studies and field trials over several years, leading to products that are standardized and designed in such a way that problems of contamination with extraneous DNA and inhibition of amplification reactions by endogenous inhibitors are minimized. Nucleic acid amplification assays perform well on smear-positive specimens, but not unexpectedly the sensitivity is lower with smear-negative

respiratory specimens, when compared with culture.[20,25,67,73,132,228,241,242,281,303,338]

It is beyond the scope of this textbook to review all the published works of other researchers in the evolution of AMTD and PCR applications to direct testing of specimens for mycobacteria, but there are many. Some of the studies for each of these FDA-approved technologies are described in the following paragraphs.

One of the early field trial studies of the Gen-Probe AMTD test (now Hologic, San Diego, CA) was published by Jonas and coauthors in 1993.[146] In a study of 758 processed sputum sediments, 119 (16%) of which were positive for *M. tuberculosis*, the Gen-Probe assay performed with a sensitivity, specificity, positive and negative predictive value of 82%, 99%, 97%, and 96%, respectively, which were comparable with the results derived from culture and better than from smear analysis.

Miller and coworkers[209] performed a retrospective evaluation of three separate respiratory specimens from each of 250 patients using the AMTD, comparing the results with those of microscopy, culture, and patient chart review. Of these patients, 198 (from whom 594 specimens were collected) were negative for *M. tuberculosis* by culture and clinical criteria, whereas 52 were positive (156 specimens). The overall specificity of the AMTD was 98.5%. Of the 156 specimens obtained from the patients with TB, organisms were recovered from 142 (91%), acid-fast microscopy was positive for 105 (67.3%), and the AMTD was positive for 142 (91%). When all three specimens from each patient were tested, AMTD detected all 52 patients that were positive for TB.

In a study of 938 respiratory specimens, Pfyffer and associates[242] found that the AMTD test performed with a

sensitivity of 93.9%, a specificity of 97.6%, a positive predictive value of 80.7%, and a negative predictive value of 99.3% after resolution of discrepant results by chart review. These authors concluded that the AMTD was highly sensitive and specific for detecting MTBC organisms within a few hours. The results of another study, however, were less encouraging.[25] Of 617 respiratory tract specimens, 590 were culture- and AMTD-negative. Twenty-one cultures (3.4%) yielded *M. tuberculosis*; of these, 15 (71.4%) were detected by AMTD and 6 were missed. *M. tuberculosis* did not grow in culture from six AMTD-positive specimens (28.6%) obtained from three patients under treatment for TB. Thus, the sensitivity, specificity, and negative and positive predictive values for AMTD were 71.4%, 99%, 99%, and 71.4%, respectively. The test was judged to be easy to perform and highly specific by these authors, but lacking in sensitivity. They suggested that inclusion of an internal amplification control may be helpful.

Vuorinen and associates[338] conducted studies that compared the performance of the Gen-Probe product with the Roche Amplicor *M. tuberculosis* Test (Roche Molecular Diagnostics, Indianapolis, IN), prior to FDA approval of the latter assay in the United States. These authors tested 256 respiratory specimens obtained from 243 patients for the presence of MTBC with both the AMTD and PCR Roche Amplicor *Mycobacterium tuberculosis* Test (Amplicor PCR). When compared with the results of culture performed in parallel, the sensitivities of staining, AMTD, and Amplicor PCR were 80.8%, 84.6%, and 84.6%, respectively. The specificities for these three tests were 99.1%, 98.7%, and 99.1%, respectively. The conclusions of these authors were that both nucleic acid amplification methods were rapid, sensitive, and specific for the detection of *M. tuberculosis* in respiratory specimens.

The following are brief summaries of a few other studies of the PCR Roche Amplicor *Mycobacterium tuberculosis* Test. Ichiyama and associates,[132] in a parallel study of 422 sputum samples obtained from 170 patients with mycobacterial infections, also found that the AMTD system and the Amplicor *Mycobacterium* system performed equally well, with 98.7% agreement. D'Amato and colleagues[67] tested the Roche Amplicor test on 985 specimens from 372 patients. The sensitivity, specificity, and positive and negative predictive values compared with culture and clinical diagnosis were 66.7%, 99.6%, 91.7%, and 97.7%, respectively, comparable with culture results. The authors cite the great advantage of having test results available approximately 6.5 hours after the specimen arrives in the laboratory. Wobeser and coworkers[362] found that the Amplicor PCR assay performed with sensitivity, specificity, and positive and negative predictive values of 79%, 99%, 93%, and 98%, respectively, in a study of 1,480 clinical specimens obtained from 1,155 patients. In smear-positive specimens, the sensitivity was 98%, versus 59% for smear-negative specimens. The sensitivity and specificity of specimens demonstrating a positive GI on the BACTEC 460 system were 98% and 100%, respectively. Delacourt and associates,[71] in a study of 68 children with various stages of tuberculosis, found that PCR was positive in 83.3% of 199 specimens obtained from children with active disease, but also in 38.9% of children without symptoms.

In 2012, a third nucleic acid amplification assay—the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA)—was cleared by the FDA for use in the United States. The assay has two components: (1) a plastic cartridge that contains liquid sample processing and PCR buffers and lyophilized real-time PCR reagents and (2) the GeneXpert instrument, which performs sample processing and real-time PCR in a hands-free manner. This assay simultaneously detects *M. tuberculosis* and rifampin resistance by PCR amplification of the 81-bp fragment of the *M. tuberculosis rpoB* gene and subsequent probing for mutations associated with rifampin resistance. The internal control is a hemi-nested molecular beacon assay to detect *Bacillus globigii* DNA. Approved specimen types are raw sputum and concentrated sediments prepared

from sputum. Total hands-on time is less than 5 minutes per specimen, and results are available in less than 2 hours. The sensitivity versus culture when testing sputum specimens was 98% to 100% for AFB smear-positive samples and 69% to 72% for smear-negative samples.[26,121,380] Specificity of the assay in these two studies was 100%.

An additional amplification assay for MTBC—the *M. tuberculosis* BD ProbeTec (BD Diagnostic)—is available for use outside the United States; it is not FDA-approved. This assay, which uses strand displacement amplification, has been used for direct detection of MTBC in respiratory specimens, as well as in positive cultures.[15,18,342] Barrett et al.[15] studied the direct respiratory specimens of 205 patients who had a high probability of having TB. Of the 109 patients with culture-proven TB, 101 were positive by the BD ProbeTec assay, yielding a sensitivity of 92.7%. There were three false-positive reactions reported in this study from patients with NTM, giving a specificity of 96%.

The use of commercial products does not preclude users from encountering problems in implementation and use of the test. Manufacturer's recommendations must be closely followed and quality control standards must be rigidly enforced. These tests are not a replacement for culture. There are still instances in which MTBC is recovered from culture but not detected by molecular methods. Furthermore, complete molecular profiling for all the determinants of resistance is not yet available, so phenotypic susceptibility testing must be performed. Culture methods must be run in parallel for each specimen received, the clinical aspects of each case must be closely monitored, and communication between the laboratory and primary care physicians must be maintained throughout. With all of these provisions in place, the ability to diagnose new cases of TB early has a major impact on therapy and infection control, limiting person-to-person transfer of disease.

Laboratory-Developed PCR Assays, Including Real-Time PCR. In addition to FDA-approved assays, numerous laboratory-developed (formerly termed "home-brew") nucleic acid amplification assays have been developed. These applications, although not approved for use by the FDA, may be important diagnostic tools in the hands of the skilled user. The potential for shortening to hours, rather than days and weeks, the detection and identification of MTBC and perhaps other *Mycobacterium* species directly in a variety of clinical specimens make these assays attractive. The cost of laboratory-developed amplification assays is often considerably less than comparable commercially available products, but the appropriate validation of the assay resides with the user of the test.

Nolte et al.[226] developed one of the earliest PCR assays for the rapid diagnosis of pulmonary TB; in this paper the authors described important background information that would be of interest to those who wish to learn more about developing and setting up a PCR assay. Some of the potential problems that must be addressed with nucleic acid–based amplification assays are inhibition of amplification and the reproducibility of the assay. These were addressed in a multi-laboratory study reported by Noordhoek and coauthors.[227] They circulated 200 sputum, saliva, and water samples containing known numbers of *M. bovis* BCG cells along with negative controls for PCR analysis, among seven laboratories that used the IS*6110* insertion sequence as the target for DNA amplification. Each laboratory used its own protocol for pretreatment, DNA extraction, and detection of the amplification product. High levels of false-positive PCR results were found among the participating laboratories, ranging from 3%to 20%, with an extreme of 77% in one laboratory. This relatively poor performance resulted from lack of monitoring of each step of the procedure and underscores the necessity for careful quality control during all stages of the assay. In addition, inhibition of amplification and the erroneous reporting of false-negative results may be a problem with PCR. For this reason, the use of an internal amplification control, or the documentation and monitoring of the false-negative rate of an assay, is required for laboratory accreditation by the College of American Pathologists for laboratories that use these tests.

Real-time nucleic acid amplification is a more rapid method of performing PCR or other methods of amplification, wherein amplification and detection occur in the same reaction vessel. This vessel is closed, which significantly decreases the chance of amplicon contamination of the laboratory. Several assays have been designed for the rapid detection of *M. tuberculosis*, using most of the available formats.

Assays have been described using the LightCycler system (Roche Diagnostics, Indianapolis, IN). Some of these are specific for *M. tuberculosis* using a single set of fluorescence resonance energy transfer (FRET) probes.[166,208] Miller et al.[208] examined 135 AFB-positive specimens, of which 105 grew *M. tuberculosis*. Both the LightCycler assay and the Amplicor *M. tuberculosis* PCR test to which it was compared detected 103 of 105, yielding a sensitivity of 98.1%, each. They also studied 232 BacT/Alert MP culture bottles of respiratory specimens, 114 of which were positive for *M. tuberculosis*. All the positive cultures were detected by the LightCycler assay.

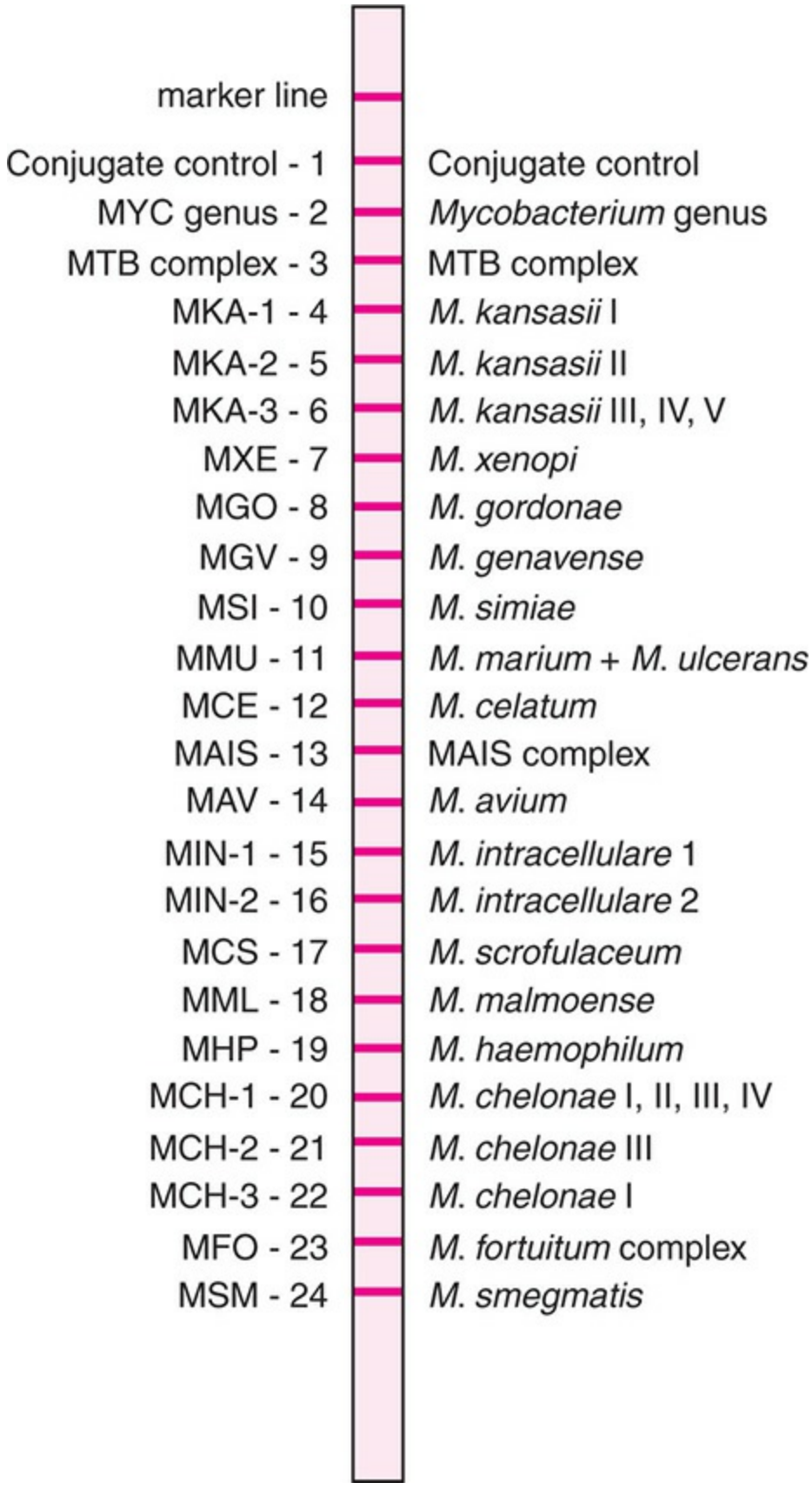
Shrestha et al.[295] took another approach and designed a broad-range PCR to detect all mycobacteria, but to differentiate *M. tuberculosis* from NTM based on postamplification melt-curve analysis. After amplification is complete, melt curves may be generated with some types of real-time amplification assays. Assays that use FRET probes produce very distinct melt curves that distinguish even a single nucleotide difference in the probe hybridization sites. Shrestha et al. used this feature to

differentiate *M. tuberculosis* from NTM using both 186 cultured isolates and 50 clinical specimens that were culture-positive for *M. tuberculosis*. Both the LightCycler assay and the Amplicor *M. tuberculosis* PCR test to which it was compared detected 48 of 50 specimens.

The results of real-time PCR assays are innately quantitative, and when used with standards the definitive amount of an organism present may be determined. Kramme and associates[165] describe a real-time PCR assay, wherein not only the presence of the organism is detected, but an indication of the amount of organism present could be determined. Similarly, Rondini et al.[274] have developed a hydrolysis probe–based assay (i.e., a Taqman assay) for the detection of another important cutaneous mycobacterial pathogen, *M. ulcerans*.

Postamplification Analysis

Reverse Hybridization/Line Probe Assays. Reverse hybridization is similar in many ways to the traditional Southern blot, wherein the amplicon-probe hybridization reaction occurs on a nitrocellulose or similar substrate. In this technology, the target sequences are first amplified by PCR using biotinylated primers. The multiple probes are immobilized on a nitrocellulose strip, and the amplified product is applied to the strip, which is the reverse of a Southern blot. Lines form at the site of amplicon-probe hybridization. When this pattern is compared with a key, one can interpret the results of this reaction (Fig. 19-2). The advantage over traditional Southern blotting is that numerous probes are assayed simultaneously and radioisotopes are not used.


marker line
Conjugate control - 1
Conjugate control
MYC genus - 2
Mycobacterium genus
MTB complex - 3
MTB complex
MKA-1 - 4
M. kansasii I
MKA-2 - 5
M. kansasii II
MKA-3 - 6
M. kansasii III, IV, V
MXE - 7
M. xenopi
MGO - 8
M. gordonae
MGV - 9
M. genavense
MSI - 10
M. simiae
MMU - 11
M. marium + M. ulcerans
MCE - 12
M. celatum
MAIS - 13
MAIS complex
MAV - 14
M. avium
MIN-1 - 15
M. intracellulare 1
MIN-2 - 16
M. intracellulare 2
MCS - 17
M. scrofulaceum
MML - 18
M. malmoense
MHP - 19
M. haemophilum
MCH-1 - 20
M. chelonae I, II, III, IV
MCH-2 - 21
M. chelonae III
MCH-3 - 22
M. chelonae I
MFO - 23
M. fortuitum complex
MSM - 24
M. smegmatis

■ **FIGURE 19-2** The INNO-LiPA MYCOBACTERIA V2 (Innogenetics, Gent, Belgium) reverse hybridization assay is demonstrated. This strip contains a conjugate control line, and hybridization probes to determine the presence of mycobacteria at the genus level and identification information for 16 different species. (The photograph is from the company package insert.)

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Reverse hybridization technology is commercially available as line probe assays for the rapid identification of mycobacteria. These assays perform best using colonies on a solid medium or broth from a positive liquid culture, although direct testing of AFB smear-positive specimens is possible. The first-version reverse hybridization assay examined was the LiPA Mycobacteria assay (Innogenetics). This assay used a broad-range PCR directed against the 16S to 23S spacer region and probes for MTBC, MAC, and the following mycobacterial species: *M. avium, M. intracellulare*, *M. kansasii*, *M. chelonae* group, *M. gordonae*, *M. xenopi,* and *M. scrofulaceum*. Miller et al.[210] studied 60 clinical isolates of mycobacteria from 59 patients using this product and found no discrepancies with routine laboratory identification with the majority of the isolates, although some were not identified to the species level. Of the isolates tested, 26 were MTBC, 9 *M. avium*, 3 MAC, 3 *M. kansasii*, 4 *M. gordonae,* and 5 *M. chelonae* group isolates. Three patient samples had positive reactions for MAC and were subsequently determined to have *M. intracellulare* by PCR–restriction-fragment-length polymorphism (RFLP) analysis. Seven additional mycobacterial species were identified only to the genus level (*Mycobacterium* spp.); six of these were *M. fortuitum*, and one was *M. szulgai*. Several other groups have examined this product with similar results, and during these studies they demonstrated minor problems that led to the development of a new, improved version.[315,324,325] PCR followed by reverse hybridization has also been used on direct clinical specimens, with excellent results from smear-positive specimens.[145] The newer version, the INNO-LiPA Mycobacteria v2, has expanded the number of identifiable mycobacterial species to 16. Tortoli et al.[324] tested 197 mycobacteria belonging to 81 taxa with this product and found 100% specificity and sensitivity for 20 out of 23 probes. The probes specific for *M. fortuitum* complex, for the MAC–scrofulaceum group, and for *M. intracellulare* type 2 demonstrated cross-reactivity with several mycobacteria that are rarely isolated from clinical specimens. The overall sensitivity was 100% and the overall specificity was 94.4%.

Reverse hybridization assays have also been shown to be useful for the detection of the genetic determinants of resistance to various antituberculosis agents.[4,42,70,144,293,305] As with species identification, detection of the genetic

determinants of resistance has also been achieved directly from the clinical specimen.[144] Although performance is good, especially for rifampin, sensitivity and specificity are less than 100%, so susceptibility testing using a phenotypic method still must be done.

DNA Sequencing. DNA sequencing for the analysis of an amplified product is now a common method of postamplification analysis. Although useful, this technology is more complicated than simple probe hybridization and often requires the user to have experience with sequence alignment and editing software, and genetic databases. Analysis of these variable regions interspersed between conserved regions that serve as broad-range primer hybridization sites becomes a powerful tool for microorganism identification.[29,94,101,106,176,259,289,372]

Traditional DNA sequencing, also known as Sanger sequencing, was once used solely in research laboratories, but it has also become commonplace in many molecular pathology and molecular microbiology laboratories. These methods have been used successfully for the identification of bacteria, mycobacteria, nocardia, and fungi. The genes that encode for the ribosomal subunits of these organisms are the most commonly used genetic targets of sequence-based identification.

This technology is revolutionizing the laboratory identification of slow-growing microorganisms such as mycobacteria and nocardia.[52,115,150,203,233,239] One of the most useful regions of the 16S gene complex for the identification of mycobacteria involves the hypervariable A region. This has been used to achieve rapid and accurate sequenced-based identification of the most common clinically relevant mycobacteria.[75,119] It has also been used to help identify and differentiate more difficult to characterize isolates.[308] This region has also been used successfully for the identification of most clinically relevant mycobacteria using pyrosequencing.[14]

MicroSeq (Applied Biosystems, Inc. [ABI], Foster City, CA) is a commercially available system for the identification of microorganisms by DNA sequencing. After cultivation, a PCR is performed using the primers provided. The sequence is then determined through ABI capillary-based sequencing, and the product is submitted as a query to a genetic database that is maintained and updated by the manufacturer. Hall et al.[115] examined this database using 59 ATCC strains and 328 clinical isolates. Sequence-based identification correctly identified, based on what is called a distance score of <1%, 98.3% (58 of 59) of the ATCC strains to the correct group or complex and 90.1% (219 of 243) of the clinical isolates. Of the remaining isolates that had distance scores of >1%, 41.1% (35 of 85) were identified to the appropriate species, complex or group level, whereas 15.3% (13 of 85) were identified to the species level. Most significantly, they report a turnaround time for mycobacteria identification of less than 24 hours, which is phenomenal when one considers the time to identification of

traditional phenotypic methods.

The *rpoB, hsp,* and *tuf* genes have also been used successfully as the genetic targets for sequence-based identification for a variety of bacteria.[79,80,150,174,229,231,267] The *rpoB* gene, which encodes for DNA-dependent RNA polymerase, has been used by several groups for the identification of mycobacteria.[157,158,181,329] Sequencing this genetic target is popular, since it not only provides identification information, but also provides information regarding the susceptibility of the isolate to rifampin.[305,336]

Microarray Analysis. Microarrays, devices commonly referred to as gene chips, have been used extensively for research. These have also been used for the identification of mycobacteria and the detection of the genetic determinants of resistance. Troesch et al.[329] describe the use of a microarray that examined two genetic regions, the 16S rDNA and the *rpoB* gene. They examined 70 mycobacteria representing 27 different species and 15 rifampin-resistant isolates of *M. tuberculosis* with this microarray and were able to identify 26 of 27 species and all of the resistant mutants. Microarrays hold great promise, but they are expensive, and currently remain a research tool.

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Strain Typing and DNA Fingerprinting. The advent of molecular techniques permits strain-specific analysis of *M. tuberculosis* isolates for purposes of epidemiologic studies. The insertion sequence, IS*6110*, was specifically identified as the target of a DNA probe to be used in fingerprint analysis.[40,41] The IS*6110* was conserved in all of the *M. tuberculosis* strains studied and was present in high copy numbers. The specific fingerprint technique described involved digestion of the genomic DNA with the restriction endonuclease *Bam*HI, followed by separation of the fragments by agarose gel electrophoresis, transferring the DNA fragments to a nylon membrane and hybridizing the membrane with cloned DNA segments representing two different portions of IS*6110*.

Several workers have used the insertion sequence IS*6110* as the target for conducting epidemiologic studies. Yang and associates[377] compared the fingerprinting patterns of 68 mycobacterial isolates obtained from HIV-seropositive patients with TB in the Dar es Salaam region of Tanzania, with 66 isolates recovered from HIV-negative patients living in the same region. They observed 101 fingerprint patterns among this group of patients, with the level of diversity equal between the two. Of these isolates, 8.8% showed resistance to at least one drug, again, with no tendency to cluster within either of the groups. In a study of the IS*6110* fingerprint patterns of *M. tuberculosis* recovered from 64 patients living in French Polynesia, 11 separate clusters were identified.[322] Clustering of strains with identical patterns were identified among family

groups, indicating that active transmission plays as significant a role as reactivation disease in French Polynesia. Similarly, in a subsequent study, a high degree of likeness was found in IS*6110* fingerprint patterns among closely related individuals.[376] One of the prevalent IS*6110*-defined clusters in Greenland accounted for 91% of 245 cases of TB in Denmark collected during the same period. These cases were traced to a group of immigrants from Greenland living in a small, defined geographic region in Denmark.

van Soolingen and associates[334] detected 43 different IS*6110* fingerprint patterns of 153 *M. bovis* strains originating from cattle and humans in the Netherlands and Argentina, various animals in Dutch zoos, in a wild park in Saudi Arabia, and from diseased seals and cats in Argentina. Strains presenting only a single band were characteristic for the strains recovered from cattle. Of the 20 human isolates from Argentina, 18 showed a single band, similar to that seen in the cattle strains, strongly suggesting bovine-to-human transmission. The fingerprint patterns of human *M. bovis* isolates from the Netherlands were diffuse, except for similar patterns in five patients, all living in Amsterdam and three of whom were from the same family. Cave and coworkers[40] found that the IS*6110* fragment-length polymorphism patterns among 6 *M. bovis* isolates from 1 patient and 42 *M. tuberculosis* repeat isolates from 18 patients remained stable over a period of 8 months to 4.5 years and were not altered by changes in drug resistance profiles. These studies illustrate the usefulness of restriction endonuclease analyses in epidemiologic studies of human and animal TB.

Several other DNA repetitive elements have been used as markers by a large number of researchers for the strain typing of *M. tuberculosis* and other *Mycobacterium* species. Hermans and coworkers[122] discovered a complex-specific mycobacterial DNA insert that hybridized specifically with DNA of MTBC strains. A nonrepetitive 158-bp fragment of this sequence was amplified with PCR and used for the selective detection of mycobacteria from the MTBC directly in pleural fluid, bronchial washings, and biopsies, to a lower limit of sensitivity of 20 bacterial cells (about 10^3 cells in a sputum sample). Wiid and associates[356] used oligonucleotide GTG5 as a marker for the identification of *M. tuberculosis* strains, which they found useful in cases in which certain *M. tuberculosis* strains have few or no insertion elements such as IS*6110*. Friedman and colleagues[92] used PCR amplification of DNA segments located between two copies of repetitive elements IS*6110* and the polymorphic GC-rich repetitive sequence. A PCR-amplified 439-bp segment of the 65-kDa heat-shock protein (HSP) gene was used to develop RFLP patterns of several rapidly growing *Mycobacterium* species, including *M. fortuitum*, *M. chelonae*, *M. smegmatis,* and *M. mucogenicum*.[310] The repetitive insertion sequence element IS*1245* was used in a study of human and animal isolates of *M. avium*.[112] In another study, the repetitive insertion sequences IS*1311* and IS*900* were used as DNA probes in assessing the RFLP of 75 clinical isolates of *M. avium*.[273] Two markers, one of

which encodes for a 40-kDa protein (p40) and the insertion sequence IS*901*–IS*902,* have been used as molecular markers in the typing of 184 field strains of MAC.[5]

Pulsed-field electrophoretic separation of restriction fragments generated by digestion of chromosomal DNA was used in the typing of 16 strains of *M. haemophilum*, 12 of which showed similar patterns, including 6 from the same hospital.[374] This technique has also been used to identify patients who were infected with more than one strain of MAC.[302] Random amplified polymorphic DNA (RAPD; also referred to as arbitrary primer PCR) has been used in the study of several primers to determine which are most discriminatory in providing reproducible fingerprints in the strain typing of *M. tuberculosis*.[187] This technique has also been used to type strains of *M. tuberculosis*, with a PCR-amplified region separating the genes coding for 16S and 23S rRNA serving as the marker.[1] These authors present molecular typing by RAPD as being more rapid and less technically demanding than most other molecular-typing methods. Also, smaller quantities of purified DNA are required than for other methods, allowing earlier analysis of young primary isolates of *M. tuberculosis*.

Kirschner and colleagues[161] have developed libraries of restriction-fragment profiles of 16S rRNA subunits extracted from several *Mycobacterium* species that were previously characterized by biochemical tests or nucleic acid probes. The specific restriction endonuclease profile of an unknown *Mycobacterium* species can be easily compared with the library profile, providing a rapid nonculture means for making an accurate identification. Similarly, Avaniss-Aghajani and associates[11] developed a method for identifying mycobacterial isolates directly from water and clinical specimens. PCR was first used to amplify a portion of the small-subunit rRNA (SSU) from 13 different species of mycobacteria, using a 5′ PCR primer that carried a fluorescent label to allow detection of the amplified product. The PCR product was digested with restriction endonucleases, and the sizes of the labeled restriction fragments were determined by an automated DNA sequencer. A library of 5′ restriction fragment lengths produced by five restriction endonucleases has

been developed that can categorize 20 *Mycobacterium* species. Each *Mycobacterium* species has a unique 5′ restriction-fragment length for each specific endonuclease, selections from which can be used to make identifications of unknown species. Advantages of this technique over rapid PCR methods include lower cost and the ability to characterize several *Mycobacterium* species and to detect more than one *Mycobacterium* species in the same sample.

Strain-typing methods have also been used in the detection of drug-resistant strains

of *M. tuberculosis*. In separate studies, Whelen and coworkers[353] and Felmlee and associates[85] used unique methods to detect mutations in the beta subunit of *M. tuberculosis* RNA polymerase (*rpoB*) to detect mutations most frequently associated with rifampin resistance. Plikaytis and coworkers,[249] in the study of an outbreak of MDR-TB in New York City, used a multiplex PCR assay targeting a direct repeat of IS*6110* with a 556-bp intervening sequence (NTF-1), to identify patients who were infected with what they designated the multidrug-resistant "W" strains of *M. tuberculosis*. Their assay correctly identified all 48 strain W isolates of *M. tuberculosis*, among a total of 193 strains studied. These studies indicate the degree of sophistication achieved by molecular techniques and the practical applications for which they are being used.

Susceptibility Testing

Mycobacterium tuberculosis *Complex*

The need for prompt and accurate susceptibility testing of MTBC is essential due to the emergence of multidrug-resistant strains. Susceptibility testing should be performed on the first isolate of MTBC recovered from every patient and should be repeated after 3 months of appropriate therapy if cultures remain positive. Initial testing should include all primary drugs: INH at the critical concentration, rifampin, ethambutol, and PZA.

The reference method for susceptibility testing of MTBC to INH, rifampin, and ethambutol is agar proportion. Recommended concentrations in Middlebrook 7H10 agar are as follows: INH, 0.2 µg/mL; rifampin, 1.0 µg/mL; and ethambutol, 5.0 µg/mL. Agar proportion, however, cannot be used to test PZA. Moreover, agar proportion is not a rapid method; results are reported after incubation for 3 weeks, if growth is sufficient. To ensure the earliest possible detection of resistance, a broth method with a short incubation time is recommended.[367] The CDC suggests that mycobacteriology laboratories strive to report results of first-line testing within 15 to 30 days of receipt of the specimen in the laboratory.[317] Ideally, susceptibility results for the primary drugs should be available within 7 to 14 days after isolation of MTBC. Second-line drugs (Table 19-9) should be tested if an isolate is resistant to rifampin or any two of the primary agents. Isolates with monoresistance to INH also should be tested for susceptibility to second-line drugs if the planned treatment regimen includes a fluoroquinolone.[367]

The frequency of drug-resistant mutants in a culture of tubercle bacilli has been estimated to be about $1:10^5$ bacteria for INH and $1:10^6$ for streptomycin. If two drugs

(i.e., INH and streptomycin) are taken together, the incidence of resistance is $1:10^{11}$, which is the sum of the two taken separately. Knowledge of the incidence of mutants becomes important because it has been determined that patients with an open pulmonary cavity may have a total bacillary population of 10^7 to 10^9 bacteria. Therefore, if these patients are treated with a single antituberculosis agent, their cultures may soon show the emergence of an isolate that is resistant to that agent, and thus treatment fails. Consequently, patients with TB must always initially be treated with the four primary drugs listed above. The failure of patients to adhere to this regimen may lead to the emergence of a specific drug-resistant tubercle bacillus (Fig. 19-3). After following a group of patients coinfected with *M. tuberculosis* and HIV, Nolan found that lack of compliance with antituberculosis drug therapy was the primary reason for treatment failure.[225] Lack of compliance may also contribute to therapeutic failure and the emergence of drug resistance.

TABLE 19-9 Second-Line Antituberculous Drugs and the Recommended Concentrations in Middlebrook 7H10 Agar

Drug	Concentration (μg/mL)
Isoniazid	1.0
Ethambutol	10.0
Amikacin	4.0
Capreomycin	10.0
Ethionamide	5.0
Kanamycin	5.0
Levofloxacin	1.0
Moxifloxacin	0.5
Ofloxacin	2.0
p-Aminosalicylic acid	2.0
Rifabutin	0.5

Streptomycin	2.0 and 10.0

A second principle of MTBC susceptibility testing is based on the in vitro correlation between the clinical response therapy and the result of in vitro susceptibility testing. If more than 1% of the tubercle bacilli present are resistant to a drug in vitro, therapy with that drug is not clinically useful. Therefore, methods for drug susceptibility testing of mycobacteria must be capable of determining the proportion of bacilli susceptible and resistant to a given drug or yield results that are equivalent when methods are compared. When using agar proportion, the inoculum should be adjusted so that the number of spontaneously resistant mutants will not mislead the laboratory worker to interpret the culture as resistant. By the same token, there must be a sufficient number of colonies on the plate so that the incidence of drug resistance in the range of 1% can be determined. This is best accomplished when 100 to 300 colony-forming units (CFUs) are present on each quadrant of a four-quadrant Petri plate. To determine the incidence of resistance, it may be necessary to inoculate two sets of susceptibility test plates, the second set with a 100-fold dilution of the inoculum used for the first set.

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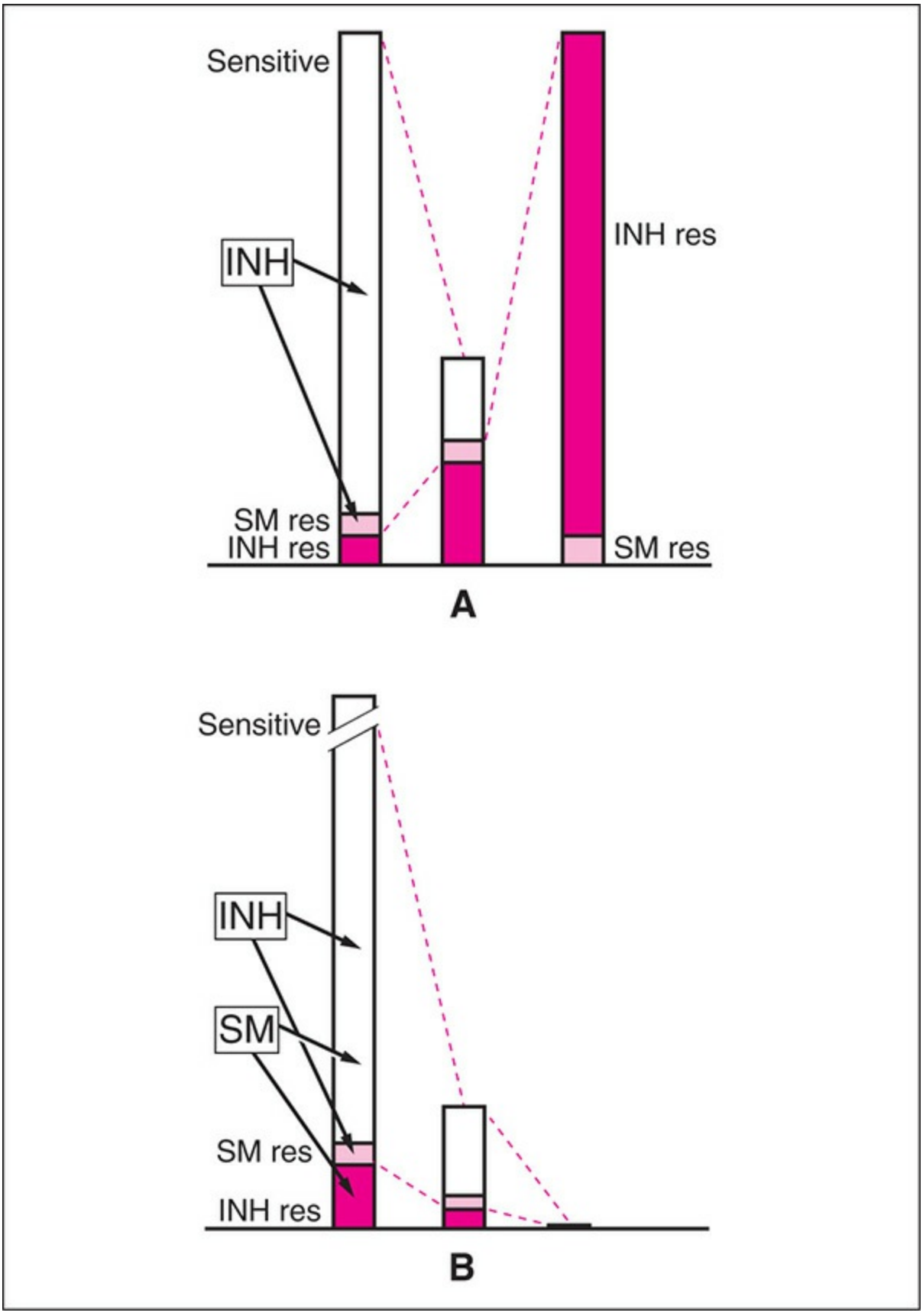
Sensitive
INH
INH res
SM res
INH res
SM res
A
Sensitive
INH
SM
SM res
INH res
B

■ **FIGURE 19-3** Emergence of mycobacterial antimicrobial resistance with one- and two-drug therapy. **A.** The patient is treated with only isoniazid (INH). Although the small number of streptomycin-resistant mutants are inhibited by the INH, the INH-resistant mutants are refractory and, in time, make up the majority of the population. This represents drug failure. **B.** The patient is treated with both streptomycin and INH. The streptomycin-resistant mutants are inhibited by the INH, and the INH-resistant mutants are inhibited by streptomycin. Thus, neither of these mutant strains can overgrow and the drug therapy is successful. (Redrawn from Crofton J. Some principles in the chemotherapy of bacterial infections. BMJ 1969;2:209–212.)

Agar proportion is performed in plastic Petri dishes divided into four quadrants. Plates can be purchased or prepared in-house. Five milliliters of agar medium are placed into each quadrant, the first with no antimycobacterial agent to act as a growth control, and the other three with varying concentrations of the drug to be tested. Either 7H10 or 7H11 agar as the base is acceptable.[367] Paper disks containing specified amounts of antituberculous drugs are placed in the individual quadrants, and drug-free agar medium is dispensed into each quadrant. Alternatively, agar medium may be prepared with liquid drug. A reference strain of *M. tuberculosis*, H37Rv (ATCC 27294), which is susceptible to all primary and second-line antituberculous drugs, is recommended for quality control of both agar proportion and the preferred broth susceptibility test methods.

The first broth-based antimicrobial susceptibility testing for MTBC was the BACTEC 460 instrument fitted with a "TB hood." The basis of this broth test is the radiometric detection of ^{14}C, released from 7H12 liquid medium containing 1-[^{14}C] palmitic acid by the metabolizing bacteria.[268,205,366,296,207] The BACTEC 460 instrument revolutionized susceptibility testing of MTBC, but it is no longer supported by the manufacturer and, therefore, is not discussed further.

In 2002, the fully automated, nonradiometric MGIT 960 system (described earlier in this chapter) was approved by the FDA for susceptibility testing of MTBC to INH, rifampin, ethambutol, PZA, and the secondary drug streptomycin. Many studies have shown that the performance of the MGIT system for testing primary drugs is comparable to that of both the radiometric BACTEC 460 and the agar proportion method, and the time to results is similar to that for the BACTEC 460.[2,17,127,170,243,278,285]Although the MGIT 960 is not FDA-approved for testing second-line agents other than streptomycin, several investigators have validated its performance for this purpose.[170,127,186,269,279]

The MGIT system uses a two-tier testing protocol: INH, rifampin, and ethambutol are in the first-tier, and PZA is tested separately. The inoculum may be prepared from either a liquid or a solid medium. To ensure accuracy and reproducibility of results, it

is critical that the manufacturer's guidelines be strictly followed. An MGIT drug susceptibility testing set includes a drug-free growth control tube and drug-containing tubes. When the GI of the growth control reaches 400 within 4 to 13 days, the system flags the completion of the testing and interprets results as follows: An isolate is called resistant to a drug when the GI of the drug-containing tube is >100; it is called susceptible when the GI is ≤100. The test is invalid if the GI of the growth control reaches 400 in <4 days or >13 days; this indicates that the inoculum is either too heavy or contains contaminants, or it is too light.

Testing PZA in the MGIT system differs slightly from testing the other three primary drugs. Inoculum preparation and interpretation of results are the same, but the broth in the MGIT PZA tube has a reduced pH, the tube has a separate supplement, and the PZA growth control tube is inoculated with a 1:10 dilution of the inoculum to PZA-containing tube. The performance of the MGIT system for testing PZA appears to be inferior to that of the other primary drugs.[44,45] There appears to be a problem with false resistance, which, based on data from one study, might be corrected by reducing the inoculum from 0.5 to 0.25 mL.[245] Some investigators have suggested that a combination of culture-based testing and *pncA* sequencing might be the most reliable approach to assessing PZA susceptibility or resistance.[298]

VersaTREK, a fully automated, continuously monitoring system described earlier in this chapter for growth and detection of mycobacteria, also is FDA-approved for testing susceptibility of MTBC to all primary drugs. As with the MGIT system, the inoculum can be prepared from either broth (VersaTREK Myco or 7H9) or a solid medium (LJ or 7H10/7H11). Inoculated drug-containing and drug-free control bottles are placed in the instrument, which automatically monitors growth every 24 hours. A susceptibility test is valid when growth in a control bottle is detected 3 to 10 days after inoculation. Drug-containing bottles are monitored for 3 days after the control bottle is positive. An isolate is resistant to a drug if growth in the drug-containing bottle is detected ≤3 days after detection of growth in the control

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bottle. An isolate is susceptible if there is no growth in the drug-containing bottle or if growth is detected >3 days after that of the control bottle. Studies have shown good agreement among the VersaTREK Myco system, BACTEC 460, MGIT 960, and agar proportion.[19,83,276]

Another broth-based method for susceptibility testing of MTBC is the TREK Sensititre MYCOTB MIC 96-well plate, which includes both first-line (except PZA) and second-line drugs. At the time of this writing, this test is not cleared by the FDA and, therefore, is for research use only. The inoculum is prepared from colonies grown on a solid medium, and results are available 10 to 21 days after the plate is inoculated.

Performance of this method compared to agar proportion is satisfactory to excellent, with categorical agreement of 94% to 100% in one study and 80% to 99% in another.[116,182] This method has the advantage of testing both first- and second-line agents simultaneously, and it does not require an instrument for interpretation of results. However, it does not include PZA, which is a major drawback, and the turnaround time is longer than automated broth systems.

Given the importance of rapid identification of drug-resistant TB, several molecular methods for detection of mutations associated with resistance have been developed. These methods can be used to test positive cultures (broth or colonies) or AFB smear-positive specimens. However, drug resistance can be caused by mutations other than those detected by these methods; therefore, failure to detect a mutation does not necessarily equate to a phenotypic result of susceptible.

Molecular methods can be divided into those that are probe-based (e.g., Cepheid's GeneXpert MTB/RIF assay using molecular beacons and HAIN's MTBDR*plus* and MTBDR*sl* assays using line probes) and those that are sequence-based (e.g., Sanger sequencing and pyrosequencing). Probe-based methods detect the presence or absence of mutations, whereas methods based on sequencing provide sequences of either wild-type isolates or mutants. Mutations are not always associated with drug resistance; therefore, detection of a mutation by a probe-based method may not in all cases equate with phenotypic resistance. Additionally, because not all mechanisms of resistance are known, failure to detect a mutation does not unequivocally exclude resistance. Therefore, although molecular-based methods provide useful information quickly, all are adjuncts to, not replacements for, phenotypic drug susceptibility testing.

The GeneXpert MTB/RIF assay was described earlier in this chapter. Detection of rifampin resistance is determined when the presence of a mutation(s) in the 81-bp rifampin resistance determining region of the *rpoB* gene is detected. The performance of this assay for detection of *M. tuberculosis* in clinical specimens was previously discussed. With regard to detecting rifampin resistance in *M. tuberculosis*, Zeka et al.[380] reported total agreement with agar proportion for 89 of the 429 specimens that were culture-positive for *M. tuberculosis*: 88 were susceptible, 1 was resistant. Marlowe et al.[195] tested 217 specimens, of which 130 were culture-positive for *M.tuberculosis*. All 130 were rifampin-susceptible by broth microdilution, but 3 were initially resistant by GeneXpert. The liquid culture of the three with discrepant results was retested by GeneXpert: two were susceptible and one remained resistant.

The HAIN probe assays, which use reverse hybridization to detect mutations, are not FDA-cleared for diagnostic use in the United States but have been used in several countries outside the United States. Both MTBDR*plus* and MTBDR*sl* assays include both wild-type probes and probes for several commonly encountered mutations. The absence of a wild-type band indicates that a mutation is present. The simultaneous presence of a mutant band indicates that a specific mutation is present in the

specimen/isolate. The absence of both a wild-type band and a mutant band indicates the presence of a mutation but does not provide information regarding the identification of the mutation. The performance of these assays varies depending on the drug, being best with rifampin and the quinolones. For both assays, specificity (the drug is susceptible by both the line probe assay and phenotypic drug susceptibility testing) is ≥95% and with the exception of rifampin, is greater than sensitivity (drug resistant by both the line probe assay and phenotypic drug susceptibility testing).[155,212,333]

Investigators at the CDC evaluated DNA sequencing for detection of first- and second-line drug resistance in 314 isolates of *M. tuberculosis*.[38] Phenotypic testing was done by agar proportion, except for PZA, which was tested by the BACTEC 460 or MGIT 960. Genetic loci examined were drug resistance determining regions of *rpoB, embB,* and *gyrA*, promoters of *inhA* and *eis*, regions of *katG* and *rrs* with established mutations, and complete open reading frames of *tlyA* and *pncA*. Specificities for first-line drugs were 86% for *pncA* (PZA), 93% for *embB* (ethambutol), 94% for *rpoB* (rifampin), and 100% for *katG, inhA,* and *katG* + *inhA* (INH). Sensitivities were 97% (*rpoB*), 91% (*katG* + *inhA*), 85% (*pncA*), and 79% (*embB*). Sensitivity and specificity for second-line drugs were 82% and 98% for ciprofloxacin and ofloxacin (*gyrA*), 86.5% and 96% for kanamycin (*rrs* or *eis*), 90% and 99% for amikacin (*rrs*), and 61% and 87% for capremycin (*rrs* and/or *tlyA*).

Nontuberculous Mycobacteria

For susceptibility testing of NTM, the Clinical and Laboratory Standards Institute (CLSI) recommends testing clinically significant isolates by broth microdilution, using cation-adjusted Mueller–Hinton broth with 5% OADC or OAD, and provides guidelines for MAC, *M. kansasii* (which also apply to other slowly growing, nonfastidious NTM), *M. marinum,* and the rapidly growing mycobacteria.[367] The general recommendations are described in the following paragraphs, but the CLSI M24 document[367] should be consulted for specific details. Both the minimum inhibitory concentration (MIC) and an interpretation should be reported. Quality control testing should be performed on each new batch of 96-well test plates and once weekly or each time testing is performed, if less than weekly, thereafter.

For susceptibility testing of MAC, the only primary drug recommended at the time of this writing is clarithromycin. However, data published by Brown-Elliott and colleagues[32] support initial testing of amikacin as well. Secondary agents, which may be useful in select patients, are moxifloxacin and linezolid. Although ethambutol, rifampin, rifabutin, and streptomycin are useful clinically, there is no correlation between in vitro susceptibility testing results and clinical

outcome of patients with MAC who are treated with these drugs. Therefore, susceptibility test results should not be reported for these agents. The translucent colony variants should be selected for testing if possible, because they are thought to be more virulent and more resistant to antimicrobial agents. Trays should be incubated in ambient air at 35°C to 37°C and examined at day 7. If growth is insufficient, trays are reincubated and examined again at days 10 to 14. The endpoint is visible turbidity. The recommended quality control strain is *M. avium* ATCC 700898, with an acceptable alternative being *M. marinum* ATCC 927. Susceptibility testing should be repeated if cultures remain positive after 3 months of therapy for patients with disseminated disease and after 6 months for patients with chronic pulmonary disease.

Standard therapy for *M. kansasii* pulmonary disease consists of rifampin, INH, and ethambutol, of which rifampin is essential for a successful outcome. An alternative, equally effective regimen is rifampin, ethambutol, and clarithromycin. CLSI recommends that all initial isolates of *M. kansasii* be tested for susceptibility to rifampin or, if the alternative regimen is planned, to rifampin and clarithromycin.[367] Testing susceptibility of *M. kansasii* to INH is not recommended because in vitro results do not correlate with clinical outcome and, therefore, could cause a false impression of resistance. Microdilution trays are incubated at 35°C to 37°C in ambient air for 7 to 14 days. Acceptable QC strains are *M. kansasii* ATCC 12478, *M. marinum* ATCC 927, and *Enterococcus faecalis* ATCC 29212. *M. kansasii* clinical isolates that are resistant to rifampin (MIC ≥1 μg/mL) should be tested for susceptibility to rifabutin, ethambutol, clarithromycin (if not initially tested as a primary drug), amikacin, ciprofloxacin, trimethoprim–sulfamethoxazole, linezolid, and moxifloxacin. Testing streptomycin also should be considered; however, breakpoints for susceptibility and resistance have not been established, so only the MIC should be reported.

Routine susceptibility testing of *M. marinum* is not recommended, because isolates are consistently susceptible to rifampin, ethambutol, doxycycline/minocycline, trimethoprim–sulfamethoxazole, and clarithromycin. However, if a patient does not respond to appropriate therapy after several months and remains culture-positive, susceptibility testing should be considered. Microdilution trays are incubated at 28°C to 30°C for 7 days. Drugs to test are rifampin, clarithromycin, amikacin, doxycycline or minocycline, ciprofloxacin, moxifloxacin, and trimethoprim–sulfamethoxazole.

Treatment of skin and soft tissue infections caused by rapidly growing mycobacteria should include clarithromycin, if the isolate is susceptible, and at least one additional agent, selected based on results of susceptibility testing. Antimicrobial agents that should be tested are amikacin, cefoxitin (up to 256 μg/mL), ciprofloxacin, clarithromycin, doxycycline or minocycline, imipenem, linezolid, moxifloxacin,

trimethoprim–sulfamethoxazole, and tobramycin.[367] Tobramycin is used to treat *M. chelonae* infections, but not infections due to *M. abscessus* or *M. fortuitum* group. Microdilution trays are incubated at 28°C to 30°C in ambient air for 72 hours. If growth is insufficient at that time, trays are examined again on days 4 and 5. This is the final read for all drugs except clarithromycin. To ensure detection of inducible macrolide resistance, clarithromycin wells are read at days 7 to 10 and again at day 14, unless the isolate is resistant at an earlier read. The MIC is the lowest concentration of an antibiotic that completely inhibits visible growth, except for trimethoprim-sulfamethoxazole, to which the rapidly growing mycobacteria exhibit trailing. For this drug, the MIC is read at about 80% inhibition of growth. The recommended QC strain is *M. peregrinum* ATCC 700686, although *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 17853, and *E. faecalis* ATCC 29212 are acceptable alternatives.

Although broth microdilution is considered the standard method for testing rapidly growing mycobacteria, the Etest (bioMerieux) may be a viable option. Biehle and associates[23] performed Etests for 100 clinical strains of rapidly growing mycobacteria against six drugs: amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, and imipenem. These authors found 85% agreement between the Etest results and agar dilution MICs within 1 $\log_2$ and a 97% agreement at 2 $\log_2$ dilutions. The rates of major and minor errors were 2.2% and 11.7%, respectively; there were no very major errors (defined by the authors as disagreement of 3 $\log_2$ dilutions or more). Interlaboratory agreement between Etest MICs determined in two separate laboratories was 81% within 1 $\log_2$ dilution and 92% within 2 $\log_2$ dilutions. These data suggest that Etest may be a reasonable alternative method for determining the susceptibility of rapidly growing mycobacteria, but additional studies testing all drugs recommended by CLSI and comparing results to broth microdilution are needed.

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CHAPTER 20

The Spirochetal Infections

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Rat-Bite Fever (Sodoku)

Spirillum minus

Spirochaetes

Human pathogens within the class *Spirochaetes* cause a significant burden of illness worldwide, but laboratory confirmation of infection may be difficult because some organisms have been cultured only in vivo, and some remain noncultivatable in the clinical laboratory. Although laboratory diagnosis of spirochetal infections has

historically relied on host serologic tests, molecular methods are increasingly being developed to improve the sensitivity of detection. The focus of this chapter is the clinical manifestations and laboratory diagnosis of *Treponema*, *Borrelia*, *Leptospira,* and *Spirillum* infections. *Treponema pallidum* subspecies *pallidum,* the causative agent of syphilis, has for centuries been a major sexually transmitted infection (STI) and has staged a dramatic comeback in the later part of the 20th century, in part due to coinfection with HIV.[128,215,514] Conversely, *Borrelia burgdorferi* sensu lato, the causative agent of Lyme disease, was discovered only 25 years ago, but has subsequently become the most common spirochetal infection in the United States,[75,80] and many parts of Europe where the *Ixodes* tick vectors reside and play a key role in animal-to-human spirochete transmission.[440,441] Tick-borne relapsing fever (TBRF) caused by *Borrelia* spp., leptospirosis caused by *Leptospira* spp., and rat-bite fever (RBF) caused by *Spirillum minus* are also classic zoonotic diseases acquired through contact with animal reservoirs due mainly to occupational exposure.[36,125,157]

Taxonomy

Spirochaetes (spirochetes) belong to a distinctive group of long gram-negative bacteria that are helically coiled, resulting in spiral-shaped cells that measure 0.1 to 3.0 µm in diameter by 5 to 120 µm in length (Fig. 20-1).[345] Spirochetes are chemoheterotrophic and motile via a unique mechanism.[87,345] They are extremely motile via endoflagella that give these organisms their characteristic corkscrew and oscillating movement. Spirochetes have axial filaments that are flagella-like organelles enclosed within an outer sheath, which spirally wrap around the cell wall of the bacteria and facilitate their rapid rotational corkscrew motility even in high-viscosity liquids.[177,231,345] Axial filaments are attached to the cell wall by insertion disks located at the end of cells.[231,345]

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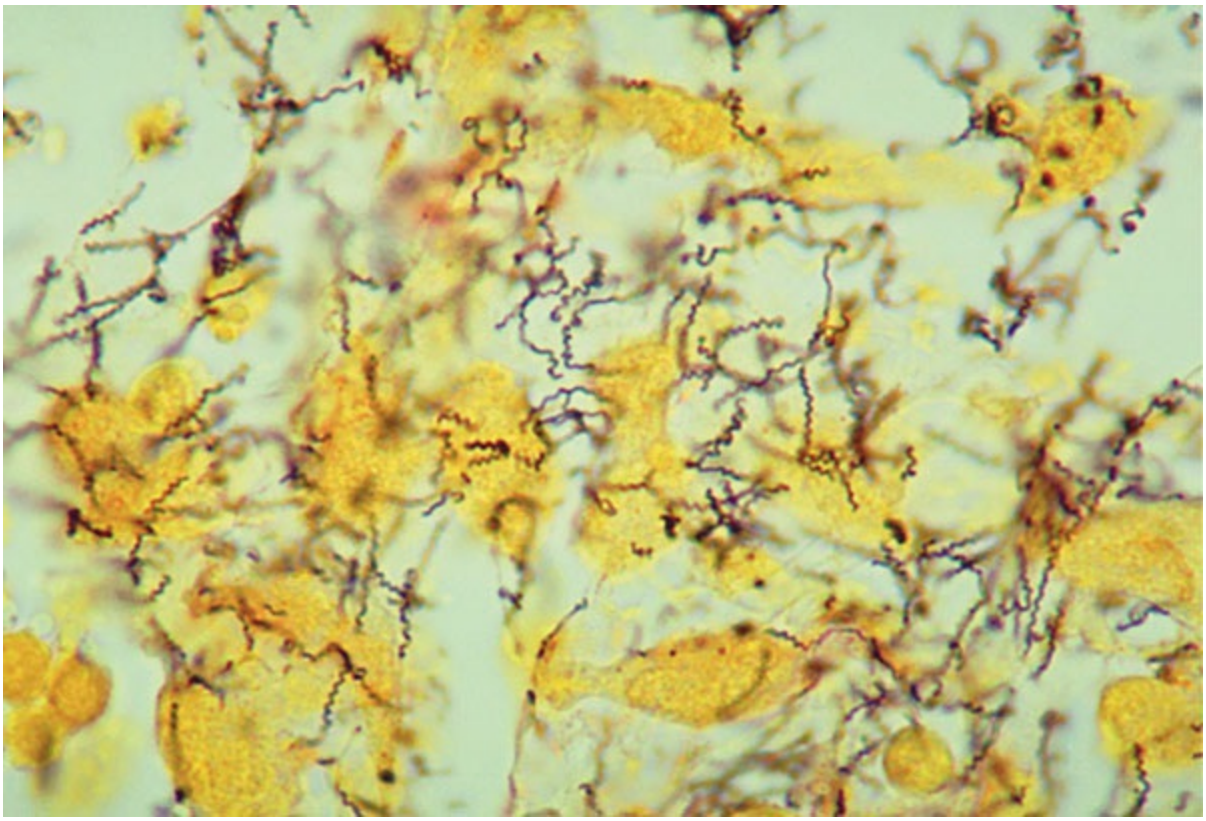

■ **FIGURE 20-1** *T. pallidum* spirochetes. (Courtesy of www.Glown.com.)

The number of axial filaments and insertion disks along with phenotypic metabolic characteristics are used to broadly differentiate genera within the family *Spirochaetaceae*.[345] *Treponema* are slender organisms with tight coils (6 to 10 axial filaments attached by a single insertion disk), *Borrelia* appear thicker with fewer and looser coils (30 to 40 axial filaments attached by two insertion disks), and *Leptospira* closely resemble *Borrelia* except they have hooked ends (two axial filaments attached by 3 to 5 insertion disks).[320,345] The genus *Spirillum*, however, has an external flagellum for motility.

The classification of the bacteria within the *Spirochaetes* phylum is shown in Table 20-1. All of the human pathogens within the *Treponema*, *Borrelia,* and *Leptospira* genera are classified in the *Spirochaetales* order, and further within the *Spirochaetaceae* and *Leptospiraceae* families.[345] *Brevinema andersonii* infects short-tailed shrews (*Blarina brevicauda*) and white-footed mice (*Peromyces leucopus*),[108] while *Cristispira* has been found in a variety of aquatic organisms including freshwater mollusks.[346]*Spirochaeta* are free-living spirochetes found in aquatic or marine environments.[345] *S. plicatilis* has been found in deep-sea sediments, while *Spirochaeta americana* is a haloalkaliphilic and obligately anaerobic bacteria that

thrives in bleach-like alkaline, salty, deep waters of California's Mono Lake.[197,346] Some *Brachyspira* spp. (*Serpulina*)within the *Brachyspiraceae* family are important causes of diarrheal illness in humans, animals, and birds.[345] *B. aalborgi* affects only humans and high primates.[49,196,424] *B. pilosicoli* causes intestinal spirochetosis and diarrhea in pigs, and may be transmitted to man through the fecal–oral route.[424] Most cases of colonic spirochetosis occur in the developing world, and human colonic spirochetosis due to either *B. pilosicoli* or *B. aalborgi* manifests as diarrhea, rectal bleeding, and abdominal cramps.[424,475] *B. hyodysenteriae* causes dysentery in swine, whereas *B. alvinipulli* is an enteropathogen in chickens.[424] The type species of *Leptonema* is *L. illini*, which was separated from the *Leptospira* genus because of its unique structure and DNA base composition.[345,346] The pathogenicity of *L. illini* remains unknown.

Additional novel spirochete species may be found that have pathogenic potential in animals and humans. Several novel spirochetes have recently been isolated from mosquitoes and black flies in the Czech Republic, and 16S rRNA gene sequencing shows that they differ from *B. burdorferi*

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sensu lato as well as other described members of the *Spirochaetales* order.[420] Many other "noncultivatable" spirochetes remain to be discovered and classified. The phylogenetic diversity and pathogenicity of spirochetes will continue to evolve as investigators study the microbiotome of the gastrointestinal tracts of mammals, birds, and insects, and aquatic and marine environments.

TABLE 20-1 Classification of Spirochetes

Organism	Geographic Location	Disease
Order *Spirochaetales*		
Family *Spirochaetaceae*		
Genus I. *Spirochaeta*		
Genus IV. *Treponema*		
T. pallidum subspecies *pallidum*	Worldwide	Syphilis, congenital syphilis, neurosyphilis

T. pallidum subspecies *pertenue*	Tropical Asia, Africa, South and Central America	Yaws
T. pallidum subspecies *endemicum*	Africa, SE Asia, Middle East, Yugoslavia	Endemic nonvenereal syphilis
T. carateum	Central and South America	Pinta
T. denticola and other "*T. pallidum*-like oral spirochetes"	Worldwide	Necrotizing gingivitis, odontogenic infection
Genus V. *Borrelia*		
Borrelia burgdorferi/afzelii	North America, Europe	Lyme disease, erythema chronicum migrans, neuroborreliosis
Borrelia hermsii/duttoni/parkeri	Worldwide	Tick-borne relapsing fever
Borrelia recurrentis	South America, Europe, Africa, Asia	Louse-borne relapsing fever
Family *Leptospiraceae*		
Genus I. *Leptospira*		
Leptospira interrogans	Worldwide	Leptospirosis

Adapted from reference 345.

Treponema

Table 20-2 describes the four major pathogens in the genus *Treponema*.[345,346] Although none of the *Treponema pallidum* subspecies are genetically related, they produce very distinct diseases. None have been cultivated indefinitely in vitro. *T. pallidum* subspecies *pallidum* (hereafter called *T. pallidum*) is the main "venereal" pathogen among the spirochetes that is transmitted by sexual contact. *Treponema pallidum* subspecies *pertenue* (hereafter called *T. pertenue*) is not sexually transmitted and causes a cutaneous disease called yaws that differs in most aspects from syphilis.[226] The entire genome of the Nichols strain *T. pallidum* (isolated in 1912) was initially sequenced in 1998,[155] but recent sequencing of the complete genome and

annotation of the Chicago strain of *T. pallidum* (isolated in 1951) that has been extensively used in syphilis research show important differences among strains.[162] Alignment of the *T. pertenue* genome with that of *T. pallidum* shows almost complete genome homology between these two spirochetes.[179] A difference of only one nucleotide in the gene coding for a 19-kDa protein was reported by one study, but the immunoreactivity of this protein was identical in the two subspecies.[321,322] Endemic syphilis (bejel) is caused by *T. pallidum* subspecies *endemicum* (hereafter called *T. endemicum*); and *T. pallidum* subspecies *carateum* (hereafter called *T. carateum*) is responsible for pinta.[15,226] These important *Treponema* subspecies have yet to be completely sequenced.

Spirochetes are also an important part of the human oral flora; *Treponema denticola*, *Treponema pectinovorum*, *Treponema socranskii,* and *Treponema vincentii* are the only four species that have been cultivated, but a large variety of other oral spirochetes are microbiota and likely important opportunistic pathogens in periodontal disease.[95,112] The human oral microbiome project has recently shown that ~8% of the total diversity is made up 49 taxa of oral treponemes within the *Spirochaetes*: 11 named species, 3 taxa that are cultivated but unnamed, and 35 taxa that have not yet been cultivated or named.[112] As the type species, *T. denticola* has been shown to attach to human gingival fibroblasts, basement membrane proteins, and other substrates, resulting in cytotoxicity and cell death.[85] Along with other oral anaerobes, *T. denticola* may disseminate from the oral cavity and seed atherosclerotic plaques.[70]

Syphilis

Treponema pallidum

T. pallidum, the cause of venereal syphilis, has been infamous for 500 years, although the origin of this infection remains uncertain.[107,303,384] The disease did not appear prominently in Europe until the 16th century. Fornaciari and colleagues[153] studied a Renaissance mummy that contained structures identified by immunofluorescence and electron microscopy as *T. pallidum.* Some investigators believe that Columbus and his sailors brought the disease back from the New World (the Caribbean and South America),[385] whereas others believe that urbanization spread endemic syphilis in Europe.[107,384,472] A pandemic designated as the "Great Pox" (variola virus produced the "Small Pox") occurred in Europe shortly after Columbus returned from America, but it also occurred simultaneously with the increased movement of armies and people

in Europe.[472] Bone changes attributed to syphilis have been described in New World skeletons,[385] but similar lesions have also been reported from pre-Columbus skeletons from the Old World. Recent phylogenetic studies of available *Treponema* sequence data appear to support the import of syphilis from the New World.[179] More interesting is the question of how limited divergence between *Treponema* species and subspecies results in the differences in disease pathogenesis. A genome-wide microarray analysis and whole-genome restriction mapping of *T. pallidum* (Nichols strain) compared with *T. paraluiscuniculi* (strain Cuniculi A) showed that this closely related spirochete that causes venereal spirochetosis in rabbits has limited sequence differences that occur primarily in the *tpr* genes or nearby genes.[171] It is

possible that evolution of genes within the *tpr* locus is primarily responsible for the differences in disease manifestations and host susceptibility.[171,231,461]

TABLE 20-2 Transmission of Spirochetes

Organism	Transmission
T. pallidum	Sexually transmitted infection Vertical transmission from mother to infant Blood transfusion (human only)
T. pertenue	Direct skin contact (human only)
T. carateum	Direct skin contact (human only)
T. endemicum	Direct mucosal contact (human only) Contaminated eating or drinking vessels
B. recurrentis	Human host, human louse vector (*Pediculus humanus humanus*)
B. hermsii/duttonii/parkeri	Rodents, primates, human host; tick vector (*Ornithodoros, Rhipicephalus*)
B. burgdorferi/afzelii	Rodent, deer host; tick vector (*Ixodes*)
L. interrogans	Rat hosts; contaminated water

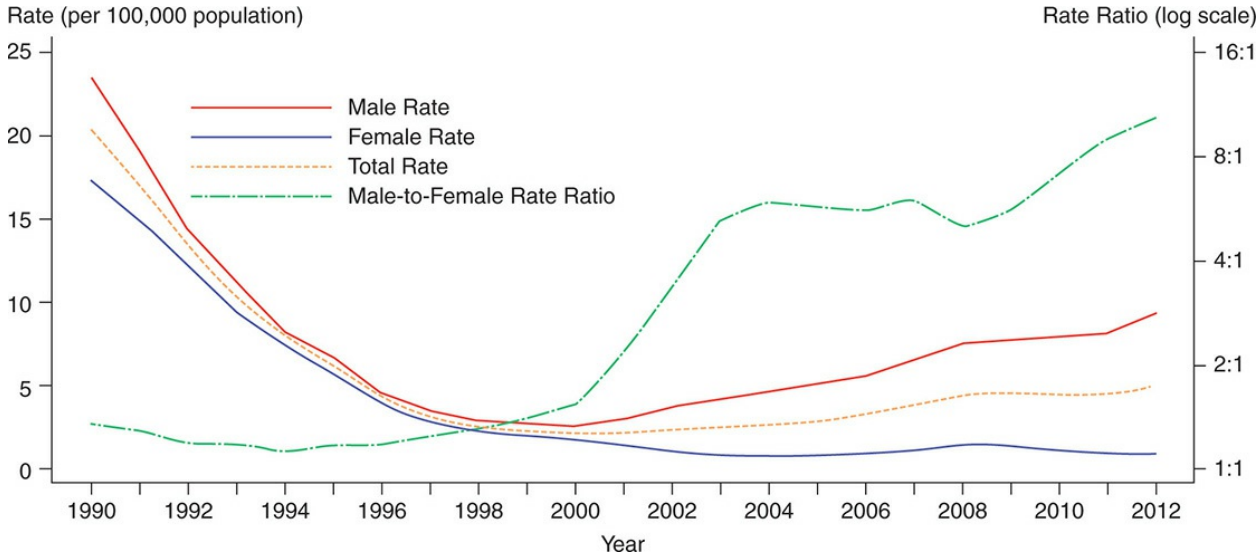
Epidemiology. Treponemes are inoculated and penetrate into the body through a skin

or mucous membrane defect. Syphilis may therefore be transmitted by sexual contact,[378] direct inoculation into the vascular system by shared needles or transfusions,[84] direct cutaneous contact with infectious lesions, or transplacental transfer of spirochetes (Table 20-2).[231] However, sexual contact with an infected person in an early stage of syphilis (stage I or II) is the most common mechanism of disease spread.[77,185] Men are usually diagnosed during the primary stage, whereas the primary lesion is often not detected in women, who are diagnosed during the early latent phase unless they are screened for another reason (e.g., pregnancy).[240] On average, one-third of patients become infected from sexual contact with an infected person, but the risk varies and may be higher.[231,393] Congenital syphilis (CS) is acquired by transmission of treponemes to the infant via the placenta.[74,256,296] Screening used by modern blood transfusion services has made the transmission of syphilis via this route extremely rare. Experimental studies in human volunteers have documented that the ID_{50} for *T. pallidum* (the number of organisms needed to infect 50% of those exposed) is as few as 57 organisms.[273] Shortly after inoculation, the spirochetes disseminate throughout the body, where they may eventually cause disease. The incubation period varies from 3 to 90 days, with a mean of 3 weeks.[231]

Syphilis Prevalence, Risk Groups, and HIV Coinfection. Despite the introduction of effective therapy for *T. pallidum* and prevention programs, the sexual spread of syphilis continues to be a problem,[382] and rates in the United States have increased.[71] Although epidemiologic data from the United States are presented herein, similar trends in increasing primary and secondary syphilis (P&S) cases, particularly among men who have sex with men (MSM), have occurred in many other jurisdictions.[214,239,431,470,503] Figure 20-2 shows the overall prevalence of P&S and late syphilis cases reported to the Centers for Disease Control and Prevention (CDC), Atlanta, GA, from 1941 when surveillance began until 2012.[71] Prior to the introduction of penicillin therapy in 1941, the rate of syphilis was ~350 to 400 cases per 100,000 persons in the United States.[71] Although effective therapy, contact tracing programs, and treatment of contacts of infected cases subsequently decreased and stabilized the syphilis rates for several decades, the aim to eradicate syphilis was never realized.[389] A syphilis epidemic occurred from 1981 to 1989 in the United States when the incidence of P&S syphilis cases among heterosexuals and minority populations grew from 13.7 to 18.4 cases per 100,000 persons, an increase of 34%.[379] During the 1990s, the rate of P&S syphilis cases then declined again due to implementation of effective control measures among all racial and ethnic groups.[71] Since 2001, the syphilis rate has begun to increase again, mainly due to spread among MSM who use

unsafe sexual practices.[41,56,71,89] The syphilis rate between 2008 and 2012 has increased among all racial and ethnic groups except American Indians/Alaska natives (Fig. 20-3).[71] Overall, the rate of P&S cases for blacks in 2012 was 6.1 times higher than those for whites, with 16.4 cases/100,000 occurring in blacks and 2.7 cases/100,000 occurring in whites.[71] The highest rates in 2012 occurred in black men aged 20 to 24 years (96.7/100,000), followed by black men aged 25 to 29 years (89.2/100,000).[71]Trends in syphilis rates in young black men are concerning because of the high HIV incidence in this population.[79] Similar trends occurred for black women aged 20 to 24 years of age; this group had the highest incidence among women with 19.1 cases/100,000 women.[71] In 2012, rates were 17 times higher for black women aged 20 to 24 years than white women in this same age group.[71]

From 2008 to 2012, the rate of P&S syphilis increased 40.9% among Hispanics (from 2.4 to 2.9 cases/100,000), 17.8% among American Indians/Alaska natives (from 2.9 to



■ **FIGURE 20-2** Distribution of syphilis cases by gender, United States (1994–2012). (Adapted with permission from reference 71.)

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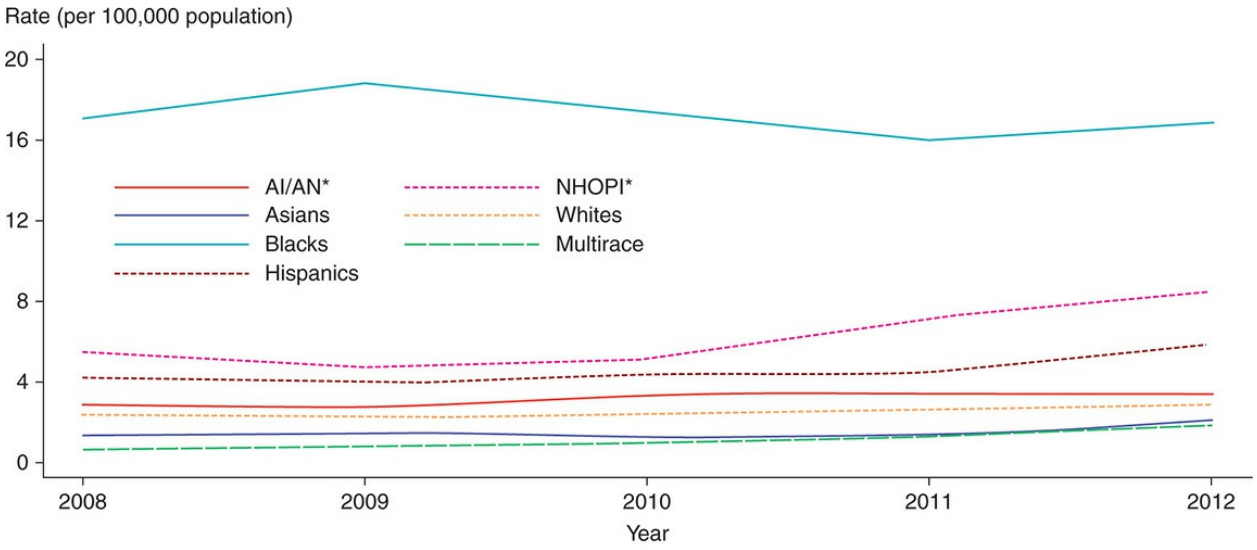

*AI/AN = American Indians/Alaska Natives; NHOPI = Native Hawaiian and Other Pacific Islanders.

■ **FIGURE 20-3** Distribution of syphilis cases by ethnicity, United States (1994–2012). (Adapted with permission from reference 71.)

3.4 cases/100,000), 21.4% among whites (2.4 to 2.9/100,000), and 55% among Asians (from 1.4 to 2.1/100,000).[71] The rates decreased within this time period by 0.7% for blacks (from 17.1 to 16.9 cases/100,000).[71]

CS rates rose by 18% during the period from 2006 to 2008. This was followed by a period of decline wherein the rate of CS decreased by 25% from 2009 to 2012 (i.e., a decrease from 10.4 to 7.8 cases/100,000).[71] The highest rates of CS continue to occur in the South, but these rates have declined from 16.7/100,000 in 2008 to 12.7/100,000 in 2012.[71] Significantly, there has been a substantial decrease in CS rates in Puerto Rico; in 2008 there were 17.5 cases/100,000, whereas in 2012 this rate declined to only 2.2 cases/100,000.[71] Overall rates of syphilis contraction by women of childbearing age must be controlled in order to abate this upward trend in CS. Early access to prenatal care for women of all races/ethnicity is required in order for syphilis screening to contribute to the prevention of maternal fetal transmission.

Several other factors besides age and race/ethnicity have been clearly associated with higher rates of syphilis transmission in the United States and elsewhere. *T. pallidum* and HIV interact in several ways.[470,513,514] It has been suggested that genital ulcers of a variety of etiologies facilitate the acquisition of HIV.[439,471] In healthy individuals, serologic tests are uniformly reactive during the secondary phase of syphilis, but immunosuppression due to HIV infection may affect the serologic response to syphilis.[514] Biologic false-positive reactions in nontreponemal tests are also more common in HIV-infected patients, particularly those with hepatitis C virus (HCV)

coinfection.[16,417] Haas and colleagues[175] evaluated the sensitivity of treponemal tests as a marker of prior syphilis in a cohort of 109 homosexual men with HIV infection who had a documented history of treated syphilis. Individuals receiving antiretroviral therapy maintained reactivity to treponemal tests, but 7% of the symptomatic men who had subclinical HIV infection and 38% of those with HIV disease lost reactivity to these antigens.[175] Other confirmatory tests besides the FTA-ABS may have to be used to diagnose syphilis in HIV-infected individuals when the initial test is negative.[136]Although physicians should be aware of the potential for unusual serologic responses in HIV-infected patients, the interpretation of nontreponemal and treponemal serologic test for syphilis is the same as those who do not have HIV infection.[71]

Patients who are infected with HIV are more likely to present with secondary syphilis and with persistent chancres than non–HIV-infected patients.[513,514] Some experts have documented that the HIV epidemic has been accompanied by an increased risk of developing neurosyphilis.[152,468] A correlation between abnormal cerebrospinal fluid (CSF) findings consistent with neurosyphilis and advanced HIV disease has also been reported.[277,279] Unsuccessful treatment of syphilis in patients infected with HIV has been reported by several investigators.[161,166,198]

Prevention and Control. Traditional epidemiologic approaches used to control syphilis include reporting of all cases to public health who then does contact tracing and partner notification. Active treatment of all laboratory confirmed cases and follow-up as outlined above is also critical to stop ongoing transmission. Traditional epidemiologic techniques alone, however, are proving inadequate,[69] and electronic communication tools are increasingly being used to communicate with and educate persons at risk of acquiring disease as well as to enhance partner notification within sexual networks.[220,221,291] Syphilis should also be tested for in all patients diagnosed with HIV or another STI.[71]Molecular epidemiology should also enhance contact tracing and identification of transmission clusters. In several reported applications of molecular epidemiology, however, numerous genetic subtypes have been identified in a geographic area.[278,281]

Elimination of CS requires additional strategies to reduce mother-to-child transmission. Adverse pregnancy outcomes due to syphilis can be averted by early identification of maternal infections through antenatal screening programs,

and the prompt treatment of women who test positive.[256] Although the United States

and almost all other countries have existing policies recommending universal syphilis screening during pregnancy, significant barriers exist within many healthcare jurisdictions that limit effective screening and treatment.[71,516] This is particularly true for some of the most high-risk patients including those in lower socioeconomic classes, injection drug users, and some racial-ethnic groups.[71] These women often have limited access to early antenatal clinical services, not only in resource-poor countries,[230,476] but also in developed nations where universal healthcare coverage is not provided. Treatment may also be delayed when laboratory results are not relayed in a timely fashion, or when pregnant women have to travel to another clinic location for syphilis-specific treatment.[304] Although epidemiologic data is collected on maternal syphilis screening and CS cases, access to healthcare services and the provision and timing of treatment should also be included in order to identify deficiencies in existing programs.

Clinical Disease and Treatment. Syphilis is known as "the great imitator" because the varied and complex clinical manifestations of this illness can mimic many other diseases.[349] Sir William Osler, one of the founders of modern medicine stated in 1891, "Know syphilis in all its manifestations and relations, and all other things clinical will be added into you." Although untreated syphilis is a chronic progressive systemic infection, somewhat arbitrary overlapping clinical stages are defined that are used to guide assessment, treatment, and follow-up (Table 20-3).

Early Disease (Stages I and II)

Primary syphilis (Stage I). The first sign of syphilis is the development of a primary ulcerated lesion called a chancre at the site of inoculation, which results from an intense inflammatory response. A localized erythematous, painless, indurated papule initially develops, and then evolves within days with necrosis of the skin surface into a typical chancre.[86,421] The typical chancre is a painless ulcer with minimal exudates that has a clean, smooth base with a well-circumscribed raised, firm edge. Secondarily infected lesions, however, will have surrounding erythema, and be painful and purulent. Patients suspected of having syphilis should have a thorough examination of all skin and mucosal surfaces for a primary chancre. A solitary chancre is usually located on the genitalia in otherwise healthy men (i.e., most commonly the glans, the coronal sulcus, and the foreskin of the penis) and women (i.e., the labia, fourchette, and perineum).[10,86,421] Extra-genital lesions occur in <2% of cases, and are mainly found in the oral cavity and on the lip, but other locations have been described (i.e., digits, limbs, trunk, nipples, and eyelids).[8,365] MSM may present with multiple ulcers and symptoms of anorectal infection, including rectal pain and nodular popular lesions surrounding the peri-anal area.[363,382,514] A diagnosis of syphilis may be delayed, particularly in women who have a vaginal or endocervical lesion, MSM with atypical anal lesions, or those with nonerosive ulcers.[302,421,437] The chancre base

contains spirochetes that can be visualized after scraping the lesion and making a smear by darkfield microscopy or direct immunofluorescence.[195,235] Firm, rubbery, painless bilateral regional lymphadenopathy may also occur simultaneously with the development of the primary chancre, but this is not always the case. The chancre heals in 3 to 6 weeks (range, 1 to 12).

Secondary syphilis (Stage II). Approximately one-half of untreated patients with primary syphilis will develop symptoms of the second stage within 6 to 8 weeks after the appearance of a chancre, whereas the other half enter directly into the latent phase. HIV coinfected patients may present with symptoms that overlap the primary and secondary phases of the disease.[215] The secondary stage is the most florid part of the disease and is the result of hematogenous dissemination of *T. pallidum*. Most patients present with a widespread rash,

which may be macular, maculopapular, papulosquamous, or pustular, but not vesicular.[236] HIV-positive patients often have a more severe secondary course with extensive pustular, granulomatous, or nodular variations.[236,514] The rash in syphilis characteristically involves the trunk, face, extremities, and the palms of the hands and soles of the feet. Palmoplantar exanthems may become hyperkeratotic (clavi syphilitici) while lesions around the hairline form a crown-like pattern called corona veneris.[236] In moist intertriginous areas, broad, moist, gray-white plaques called condylomata lata may be found, which are teeming with infectious spirochetes, and can be visualized by scraping the lesions and making smears for darkfield microscopy or direct immunofluorescence.[195,235] Similarly, infectious lesions called mucous patches are found on mucous membranes. There may be loss of hair or thinning of the eyebrows.

TABLE 20-3 Definition of the Stages of Syphilis

Stage	Duration	Manifestation
Incubation period	3 weeks (9–90 days)	
Primary syphilis (stage I)	6 weeks	Chancre formation at the site of inoculation, regional infection
		Various skin eruptions, mucous membrane lesions, generalized

Secondary syphilis (stage II)	Months	lymphadenopathy, organ involvement, hematogenous dissemination
Early latent	<1 year (CDC), <2 years (WHO)	Seropositivity, asymptomatic phase
Late latent	>1 year (CDC), >2 years (WHO)	Seropositivity, asymptomatic phase
Tertiary syphilis	Years (1–50)	Late benign syphilis (granulomatous nodules, psoriasitiform plaques, gummas, tubero-serpiginous syphilis). Gummatous involvement of various organs, cardiovascular syphilis, neurologic involvement.
Neurosyphilis	Years	
Early (in stage II of latent syphilis)		Acute syphilitic meningitis, meningovascular syphilis (symptomatic or asymptomatic), gummatous neurosyphilis
Late (in tertiary syphilis, sometimes referred to as "metasyphilis")		Late parenchymatous neurosyphilis (tabes dorsalis, general paresis)

WHO, World Health Organization.
Adapted from Syphilis—Physician's Pocket Guide, Centers for Disease Control and Prevention (CDC).

Systemic symptoms include fever, malaise, sore throat, headache, myalgias, and a generalized lymphadenopathy. Virtually any organ may be involved in secondary syphilis, but the most common involvement occurs in the eyes (uveitis), ear (sudden deafness), certain visceral organs (hepatitis, nephritis), and the central nervous system (meningitis).[421] Although infection of the central nervous system (CNS) occurs at any stage of syphilis, this is most common during the secondary phase.[390] Aseptic meningitis presents with headache and meningismus. Spirochetes have been cultured from the CSF, however, without any evidence of inflammation and without clinical

disease.[264] Secondary syphilis typically resolves spontaneously after 4 to 12 weeks, but relapse may occur in untreated patients within a year after the initial presentation. HIV coinfected patients may relapse despite treatment.[39]

Latent and Late Disease (Stage III)

Latent syphilis. After the primary/secondary stages, the disease becomes latent, and is characterized by the presence of positive syphilis serology and an absence of symptoms. Patients in whom the primary and/or secondary manifestations of syphilis went unnoticed and untreated may continue to be asymptomatically infected until symptoms of the tertiary stage develop. This period has been arbitrarily divided into early- and late-latency stages by public health agencies. The CDC defines early latency as a duration of infection up to 1 year, while infection of >1 year is considered to be late latency when the patient is no longer considered to be contagious.[71] Although the World Health Organization (WHO) considers 2 years to be the dividing line between the early and late latent periods of syphilis, these distinctions are often not clinically possible and both latency phases are similarly treated. Neurosyphilis must be excluded in patients with latent infection, and a lumbar puncture should be done in patients who present with neurologic, ophthalmologic, or otologic symptoms, particular those with HIV coinfection.[71,470,514]

Late syphilis (Stage III). Approximately one-third of untreated patients will progress to late-stage or tertiary syphilis. This stage of the disease is rarely seen in the developed world, but cases are increasingly being diagnosed worldwide due to HIV coinfection.[470,514] The three different forms of tertiary syphilis are late "benign" syphilis, cardiovascular syphilis, and neurosyphilis.

Late "benign" syphilis. This manifestation occurs in one-half of syphilis cases. Most patients present with various skin lesions, but the formation of nonspecific granulomatous lesions called gummata occurs in ~15% of untreated patients. The formation of the granuloma indicates a fully active cellular immune response. Gummata may destroy surrounding tissue as it enlarges[377]; clinically, they are destructive mass lesions that can occur in virtually any organ and may be mistaken initially for carcinomas.

Cardiovascular Syphilis. Syphilitic aortitis occurs in approximately 1% of untreated cases. It is caused by inflammation in the small vessels that supply the aorta (syphilitic endarteritis) and primarily affects the ascending aorta.[421] Two complications may result: an aortic aneurysm and dilatation of the aortic ring causing insufficiency and regurgitation of blood through the aortic valve. Aortic aneurysms may grow to such a size that they erode through the sternum and are visualized under the skin of the chest.

Neurosyphilis. The interval between primary disease and neurologic complication is 5 to 10 years for meningovascular syphilis, 15 to 20 years for general paresis, and

25 to 30 years for tabes dorsalis. Late neurovascular syphilis may be symptomatic or asymptomatic.[390] Asymptomatic disease is characterized by CSF abnormalities in the absence of symptoms. Pleocytosis, elevated protein levels, or depressed glucose levels are often found in the CSF. A positive CSF serologic test, classically the Venereal Disease Research Laboratory (VDRL) assay, defines the disease, although spirochetes may rarely be demonstrated by culture or molecular methods.[235,274,276] Symptomatic infection is either meningovascular or parenchymatous, but there is considerable overlap in the categories. Meningovascular syphilis resembles the aseptic meningitis of the secondary stage. Any cranial nerve may be affected by the inflammation, and deafness or visual impairment may result. Parenchymatous disease may involve the neurons of either the cerebrum or the spinal cord. Cerebral involvement is manifested as a wide variety of neuropsychiatric disturbances, including physical changes, such as paralysis, and psychiatric problems, such as delusions of grandeur ("general paresis of the insane"). The posterior columns (sensory tracts) of the spinal cord are preferentially affected, causing severe pains and inability to perceive sensory impulses from the extremities. The disease, called tabes dorsalis, includes a peculiar "slapping" gait and deformed knees (Charcot's joints), which are caused by the lack of proprioception and the feedback loop that allows for appropriate use of force during ambulation.

Patients with HIV coinfection may have an increased risk for neurologic complication even in the early stages of syphilis,[470,477,514] and also may have higher rates of serologic treatment failure with currently recommended regimens.[470] Persons coinfected with HIV and syphilis manifest clinical and CSF abnormalities consistent with neurosyphilis with a CD4 count of ≤350 cells/mL and/or an rapid plasma reagin (RPR) titer of ≥1:32.[514]

Treatment. Comprehensive guidelines for the treatment of STIs including syphilis have recently been published by the CDC.[71] Parenterally administered penicillin G is the preferred agent for treating all stages of syphilis. Syphilis regimens used to treat non–HIV-infected patients are also effective in preventing neurosyphilis in HIV-infected patients.[71] Benzathine penicillin administered by IM injection is the recommended drug preparation for treatment of

both adults and children diagnosed with syphilis regardless of their HIV infection status who do not have CNS involvement. Adults confirmed to have primary or secondary syphilis should be treated with benzathine penicillin G 2.4 million units IM as a single dose, while infants and children should receive a dose of 50,000 units/kg IM in a single dose, but the total amount given should not exceed the adult dose (i.e., 2.4

million units).[71] Although latent syphilis is not transmitted sexually, patients should still be treated with this stage of disease to prevent complications. Adults with early latent syphilis should be treated with benzathine penicillin G 2.4 million units IM as a single dose, while infants and children should receive 50,000 units/kg IM as a single dose, but the total amount given should not exceed the adult dose (i.e., 2.4 million units).[71] Adults with late latent syphilis of unknown duration should be treated with higher doses of benzathine penicillin G 7.2 million units total, administered as 3 doses of 2.4 million units IM each at 1-week intervals. Similarly, children at this stage should receive three weekly doses of 50,000 units/kg IM each, up to the adult dose (a total of 150,000 units/kg up to the adult total dose of 7.2 million units).[71]

If patients have clinical evidence of neurologic involvement (e.g., cognitive involvement, motor or sensory deficits, ophthalmic or auditory symptoms, cranial nerve palsies and symptoms of signs of meningitis) or syphilitic eye disease (e.g., uveitis, neuroretinitis, and optic neuritis), a CSF examination should be performed. All patients with neurosyphilis and/or syphilitic eye disease should be treated with aqueous crystalline penicillin G 18 to 24 million unit per day administered as 3 to 4 million units IV every 4 hours or continuous infusion, for a duration of 10 to 14 days.[71]

Patients with primary and secondary disease with a severe allergy to penicillin may be treated with an alternative regimen.[71] The effectiveness of alternatives to penicillin in the treatment of latent syphilis is not well documented. Nonpregnant patients diagnosed in the early latent stage of disease should respond to the alternative antibiotic regimens recommended for the earlier stages of syphilis, but they should be closely followed both clinically and serologically after the treatment course is completed. Patients with late latent syphilis must be treated with penicillin after undergoing desensitization.[71] Pregnant patients should also be desensitized and treated with the penicillin regimen appropriate for their stage of infection.

Congenital Syphilis. CS occurs when fetal *T. pallidum* infection is acquired from a mother with untreated or inadequately treated syphilis. Because most infants at risk for CS can be easily identified by a positive serologic test in the mother,[493] it is tragic that CS rates have steadily increased since 1983 along with those in the general population.[71,74,120] Transplacental infection is most likely to occur during the primary or secondary stages of syphilis (Image Plate 20-1A), but spirochetes may infect the fetus at any time during pregnancy. The estimated rates of vertical transmission in untreated women are 70% to 100% for primary syphilis, 67% for secondary syphilis, and 40% to 83% for early latent syphilis.[35] Mothers with late latent syphilis may also transmit infection, but the rate is low (10%).[35] Infection may also be transmitted at the time of delivery in women who have active genital lesions, if there is a short interval (<4 weeks) between treatment and delivery.[35,71]Symptoms and signs of early CS can occur at any time during the first 2 years but seldom later than 3 to 4 months after

birth. Early CS cases include stillbirth due to in utero spirochete infection.[493] In some areas, CS is the most common cause of nonimmune hydrops, a disease of the placenta that causes fetal death.[28] Although the likelihood of clinical disease increases as the pregnancy progresses, more than half of all infected infants are asymptomatic at birth, and the signs in symptomatic infants may be nonspecific. The most common clinical presentation of early CS include premature and low birth weight infants (10% to 40%), hepatomegaly and/or splenomegaly (33% to 100%), a skin rash that may resemble the lesions of secondary syphilis (40%) and deformed tibias (saber shins) or teeth (mulberry molars) (75% to 100%) with associated radiologic changes, among other findings.[254,497] Pseudoparalysis, respiratory distress, bleeding, anemia and fever may occur in early CS.[149,497] Late CS may occur if the diagnosis of syphilis is missed in the neonatal and early childhood period. Late CS cases present after 2 years of age, and the clinical symptoms mainly affect the bones, teeth and nervous system.[208,294,497] CS may be prevented by clinically screening all pregnant women for syphilis infection. In high-risk populations, maternal and neonatal sera should undergo serologic testing for antibodies to *T. pallidum* at delivery. Cord blood is inferior to neonatal sera.[90]

Treatment. Interpretation of reactive serologic tests for syphilis in infants may be difficult because of the transplacental transfer of maternal nontreponemal and treponemal IgG antibodies to the fetus. Infants at risk of contracting CS should be treated even if a definitive laboratory diagnosis cannot be made due to limitations of the available tests (see Laboratory Diagnosis of Treponemal Diseases—CS section). Treatment decisions are therefore made on the basis of several factors, including: (1) diagnosis of syphilis in the mother, (2) adequacy of maternal treatment, (3) presence of clinical, laboratory, or imaging evidence of CS at delivery, and (4) comparison of maternal (at delivery) and infant nontreponemal serologic titers using the same test done by the same laboratory. Treatment regimens using different preparations of penicillin based on a combination of these factors have been clearly outlined by the CDC guideline document,[71] and a detailed description of the various clinical scenarios, their recommended evaluation, and treatment is beyond the scope of this chapter.

Antibody Response, Serologic Profile, and Immunity. *T. pallidum* has been called the stealth organism because its outer membrane contains few proteins, such that there are few cell surface antigen targets recognized by host immune cells or antibodies. Serum IgM and IgG antibody responses to *T. pallidum* have been the focus of several animal and human studies.[263] Certain membrane lipoproteins (e.g., TpN47, TpN17, and TpN15) primarily mediate the inflammatory effects of *T. pallidum* infection.[231,263] TpN47 is highly immunogenic and activates endothelial cells, and TpN17 and TpN15 induce antibody responses.[30] Several recombinant antigens

including Tp0453, Tp92 and Gpd proteins also showed equivalent serum antibody responses for *T. pallidum* infection.[483] Both antitreponemal IgM and IgG antibodies may be detected as early as 3 days after the onset of the chancre in primary syphilis.[23] Early antibody

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responses are directed toward TpN47 and some flagella proteins, followed by TpN15 and TpN17.[231] Antitreponemal IgG3 specific responses are increased in secondary syphilis, and immune-complex may be formed by coupling of IgG1 or IgG3 antibodies and *T. pallidum* antigens.[29]

Common patterns of serologic reactivity with production of IgM and IgG antibodies are seen in patients with syphilis.[23,231] Anti-IgM treponemal antibody production starts ~14 days after exposure before a chancre appears, with titers steadily increasing until 10 to 12 weeks, after which they gradually decline. Production of anti-IgG treponemal and nontreponemal antibody production starts ~14 days after IgM production and the appearance of a chancre but the IgG antibodies persist for years after the time of infection, regardless of treatment. By the time a patient has untreated latent syphilis, there is a markedly reduced IgM but strong IgG response. Patients in an even later stage of disease have a further reduction in IgM (i.e., faint or absent), and more variability in the number of IgG reactive antigens compared to those with early latent syphilis.[23]

The cellular immune system plays an important role in resistance to repeat *T. pallidum* infection. There is firm evidence in humans that immunity to *T. pallidum* is partial or delayed in development.[231] Adolescents who had congenital syphilis and volunteers who previously had natural syphilis have been re-infected. The likelihood that reinfection will occur decreases with increasing time after primary infection.[231] A period of partial resistance develops, in which reinfection may occur without the presence of a chancre. By the late stage, the patient is completely immune to reinfection. The likelihood that reinfection will occur decreases with increasing time after primary infection.[231] A period of partial resistance develops, in which reinfection may occur without the presence of a chancre. By the late stage, the patient is completely immune to reinfection.

Endemic Treponemosis

The treponemal diseases endemic to the tropics include yaws, pinta, and bejel caused by the different *Treponema* spp. (Table 20-1). All of these diseases have prominent

cutaneous manifestations, occur primarily in children, and are spread through lack of clothing, poor hygiene, crowded conditions, and poor access to health care. Epidemiologic and clinical features of these interesting conditions are reviewed in detail elsewhere and only briefly described herein.[15,226]

Treponema pertenue

Yaws is caused by *T. pertenue* and is the most prevalent nonvenereal treponematosis (Table 20-1).[15] It is endemic in tropical regions that have heavy rainfall and annual temperatures at or above 27°C (80°F), including central Africa and parts of the Indian subcontinent, South America, and southeast Asia. Yaws is a chronic disease that most commonly affects children younger than 15 years of age. Treponemes are inoculated by contact with open skin lesions, most commonly excoriations or bites.[15,138,226]There are primary, secondary and tertiary stages of the disease (Table 20-2). Primary yaws begins with a sentinel lesion (pruritic but nontender ulcer with a honey-brown crust) that develops 2 to 4 weeks after inoculation, usually on the buttocks or lower extremities. Secondary yaws begins several weeks to months afterwards, and the skin and bones are primarily affected.[15,138] Multiple cutaneous "daughter yaws" lesions develop near orifices such as the mouth and nose, but then spread diffusely, and ulcerate secreting infectious treponemes. Central resolution in these lesions mimics fungal infection and is therefore called "tinea yaws." A "crablike gait" may develop due to the formation of hyperkeratotic plaques on the palms and soles, and "pianic onychia" results from hyperkeratotic macules and papules within the nail folds.[138] Periostitis and osteitis cause severe pain in the hands, feet, and limbs. Periosteal thickening can be palpated and plain radiography demonstrated the early bone changes.[138]Tertiary yaws develops in 10% of patients 5 to 10 years after inoculation. The results of long-standing skin infection are destructive and deforming. Diffuse subcutaneous gummatous nodules cause large necrotic skin ulcers which heal by scarring and contracture.[15] Destructive osteitis results in saddle nose deformity, a rhinopharyngitis called gangosa, or bowing of the tibia or saber shins.[138,226] Periarticular sites are the most common locations for bone gummata, and a unique aspect of tertiary yaws is the development of gondou due to exostosis of the paranasal maxilla.[138] CNS disease is seldom attributed to endemic treponemes although late yaws may be associated with optic atrophy.[299] Benzathine penicillin is curative, although it may take months for the lesions to regress. Antibodies to *T. pertenue* are indistinguishable from antibodies to *T. pallidum*.

Treponema endemicum

Endemic syphilis is caused by *T. endemicum* (see Table 20-1).[15] The Arab Bedouins

called this disease bejel. This treponemal disease is geographically isolated in the dry, arid climates of the Arabian peninsula and the Sahel (southern border of the Sahara desert) and southern Africa, and it has not been eradicated due to poor hygiene in rural areas.[138,226] Bejel runs in families and primarily effects children 2 to 15 years of age. Several serologic surveys show that seropositivity ranges from 7.5% up to 27% in children in these regions.[138] Bejel mimics venereal syphilis in both clinical presentation and course. The primary skin lesions develop as painless, tiny papules or ulcers within the oral and nasopharyngeal mucosa. Spirochetes are transmitted through direct oral contact or with oral contact with contaminated food, cups, or kitchen utensils (Table 20-2). Bejel is not diagnosed until 3 to 6 months after exposure during the secondary stage because the primary oral lesions are not visible. A diffuse, nonpruritic maculopapular, papulosquamous, or annular skin rash then develops, and new lesions also develop within the oral and nasopharyngeal cavities that often move down to cause laryngitis.[138,226] Osteitis and periosteitis are also prominent signs of secondary bejel. The latent phase occurs within 6 to 9 months and is much shorter than that of yaws. Tertiary bejel starts as early as 6 months after the initial symptoms resolve, and is characterized by gummatous skin nodules that progress to form depigmented, noncontracted scars with dark margins. Nasopharyngeal destruction occurs through necrosis, and secondary infection with scarring of mucosal

gummata, and gangosa may ensue.[138,226] Bejel seldom produces the marked bony changes characteristic of tertiary yaws, but bone gummata may be seen on plain radiographs, and most patients have arthralgias and bone pain from periosteitis. Improved nutrition and earlier access to effective antibiotic treatment may attenuate the course of bejel so that leg pain due to periosteitis may be the only manifestation.[138]

Benzathine penicillin is the therapy of choice. Correction of poor sanitation and hygiene may be preventive. Patients with endemic syphilis produce antibodies that cross-react with *T. pallidum*.

Treponema carateum

T. carateum, the etiologic agent of pinta, is probably the oldest of the human treponemes.[15] Pinta is the least severe of the nonvenereal treponematoses, and causes only ulcerative or papulosquamous skin lesions that often depigment.[138,226] This disease occurs only in the western hemisphere, and remains endemic in remote regions of Mexico (states of Oaxaca, Guerrero, Michoacan, and Chiapas), and central and South America (primarily the western Amazon region of Brazil) (Table 20-1). No

recent pinta serologic survey data is available in these regions. Pinta like the other endemic treponematoses largely affects children less than 15 years of age.[138,226] Treponemes are most likely transmitted by direct skin-to-skin contact (Table 20-2). A primary sentinel skin lesion develops within 1 to 8 weeks after inoculation. A small number of papules or erythematous macules expand and coalesce into a scaling nonpruritic, nonulcerative plaque surrounded by a red halo.[138] The sentinel lesion is found on the exposed lower limbs and is rich in treponemes. Between 3 and 5 years after inoculation, a secondary skin rash occurs with diffuse, scattered lesions similar to the primary lesion called pintids.[138] Pintids development then waxes and wanes for 2 to 4 years when lesions may change color, and form larger coalescing lesions that lead to extensive depigmentation. After another 3 to 10 years, generalized pigmentary skin changes resembling vitiligo develop over bony prominences.[138] Spirochetes may be seen in histopathologic sections of skin biopsies of late hypopigmented skin, but these late-stage lesions are not infectious.

Benzathine penicillin is the therapy of choice. Pinta has no long-term systemic health effects, but the cosmetic disability is considerable. Antisera from patients with pinta cross-react with specific antigens of *T. pallidum*.[281]

Laboratory Diagnosis of Treponemal Disease

Traditional approaches to the laboratory diagnosis of syphilis have been extensively reviewed by Larsen and colleagues,[235] but more recently clinical laboratories have changed the screening algorithm, using newer highly sensitive commercial treponemal enzyme immunoassays (EIAs), immunochemiluminescence, and rapid immunochromatographic assays as the initial test.[81] This section outlines the current tests available for diagnosing syphilis, the ability of various test methods to detect infection throughout the various stages of the disease, the use and outcomes of implementing a reverse sequence testing algorithm, and the use of molecular tests.

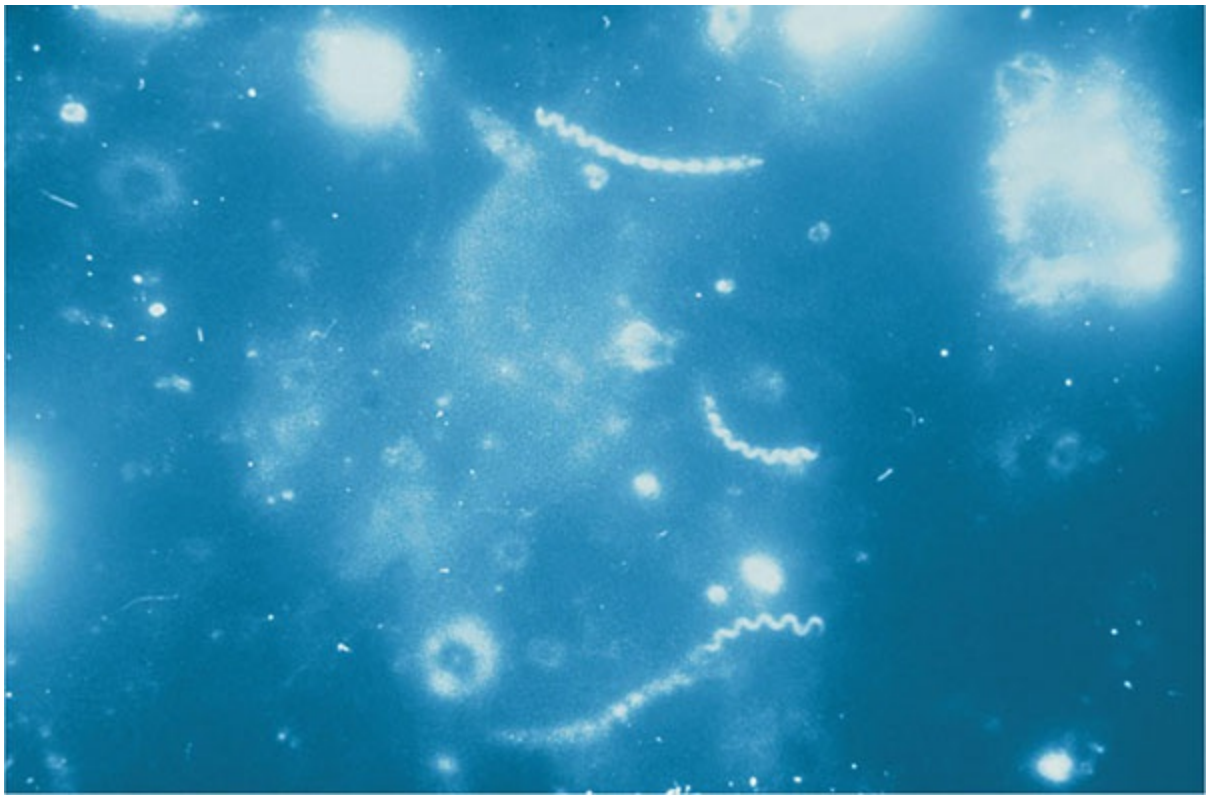
Culture. Prior to the development of serologic assays, culture was the gold standard test for diagnosis of syphilis, but is now only performed as an investigative tool (Table 20-4). Testicular tissue from rabbits was initially used by Noguchi (1911)[319] for cultivating 10 different strains of *T. pallidum* in pure culture (i.e., rabbit infectivity test [RIT]). Much later, Fieldsteel and colleagues[146] also propagated the virulent Nichols strain of *T. pallidum* in tissue culture monolayer of cottontail rabbit epithelial cells growing under an atmosphere of 1.5% oxygen. The RIT had been used to diagnose in utero fetal infection by inoculation of rabbits with amniotic fluid.[311] Molecular detection methods have now supplanted the use of the RIT, but one recent study found that polymerase chain reaction (PCR) was less sensitive for detection of *T.*

pallidum in neonatal samples due to PCR inhibitors in clinical specimens.[194]

Microscopy. The diagnosis of early primary syphilis has traditionally relied on direct detection of spirochetes in the exudates of active skin lesions (i.e., primary chancre) using darkfield microscopy (DFM) or direct histologic examination of tissue (Chart 20-1). Visualization of spirochetes (i.e., spiral organisms with pointed ends that have rapid corkscrew motility) by DFM in material scraped from the surface of a chancre is diagnostic of primary infection. DFM has a lower limit of resolution than bright-field microscopy (0.1 µm vs. 0.2 µm) so that the spirochetes, if present, may be easily visualized as luminous spiral-shaped organisms against a dark background (Fig. 20-4). Examination of oral or rectal lesions is not recommended because of the frequent presence of saprophytic spirochetes at these sites. Cutaneous and

TABLE 20-4 Culture of Spirochetes

Organism	In Vivo	In Vitro
T. pallidum	Rabbit testis; hamster; guinea pig	None
T. pertenue	Rabbit testis; hamster; guinea pig	None
T. endemicum	Rabbit testis; hamster; guinea pig	None
T. carateum	None	None
B. recurrentis	Various	Modified Kelly medium
Borrelia spp.	Various	Modified Kelly medium
B. burgdorferi	Various	Modified Kelly medium
L. interrogans	Various	Fletcher medium; Korthof's medium; Tween 80-albumin medium



■ **FIGURE 20-4** Positive darkfield examination for *Treponema pallidum.*

visceral lesions of secondary syphilis may also contain large numbers of spirochetes, which can be demonstrated by DFM of scrapings or imprints. DFM has also been used to detect fetal infection by finding spirochetes in amniotic fluid, but the specimen must be fresh and promptly examined.[194] Because of transport requirements, DFM testing is not feasible and thus not widely available in clinical laboratories. Specimens must be immediately examined after collection in order to visualize viable motile spirochetes, and up to three sequentially collected specimens should be examined before providing a negative result. Some STI clinics, however, continue to perform onsite DFM examination as a means to detect patients in the early phases of syphilis.

T. pallidum is difficult to stain using routine laboratory stains, but a direct fluorescent antibody (DFA) stain using monoclonal antibodies improves the detection of spirochetes in early skin lesions (Image Plate 20-1B).[204,235] Overall, DFM and DFA staining have a sensitivity of 74% to 86% and 73% to 100% and a specificity of 85% to 100% and 89% to 100%, respectively.[235] To perform the direct fluorescent antibody test for *T. pallidum* (DFA-TP), material from a lesion or tissue is collected as described for the darkfield examination, except that the material is allowed to dry on the slide (Chart 20-1). Alternatively, the dried smears or the exudates itself may be sent to a reference laboratory, the latter of which is collected in capillary tubes, then

sealed and stored at 4°C for transportation. The direct immunofluorescence method has also been effective for diagnosis of *T. pertenue* as well as for *T. pallidum*.[351]

Hook and colleagues[195] used polyclonal *T. pallidum* antibodies and demonstrated fluorescent treponemes in 30 patients with primary syphilis, 29 of whom also had positive darkfield examinations. More recently, Cummings and colleagues[100] compared DFM and monoclonal DFA staining to an early solid-phase enzyme-linked immunosorbent assay for detection of *T. pallidum* in lesion exudates from 188 patients, 64 (34%) of whom had lesions of early syphilis diagnosed by one of more of these methods. While DFM and the early EIA were positive in 55(85.9%) and 52(81.3%) and of the 64 patients, respectively, the DFA demonstrated the presence of *T. pallidum* in 59(92.2%) of the patients. A DFA test may give false-positive reactions even using a highly specific monoclonal antibody when testing oral lesions because oral spirochetes which cross-react with *T. pallidum* antigens are described in cases of necrotizing gingivitis.[376]

Diagnosis of the early stages of syphilis may be difficult in patients coinfected with HIV or other immunosuppressed patients because antibody titers may be so low as to be undetectable, yielding false-negative serologic tests. Syphilis may be diagnosed by biopsy of skin lesions, but characteristic morphologic features are often not present.[134,207,423] Spirochetes in formalin-fixed tissue have traditionally been detected by one of several silver impregnation stains, such as the Steiner, Warthin–Starry, or Dieterle procedures (Image Plate 20-1A).[482] Silver stains are sometimes difficult to interpret because of intense background staining.[193] Detection of spirochetes on histopathology in the appropriate clinical situation is presumptive but nonspecific evidence of syphilis. Immunohistochemistry with application of a fluorescein antibody provides improved specificity as well as sensitivity. Ito and colleagues[204] have demonstrated *T. pallidum* in formalin-fixed tissues using either polyclonal antisera or monoclonal antibodies after treatment with ammonium hydroxide or trypsin to open antigenic sites. More recently, PCR and real-time PCR have been used to improve detection of *T. pallidum* in tissue biopsies.[31]

Serology. Most cases of syphilis are confirmed by serology, followed by confirmation using either a second serologic test or another method including Western blot, or more recently a rapid immunochromatography assay or real-time PCR. The serologic tests for syphilis include nontreponemal and treponemal tests including newer commercially available enzyme EIAs, which have distinctive characteristics that make them useful for different purposes. The overall performance of the different tests for diagnosis of syphilis depending on the stage of disease is summarized in Table 20-5.[412] The US Public Health Service has published standardized procedures for performance of nontreponemal and traditional treponemal tests,[235] and the manufacturer's instructions

should be followed when performing a commercial syphilis assay.

Nontreponemal Tests. Nontreponemal antibodies directed against a tissue lipid called cardiolipin begin to be produced by the later stage of primary syphilis when the chancre appears (i.e., 21 days after inoculation), but seroconversion may occur up to 6 weeks after infection.[235] The association was recognized early in this century, and formed the basis for development of a variety of complement fixation tests.[227] Binding of cardiolipin and other host lipids to the spirochete may convert them into immunogens.[344] There are currently three tests available in the United States that use a synthetic form of cardiolipin that is complexed with lecithins and cholesterol as the antigen and rely on flocculation on reaction with IgM and IgG antibodies including: (1) VDRL, (2) RPR, (3) and the toluidine red unheated serum test (TRUST).[68,235] All of these nontreponemal tests are manually performed because none are suitable for the current methods of automation. Nontreponemal tests have been used to initially screen patients for syphilis, but they are now more commonly used as secondary confirmation tests in the newer test algorithms (Fig. 20-5) (see Reverse Testing Algorithm).

The VDRL test was the first nontreponemal test introduced for testing sera, and it remains the only syphilis serologic test that can be performed on CSF that is accepted universally for the diagnosis of neurosyphilis. Plasma and cord blood should not be used because borderline reactions may be obtained.[235] The VDRL assay is somewhat difficult to perform because of the precision required to prepare the antigen. The cessation of manufacture of the bottles required for preparation of VDRL antigen also prompted a search for an acceptable alternative.[358] Examples of VDRL reactions are shown in Figure 20-6. The procedure is summarized in Chart 20-2.

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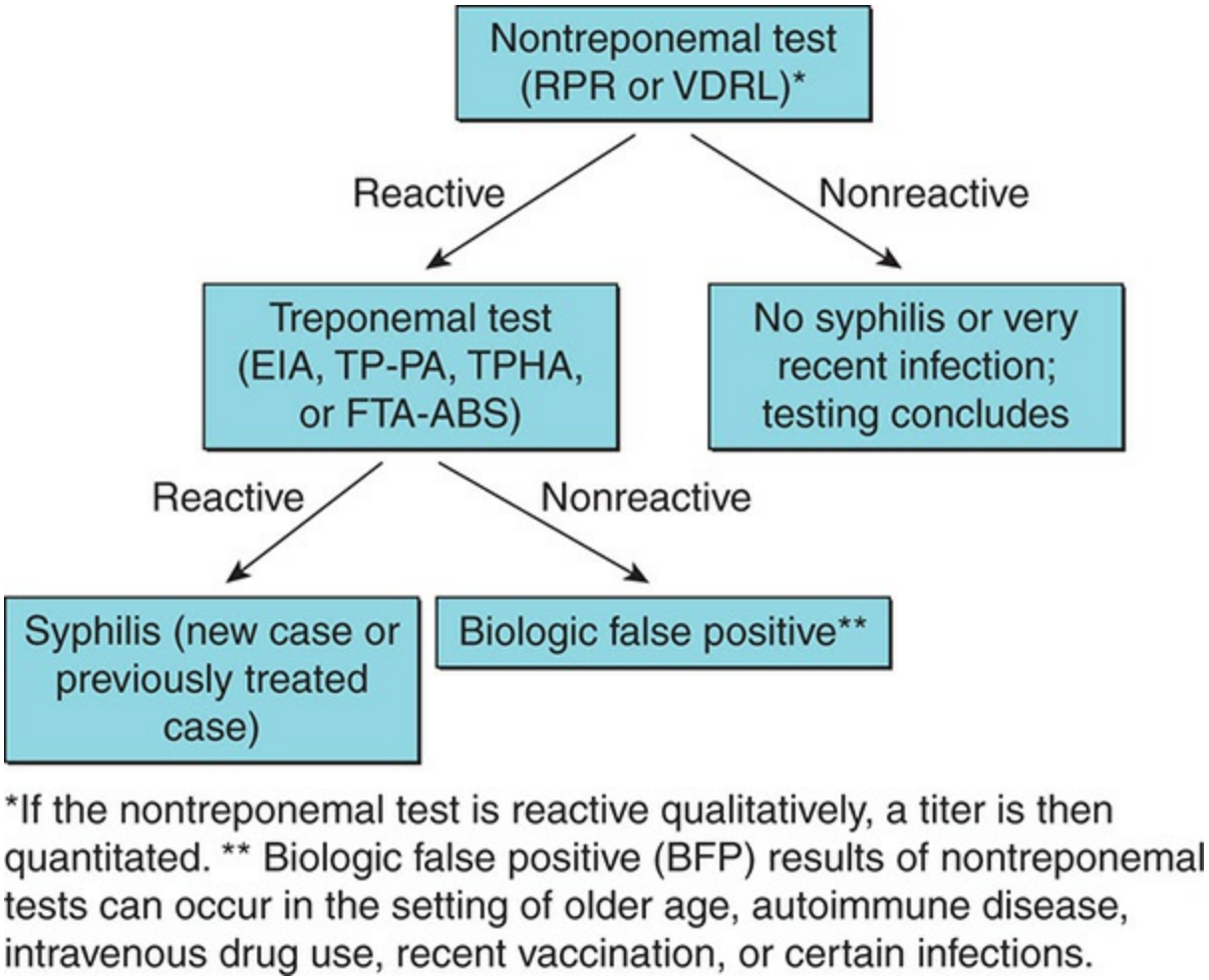

*If the nontreponemal test is reactive qualitatively, a titer is then quantitated. ** Biologic false positive (BFP) results of nontreponemal tests can occur in the setting of older age, autoimmune disease, intravenous drug use, recent vaccination, or certain infections.

■ **FIGURE 20-5** Prior conventional syphilis testing algorithm.

The RPR test is a flocculation test adapted to a card format (Fig. 20-7). The procedure is described in Chart 20-3. The visibility of the flocculation is enhanced by incorporation of charcoal particles. The RPR test is performed on sera samples, but it has not been validated for testing on CSF. Again, plasma and cord blood should not be used because of the risk of borderline reactions.[235] Due to the simplicity of the RPR test, most laboratories perform this assay as their primary nontreponemal screening test. The TRUST assay is performed in the same way as the RPR card test, but the antigen is based on the one used in the VDRL. In addition, ethylenediaminetetraacetic acid (EDTA), choline chloride, and toluidine red toner are added in order to produce a visible flocculation reaction.[352] This assay also has similar performance characteristics to the RPR test (Table 20-5).

TABLE 20-5 Sensitivity and Specificity of Serologic Tests for Syphilis

Test	Primary	Secondary	Latent	Late	Specificity
NTP Tests[a]					
VDRL	78 (74–87)	100	96 (88–100)	71 (37–94)	98 (96–99)
TRUST	85 (77–86)	100	98 (95–100)	NA	99 (98–99)
RPR	86 977–99)	100	98 (95–100)	73	98 (93–99)
Early TP Tests[a,b]					
MHA-TP	76 (69–90)	100	97 (97–100)	94	99 (98–100)
TPPA	88 (86–100)	100	100	NA	96 (95–100)
TPHA[c]	86	100	100	99	96
FTA-ABS	84 (70–100)	100	100	96	97 (94–100)
Enzyme IAS					
IgG ELISA[d]	100	100	100	NA	100
IgM EIA[e]	93	85	64	NA	NA
ICE[f]	77	100	100	100	99
ICL Assays					
CLIA[g]	98	100	100	100	99

[a]See reference 235.
[b]See reference 368.
[c]Lesinski J, Krach J, Kadziewicz E. Specificity, sensitivity, and diagnostic value of the TPHA test. Br J Vener Dis 1974;50:334–340.
[d]Castro R, Prieto ES, Santo I, et al. Evaluation of an enzyme immunoassay technique for detection of antibodies against Treponema pallidum. J Clin Microbiol 2003;41:250–253.
[e]Lefevre JC, Bertrand MA, Bauriavud R. Evaluation of the CAPTIA enzyme immunoassays for detection of immunoglobulins G and M to *Treponema pallidum* in syphilis. J Clin Microbiol 1990;28:1704–1707.
[f]See reference 509.
[g]See reference 510.
NTP, nontreponemal; VDRL, venereal disease research laboratory; TRUST, toluidine red unheated serum test; RPR, rapid plasma reagin; TP, treponemal; MHA-TP, micro-hemagglutination assay for *T. pallidum*; TPPA, *T. pallidum* particle agglutination; TPHA, *T. pallidum* hemagglutination assay; FTA-ABS, fluorescent treponemal antibody absorption assay; EIAs, enzyme immunoassays; ELISA, enzyme-linked immunosorbent assay; ICE, immune-capture EIA; ICL, immunochemiluminescence; CLIA, chemilumi-nescence assay.

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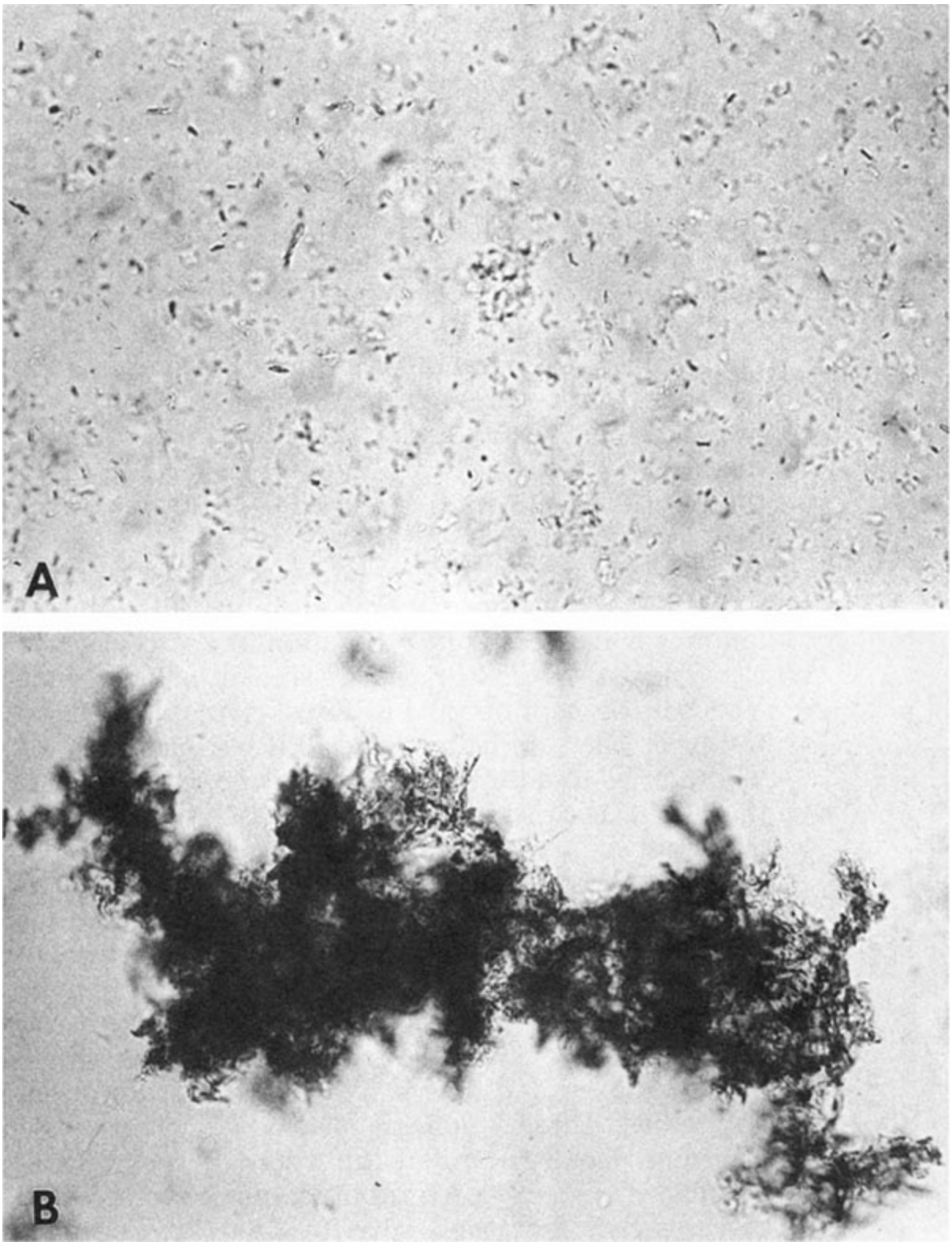
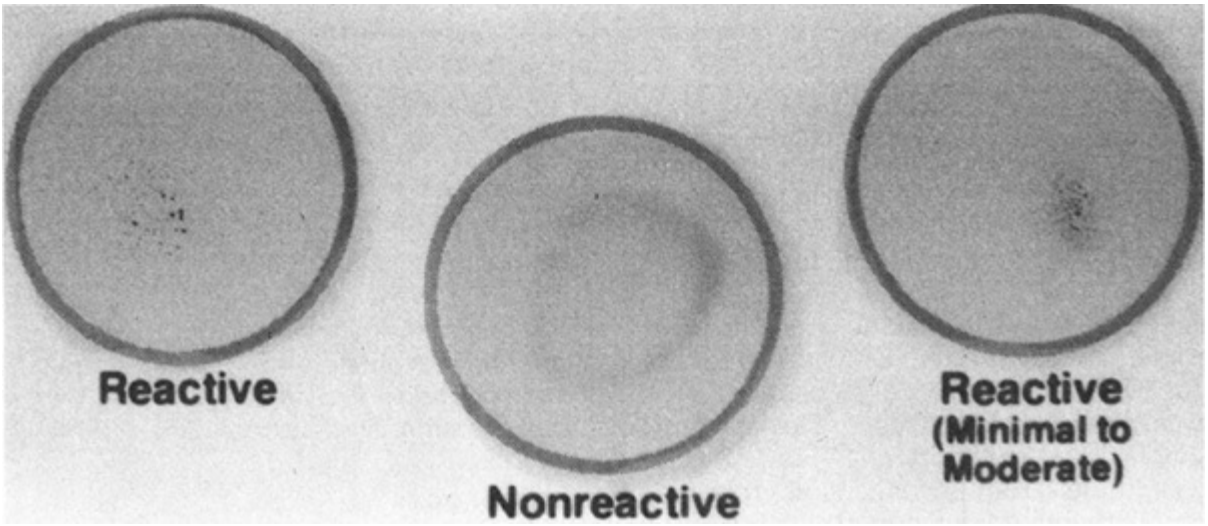


■ **FIGURE 20-6** VDRL test: The reactions in this test are evaluated microscopically. **A.** Nonreactive serum. The particles of VDRL antigen are uniform and freely dispersed without clumping. **B.** Reactive serum: The VDRL antigen particles are strongly agglutinated by this

syphilitic serum. The individual particles have aggregated into sheaves and large clumps ×100. (Courtesy of Burton Wilcke, PhD and Mary Celotti.)



■ **FIGURE 20-7** RPR card test: The reactions in this test are read with the naked eye, using incandescent light illumination. This control card contains Reactive (R), Weakly reactive (W), and Nonreactive (N) sera. The modified VDRL antigen is made visible by complexing with charcoal particles. The charcoal-antigen particles are evenly dispersed and finely distributed in the nonreactive serum and are grossly clumped. The serum with minimal-to-moderate activity produces small aggregates and clumps of charcoal-VDRL antigen particles.

The nontreponemal tests are affected by antimicrobial therapy and thus quantitative titers at baseline allow monitoring of the response to treatment.[235,23] The results of any positive test should be titered to an end-point dilution. In addition, serum from patients with large amounts of antibody may produce a prozone phenomenon resulting in a false-negative result. When a prozone occurs, the large relative concentrations of antibody to antigen prevent precipitation or flocculation from occurring. The prozone results are often marked by a "rough" appearance to the flocculated antigen. Any serum that produces this rough appearance should be titered. Serum should also be diluted and retested if it is strongly suspected on clinical grounds that a patient with negative test results has syphilis. In the general population the frequency of prozone reactions is sufficiently low that routine dilution of serum is not cost-effective.[132]

Treatment failure should also be considered if the nontreponemal antibody titer does not fall progressively as expected after an antibiotic course is given. There should be at least a fourfold decrease in antibody titer by 3 months after antitreponemal therapy. In patients who are treated in the late stages of syphilis or who are reinfected, titers that

decline very slowly or remain stable may develop. Some of these "chronic persisters" may maintain positive nontreponemal tests for life.

The nontreponemal tests have a sensitivity of 70% to 99%, depending on the stage of disease (Table 20-5).[412] Nontreponemal tests may not be positive in early syphilis, and serology should be repeated 7 days, 1 month, and 3 months after a negative result is obtained in a patient suspected of having syphilis. During the secondary phase, the sensitivity of the VDRL or RPR approaches 100%. Late syphilis, especially if treated, can also not be diagnosed using a nontreponemal test because the titer of antibody will eventually decline to undetectable levels (Tables 20-5 and 20-6).[368,412]

The lack of specificity (98%; range = 93% to 99%) of the nontreponemal tests limits their use as screening tests, particularly in low-prevalence populations (Table 20-5). Biologic false-positive reactions occur not only in patients who have other treponemal infections, but also in patients with other inflammatory conditions, including viral infections where antibody may be transiently produced, as well as pregnancy, advanced age, cancer, and autoimmune diseases (Table 20-7).[368] Biologic false-positive reactions have also been reported in intravenous drug users, a population at increased risk for contracting syphilis,[188,218] and HIV and hepatitis C seropositive patients.[16,514] A positive nontreponemal test requires confirmation by a treponemal test because of this limitation. Treponemal tests, however, may also be falsely-negative, in patients with immunosuppression or HIV coinfection.[215]

TABLE 20-6 Interpretation of Serologic Tests for Syphilis

Nontreponemal Tests	Treponemal Tests	Likely Interpretation
Positive	Positive	Syphilis, yaws, or pinta
Positive	Negative	False-positive (no syphilis)
Negative	Positive	Primary or latent syphilis; previously treated or untreated syphilis, yaws, or pinta
Negative	Negative	No syphilis, incubating syphilis

Adapted from reference 368.

TABLE 20-7 Causes of False-Positive Serologic Tests for Syphilis

Nontreponemal Tests	Treponemal Tests
Elderly	Elderly
Drug addiction/IVDU	Drug addiction/IVDU
Immunizations	Immunizations
Immunoglobulin abnormalities	Immunoglobulin abnormalities (Hyperglobulinemia)
Pregnancy	Pregnancy
Systemic infection: Bacterial endocarditis, brucellosis, chancroid, chickenpox, hepatitis, infectious mononucleosis, leprosy, lymphogranuloma venereum, measles, mumps, pinta, pneumococcal pneumonia, rickettsial disease, tuberculosis, viral pneumonia, yaws	Systemic infection: Brucellosis, genital herpes, infectious mononucleosis, leptospirosis, leprosy, Lyme disease, malaria, pinta, relapsing fever, yaws
Other inflammatory conditions: Idiopathic thrombocytopenic purpura, malignancy, polyarteritis nodosa, rheumatoid arthritis, systemic lupus erythematosis, thyroiditis, ulcerative colitis, vasculitis	Other inflammatory conditions: Cirrhosis, scleroderma, systemic lupus erythematosis, thyroiditis

Adapted from reference 368.

Treponemal Tests. Treponemal tests detect antibodies directed against surface antigens of *T. pallidum*, and antibody titers correlate poorly with disease activity or stage. Once positive, these tests remain reactive for years, regardless of treatment status (Tables 20-5 and 20-6). Treponemal tests, therefore, cannot be used to monitor the response to therapy, or to detect relapse or reinfection in previously treated patients. Treponemal tests may also be cross-reactive in patients with endemic treponemoses (yaws and pinta) and thus cannot be used to distinguish patients with syphilis from these other types of spirochete infection (Table 20-6). Traditional treponemal tests (FTA-ABS or TPPA) have mainly been used to confirm nontreponemal test reactivity, or as diagnostic tests in patients with late syphilis who have a negative nontreponemal test. More recently, however, highly sensitive

treponemal immunoassays have become available in several formats that are increasingly being used to screen low-prevalence populations for syphilis.

The first treponemal assay developed was the *T. pallidum* immobilization (TPI) test, in which the motility of live, virulent treponemes was inhibited by the presence of a specific antibody.[295] Due to the technical problems with this assay and its expense, other test formats have been progressively developed in the past 60 years. Tests formats that are currently available for the detection of specific *T. pallidum* antigens include earlier enzyme and particle immunoassays, newer commercial EIAs including some that are immunochemiluminescence assays that incorporate recombinant antigens, and rapid immunochromatographic assays that are being used in resource-limited settings.[129,130,139,176,412] This section briefly describes the principles behind each of these types of tests, including their performance and limitations.

Traditional Treponemal Tests. Earlier treponemal tests have been used for several decades to confirm the diagnosis of syphilis in patients with reactive nontreponemal test results, but these assays are now mainly used as reference tests since the implementation of highly sensitive EIAs to screen for syphilis (Fig. 20-5; see Reverse Sequence Testing Algorithm). The four treponemal tests that have been in use in the United States include the fluorescent treponemal antibody absorption assay (FTA-ABS), the *T. pallidum* particle agglutination (TPPA) (Fujirebio Inc., Japan), the microhemagglutination assay for *T. pallidum* (MHA-TP) (Fujirebio Inc., Japan), and the *T. pallidum* hemagglutination assay (TPHA).[235] The MHA-TP uses sensitized sheep erythrocytes coated with *T. pallidum* (Nichols strain), which agglutinate with antitreponemal IgM and IgG antibodies. The TPHA is a microhemagglutination assay for IgM and IgG antibodies. The MHA-TP assay and TPHA are less sensitive than the other treponemal tests for diagnosis of early syphilis (Table 20-5), and all equivocal reactions required confirmation by another treponemal test (i.e., FTA-ABS).[462] The MHA-TP and TPHA have, therefore, been replaced by more sensitive treponemal assays. Treponemal tests remain reactive for life except for patients who are diagnosed and receive therapy with primary syphilis,[235] and thus cannot be used to monitor the effects of therapy.

The FTA-ABS test is a qualitative indirect immunofluorescence test that requires pretreatment of sera samples with an absorbent to remove nonspecific antibodies (Chart 20-4). The assay uses fixed *T. palladium* to bind fluorescent IgM and IgG antibodies. Darkfield illumination was originally used, but the double-staining procedure which also applies rhodamine-conjugated IgG antitreponemal antibody as a contrasting counterstain improves location of spirochetes. The performances of the FTA-ABS traditional and double-staining procedures are comparable.[375] The FTA-ABS test is more sensitive than the MHA-TP or TPHA and the nontreponemal tests for detection of antibodies in early syphilis, but all of these tests are better at detecting patients in the later stages of syphilis (Table 20-5). The FTA-ABS test approaches a

sensitivity of 100% during the secondary stage.

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The FTA-ABS test has a high specificity which can be increased by absorption of the test sera with a nonpathogenic spirochete, the Reiter strain of *T. phagedenis.*[22,202] It has been estimated that 1% of the normal population will have a false-positive FTA-ABS test. Although false-positive FTA-ABS results are uncommon, patients with systemic lupus erythematosus or other connective-tissue diseases, and other infections may produce an unusual beaded staining reaction.[229] Cross-reactions can also occur with other pathogenic treponemes.[22] False-positive reactions may occur even with this very specific test if the FTA-ABS test is used to screen low-prevalence populations for syphilis.

Because the TPPA is less expensive and less complex to perform than the FTA-ABS test, it is one of the more commonly used traditional treponemal tests. The TPPA assay is a quantitative agglutination assay for the detection of antibodies to *T. pallidum* in sera or plasma. The TPPA uses the same treponemal antigen as the MHA-TP but instead uses colored gel particles sensitized with *T. pallidum* antigen instead of erythrocytes, thereby eliminating false-positive reactions in plasma samples.[235] The TPPA is as sensitive as the FTA-ABS test in primary syphilis but cannot be used to monitor therapy (Table 20-5).

Enzyme immunoassays. Investigators have been developing highly sensitive and specific enzyme immunoassays for syphilis since the 1970s.[485] The initial commercial EIA for syphilis, detected IgG antitreponemal antibodies using a microtiter plate with wild-type *T. pallidum* antigens coated in the wells.[508] This early EIA assay had a sensitivity of 98.4% and a specificity of 99.3% compared to the FTA-ABS and TPPA reference tests. The treponemal antigens, antibody targets and documented performance of several currently available commercial treponemal-based EIA tests, as well as an estimated range of positive predictive values based on the prevalence of syphilis in the US population are shown in Table 20-8.[412] Commercial syphilis EIA assays now mainly use one or more recombinant treponemal antigens (Tp15, Tp17 and/or Tp47) although some tests still use wild-type antigens. EIA tests that use wild-type antigens or only one recombinant antigen may be less sensitive, but this has not been verified using a large number of samples. For example, an immune-capture EIA (ICE Syphilis; Abbott Murex) which uses recombinant TpN15, TpN17, and TpN47 antigens had higher sensitivity than the CAPTIA SelectSyph-G (Centocor) that uses wild-type antigens (99% vs. 91.4%; $p<0.01$).[509]

These assays have been increasingly adopted as the major syphilis screening tests used by clinical laboratories because EIAs have comparable performance to traditional

treponemal tests (FTA-ABS and TPPA), but they are performed on automated systems that allows a higher volume of tests to be reported using less technologist time. Most EIA assays detect both IgG and IgM antibodies against one or more *T. pallidum* antigens, and are therefore unable to distinguish treated from untreated syphilis infections. Although many evaluations have been reported on the performance of individual EIA compared to reference tests, these studies have used different reference methods and testing algorithms, making it difficult to directly compare these assays. Only a small number of studies have been reported that evaluated several different EIAs using either wild type of recombinant *T. pallidum* antigens for detection of IgG and IgM antitreponemal antibodies using the same patient population. Cole and colleagues[92] compared 15 serologic assays (10 commercial EIAs vs. four TPHA and one TPPA kit) for the detection of syphilis using the same specimen panel that included 114 serum and plasma samples from patients with syphilis (40 with primary, 43 with secondary, 19 early latent stage, and 12 late latent stage infections) and 249 specimens from unselected blood donors. The sensitivity of the ten EIAs (Trepanostika TP Recombinant, bioMérieux; Syphilis EIA II, Newmarket Laboratories; Abbott Murex ICE Syphilis; Enzygost Syphilis, Dade Behring; Diesse Enzywell Syphilis; Biokit Bioelisa Syphilis 3.0; Bio-Rad Syphilis Total, Omega Pathozyme Syphilis; Mercia Syphilis, Microgen Bioproducts; Trinity Biotech CAPTIA Syphilis) and five TPHA/TPPA kits ranged from 93.9% to 99.1%, and overall, the EIAs had the highest sensitivity for detection of early syphilis cases. Only the Trinity Biotech EIA and three TPHA assays failed to detect specimens from the untreated secondary syphilis category. All specimens from all other syphilis diseases stages were detected by all other assays. All of the kits gave a final specificity of 100% except for the Abbott murex ICE Syphilis and the Biokit TPHA kits, which were repeatedly reactive with a few specimens that were negative according to supplemental tests.

Binnicker and colleagues[37] recently compared the performance of seven treponemal assays (BioPlex 2200 Syphilis IgG, Bio-Rad; Zeus Scientific FTA-ABS; Serodia TP particle agglutination [Fujirebio Diagnostics]; Trep-Sure EIA [Phoenix Biotech]; Trep-Chek EIA [Phoenix Biotech]; Trep-ID EIA [Phoenix Biotech] and Treponema ViraBlot IgG [Viramed Biotech]) using 303 serum samples submitted to a reference laboratory for the detection of syphilis. All of the samples in this study were also tested using an RPR assay and a treponemal IgM Western blot assay (Viramed VirBlot). Compared to a "consensus of the test panel" (defined as 4/7 treponemal tests in agreement) as the gold standard, the seven evaluated assays had comparable performance, with agreement ranging from 95.7% (95% CI, 92.7% to 97.5%) for the Trep-Sure EIA up to 99.3% (97.5% to 99.9%) for the Trep-ID EIA. Despite the overall performance of these assays, all of them gave discordant results. For example, when FTA-ABS results were compared to the consensus of the panel, three discordant samples were found with FTA-ABS positive, panel-negative results.[37] One of these

samples was also positive by the Trep-Sure assay but negative for all other tests including the RPR and IgM assays and was interpreted as probably false-positive FTA-ABS results.[37] Discrepant test results in this study could not be correlated to the clinical presentation or treatment history because no clinical data were submitted with these samples.

Although most commercial syphilis EIA assays have comparable performance, laboratories should validate individual assays to determine their limitations including the potential for false-positive and false-negative results in the population to be tested. Serum samples that test positive by the treponemal immunoassay but negative by the RPR assay should be analyzed with a second treponemal test that is different from the initial test used (see Laboratory Testing Algorithm).

Immunochemoluminescence assays. Two commercial immunochemoluminescence assays are currently available on high-throughput automated systems (Table 20-8). These assays use paramagnetic microparticles that are coated with

TABLE 20-8 Treponemal Antigens, Antibody Targets, and Performance of Several Treponemal-Based Tests and Their Estimated Positive Predictive Values Based on Syphilis Prevalence Ranging From 0.7% to 4.0% in the US Population

Test	Manufacturer	Treponemal Antigens	Treponemal Antibody Targets	Reference Tests	Sensitivity (%)	Specificity (%)	PPV (%)
Rapid Tests							
Syphilis fast[a]	Diesse	Recombinant (TpN15, TpN17, TpN47)	IgM, IgG	VDRL, TPHA, FTA-ABS	95.6	99.9	87.1–97.5
Determine Syphilis TP[b]	Abbott Laboratories	Recombinant TpN47	IgM, IgG, IgA	TPHA, TPPA	97.2	94.1	10.4–40.7
Espline TP[b]	Fujirebio	Recombinant (TpN15, TpN17, TpN47)	IgM, IgG, IgA	TPHA, TPPA	97.7	93.4	9.4–38.1
SD Bioline Syphilis 3.0[b]	Standard Diagnostics	Recombinant (TpN15, TpN17, TpN47)	IgM, IgG, IgA	TPHA, TPPA	95.0	94.9	11.6–43.7
Enzyme Immunoassays							
BioElisa Syphilis 3.0[c]	Biokit	Wild type	IgG	TPHA, FTA-ABS	99.5	99.4	53.9–87.4
CAPTIA Syphilis-G[d]	Trinity Biotech	Wild type	IgG	FTA-ABS	96.7	98.3	28.6–70.3
Eti-syphilis G[e]	Diasorin	Wild type	IgG	RPR, MHA-TP, FTA-ABS	99.4	100	58.4–89.2[f]
Trep-Chek IgG EIA[g]	Phoenix Biotech	Recombinant (not specified)	IgG	RPR. VDRL, TPPA, FTA-ABS	85.3	95.6	12.0–44.7
Syphilis EIA II[h]	Newmarket Laboratories	Recombinant (TpN15, TpN17, TpN47)	IgM, IgG	TPHA, TPPA	99.1	100	58.3–89.2[f]
Syphilis Total[h]	Bio-Rad	Recombinant (TpN15, TpN17, TpN47)	IgM, IgG	TPHA, TPPA	97.4	100	57.9–89.0[f]
Enzywell Syphilis Screen Recombinant[h]	Diesse	Recombinant (TpN15, TpN17, TpN47)	IgM, IgG	TPHA, TPPA	98.2	100	58.1–89.1[f]
Immunochemiluminescence							
LIASON Chemiluminescence Assay[i]	Diasorin	Recombinant TpN17	IgM, IgG	RPR, TPPA	95.8	99.1	42.9–81.6
Architect Chemiluminescence Assay[j]	Abbott Laboratories	Recombinant (TpN15, TpN17, TpN47)	IgM, IgG	VDRL, TPPA	98.4	99.1	43.5–82.0

[a]Young H, Moyes A, de Ste Croix I, et al. A new recombinant antigen latex agglutination test (Syphilis Fast) for the rapid serological diagnosis of syphilis. Int J STD AIDS 1998;9:196–200.
[b]World Health Organization. The sexually transmitted diagnostics initiative (SDI): special programme for reaserch and training in tropical diseases (TDr). Geneva, Switzerland; 2003.
[c]See reference 129.
[d]Halling VW, Jones MF, Bestrom JE, et al. Clinical comparison of the *Treponema pallidum* CAPTIA syphilis-G enzyme immunoassay with the fluorescent treponemal antibody absorption immunoglobulin G assay for syphilis testing. J Clin Microbiol 1999;37:3233–3234.
[e]Castro R, Prieto ES, Santo I, et al. Evaluation of an enzyme immunoassay technique for detection of antibodies against *Treponema pallidum.* J Clin Microbiol 2003;41:250–253.
[f]PPV calculated using a lower estimate of 99.5% due to the 95% confidence intervals around the reported specificities.
[g]Tsang RS, Martin IE, Lau A, et al. Serological diagnosis of syphilis: comparison of the Trep-Chek IgG enzyme immunoassay with other screening and confirmatory tests. FEMS Immunol Med Microbiol 2007;51:118–124.
[h]See reference 92.
[i]See reference 224.
[j]See reference 510.
EIA, enzyme immunoassay; FTA-ABS, fluorescent treponemal antibody absorption assay; MHA-TP microhemagglutination assay; TPHA, *Treponema pallidum* hemagglutination assay; TPPA, *T. pallidum* particle agglutination.
Adapted from references 412.

recombinant *T. pallidum* antigens that detect labeled anti-human IgG and IgM monoclonal antibodies. The LIASON assay (Diasorin) uses the TpN17 antigen while the Architect Syphilis Chemiluminescence Assay (CLIA, Abbott) includes Tp15, Tp17 and Tp47 recombinant antigens. These assays provide high sensitivity for detection of early syphilis but like other treponemal tests cannot distinguish recent, remote or previously treated spirochete infections. Young and colleagues recently tested 129 sera

from serologically characterized cases of untreated syphilis and 1,107 sera submitted for routine

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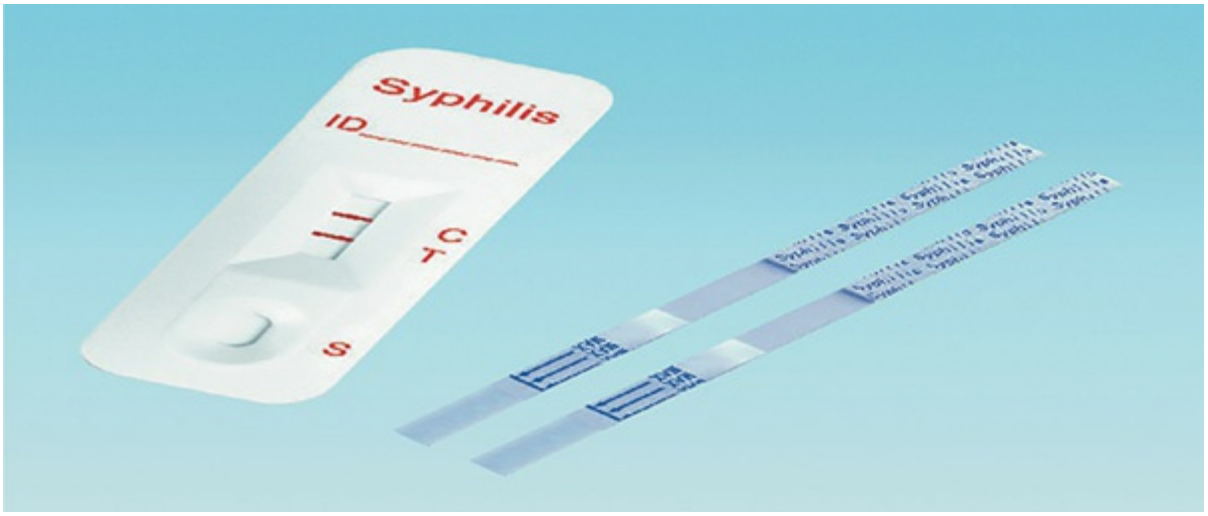
syphilis testing. The Architect Syphilis assay and the TPPA were in total agreement for all untreated infections with sensitivity of 98.4%, which was significantly higher than that of the Murex immunocapture EIA (ICE), the IgM immunoassay or the VDRL test.[510] Furthermore, the difference in sensitivity between the Architect syphilis assay and the Murex ICE assay was due to increased detection of primary stage syphilis cases (97.5% vs. 77.2%, $p<0.001$), but the specificity was higher for the Murex ICE (99.9%) versus the Architect assay (99.1%) ($p = 0.016$).[510] Knight and colleagues[224] recently showed similar performance characteristics for the LIASON assay compared with the CAPTIA Syphilis-G EIA using a testing algorithm that also included the RPR and TPPA assays. As a screening test, the LIASON compared with the EIA showed levels of agreement within the test algorithm, respectively, of 94.1% and 100% for 51 samples from patients with primary or secondary syphilis, 93.2% and 98.7% for 999 samples sent to the laboratory for routine syphilis testing, 84.5% and 94.0% for 200 samples from human immunodeficiency virus-positive patients, 98.0% and 100% for 200 samples from pregnant patients, and 94.3% and 98.3% for 992 samples from apparently healthy adults.[224] As a confirmatory test, the LIASON showed 99% agreement for 204 RPR-positive samples with the EIA. After resolution with further TPPA testing, and the discard of one inadequate sample, there was 100% agreement for the remaining 203 samples. For the total group of 2,645 samples, the overall relative sensitivity was 95.8% and the relative specificity was 99.1%.[224]

Both of the CLIA assays demonstrate excellent sensitivity as a screening test for syphilis among various patient populations, including specific populations with reportedly increased rates of false-positive nontreponemal test results. However, due to their somewhat lower specificity compared to some EIA assays (Table 20-8), a small increase in confirmatory tests may be required to exclude false-positive results in some patient populations.

Rapid tests. Commercial rapid point of care tests for syphilis are promising for syphilis screening, and are increasingly being used in resource-limited settings where other treponemal syphilis assays may not be readily available onsite. Rapid syphilis assays are available either as agglutination tests using latex particles coated with treponemal antigens or as immunochromatographic strips (ICS) where a colored line indicates a positive reaction (Fig. 20-8). These assays have been developed to meet the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid/robust, Equipment-free, and Delivered) criteria set out by the WHO for resource-limited settings. Most rapid tests can be stored at room temperature, are simple to perform in

that they require minimal training and no equipment, and the results can be read visually in less than 30 minutes. Another advantage of this format is the test can be performed with whole blood, serum, or plasma.

More than 20 rapid tests for syphilis detection are currently commercially available but none have yet been approved for use in North America. The WHO, the CDC, and other public health agencies have evaluated several rapid tests, mostly of the ICS format. Bronzan and colleagues[48] evaluated onsite rapid antenatal syphilis screening in South Africa using an ICS compared to an onsite RPR and offsite RPR and TPHA tests and found 79/1250 (6.4%) of women had syphilis according to the reference laboratory. The onsite ICS resulted in the highest number of pregnant women correctly diagnosed and treated for syphilis (89.4% vs. 63.9% for onsite RPR and 60.8% for offsite RPR/TPHA). The onsite RPR had a low sensitivity (71.4%) for high-titer syphilis, and the offsite approach resulted in the lowest client return rates for treatment. In a further study, these investigators also showed that onsite antenatal screening using an ICS was also cost-effective.[38]



■ **FIGURE 20-8** TP Syphilis Test Card—Positive Syphilis Rapid Test (Boson; LumiQuick, USA). This rapid anti-TP test employs chromatographic lateral flow test device in a cassette format. Colloidal gold conjugated recombinant antigens (Au–Ag) corresponding to TP antigens (P47, P45, P17, P15) are dry-immobilized at the end of nitrocellulose membrane strip. TP antigens are bond at the Test Zone (T) and rabbit anti-TP antibodies are bond at the Control Zone (C). When the sample is added, it migrates by capillary diffusion rehydrating the gold conjugate. If present in sample, TP antibodies (anti-TP) will bind with the gold conjugated antigens forming particles. These particles will continue to migrate along the strip until the Test Zone (T) where they are captured by TP antigens generating a visible red line. If there are no anti-TP antibodies in sample, no red line is formed in the Test Zone (T). The gold conjugate will continue to migrate alone until

it is captured in the Control Zone (C) by the rabbit anti-TP aggregating in a red line, which indicates the validity of the test. The presence of two color bands "C" and "T" within the results window indicates a positive reaction, while a single band "C" is negative.

Although limited systematic analysis of rapid tests in clinical settings has been done, clinical evaluations and in vitro laboratory research suggests that rapid treponemal tests have similar sensitivities and specificities to traditional nontreponemal tests (RPR). A large meta-analysis on syphilis testing using ICS tests to detect *T. pallidum* antibody was recently reported, because this test format has been evaluated in more clinical settings than other types of devices.[476] Tucker and colleagues[476] obtained clinical data from more than 22,000 whole blood, plasma, or fingerstick ICS tests done at STI clinics and antenatal clinics during 15 separate studies. ICS syphilis tests have a high sensitivity (median 0.86, interquartile range 0.75 to 0.94) and a higher specificity (0.99, 0.98 to 0.99) compared to the RPR nontreponemal screening test.[476]

More recently, several rapid immunochromatographic tests have been developed that simultaneously detect both nontreponemal and treponemal antibodies. The CDC recently evaluated an immunochromatographic device made by Chembio Diagnostics Systems Inc. (Medford, NY).[67] The device contains two nitrocellulose membrane strips perpendicular to each other contained within a plastic cassette that allow independent delivery of the sample (one strip) and the conjugate detecting reagents (second strip) consisting of colloidal gold particles conjugated to protein A and anti-human

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IgM antibody (Fig. 20-9). This assay has been used by the CDC to test 1,601 banked serum samples, and the results compared to the RPR and TPPA assays.[67] Compared with the RPR test, the rapid dual test had a reactive concordance of 98.4% when RPR titers of sera were ≥1:2 and the nonreactive concordance was 98.6%. Compared to the TPPA assay, the reactive and nonreactive concordances of the treponemal line were 96.5% and 95.5%, respectively.

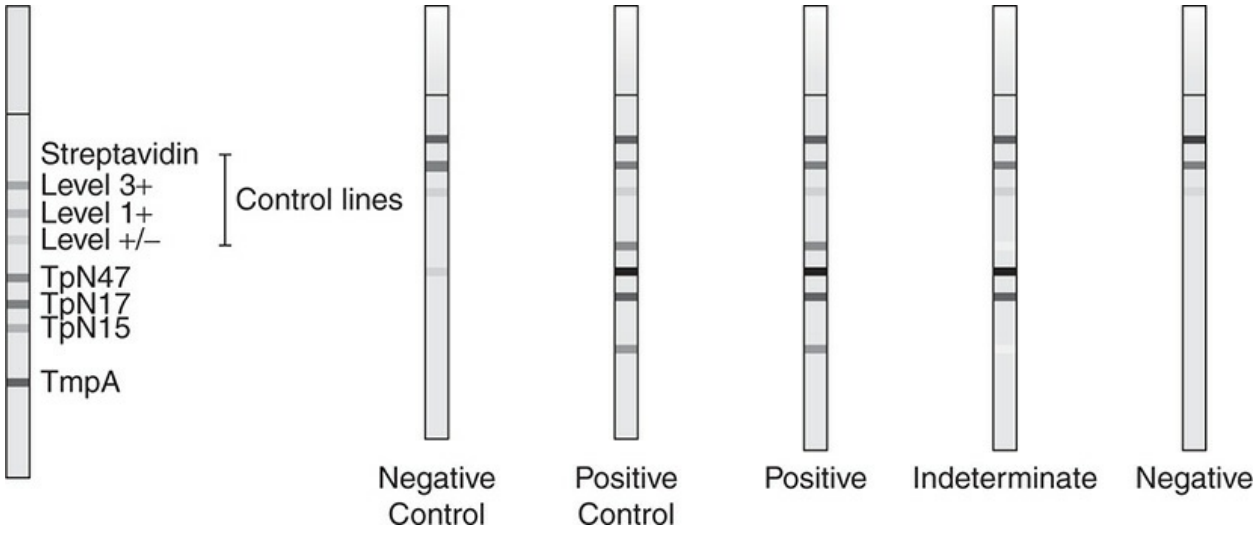
■ **FIGURE 20-9** Rapid Syphilis Test—Chembio's Dual Path Platform Technology. The recombinant *T. pallidum* antigen (T1) and synthetic nontreponemal antigen (T2) are bound to the membrane's solid phase, while a third line serves as a procedural control. Hydration of the conjugate reagents in the second well causes migration along the second strip to the test area where antibodies to treponemal and nontreponemal antigens are detected and visually read in the cassette window as red/magenta-colored lines within 15 minutes.

These data and the results of other evaluations of rapid tests suggest that these assays could be used for the serologic diagnosis of syphilis in primary healthcare clinics or resource-poor settings to improve rate of treatment where patients are unlikely to return for their laboratory results.[476] More research is needed to evaluate ICS and other rapid syphilis tests among patients with primary syphilis and patients infected with HIV before deploying these rapid tests widely in syphilis screening programs worldwide.[476]

Immunoblots. Immunoblots have also been used primarily as a confirmatory test for syphilis.[63] An advantage of this technology is that purified antigen can be used as the substrate, and the pattern of reactivity of sera can be used to determine positivity. If the criteria for determining positive immunoblot tests are not standardized, however, the variation in interpretation among laboratories can cause diagnostic problems. Western blot (immunoblot) assays for the detection of antibodies to *T. pallidum* were originally developed using the whole-cell organism (*T. pallidum* Nichols strain) as the antigen,[114,324,388] and detected the major surface antigens of *T. pallidum* (TpN15,

TpN17, TpN44.5, and TpN47). The sensitivity of one early immunoblotting assay for detection of IgM antibodies in 14 symptomatic neonates was 92%, whereas only 83% of 12 asymptomatic neonates who were later proved to be infected were detected by Western blotting.[388]

Commercial immunoblot assays have subsequently become available as confirmatory assays for *T. pallidum* antibodies that have comparable performance as a single multi-parameter test compared to confirmation using a combination of serologic assays. The INNO-LIA Syphilis Kit (Innogenetics NV, Ghent, Belgium) is a line immunoassay (LIA) that uses three immunodominant membrane proteins (TpN15, TpN17, and TpN47) derived from *T. pallidum* (Nichols strain) and one synthetic peptide (TmpA) derived from transmembrane protein A.[130,176,232] In addition to the syphilis antigens, control lines are used for a semiquantitative evaluation of the results and to verify that the reagents and sample have been added. Figure 20-10 shows the layout of the INNO-LIA syphilis kit strip. The interpretation algorithm of this immunoblot assay has been optimized for visual reading using a set of known negative and positive samples. Several representative INNO-LIA syphilis kit reactivity patterns are shown in Figure 20-10. Briefly, a sample is *T. pallidum* antibody-negative if no band or an isolated band with a minimum intensity equal to 0.5 is present. If multiple bands with a minimum intensity equal to 0.5 are visible, the sample if considered positive. A sample is considered indeterminate if a single band is visible with a minimum intensity equal to 1. Ebel and colleagues[130] reported a sensitivity of 99.6% (95% confidence interval [CI], 98.5% to 99.9%) and a specificity of 99.5% (95% CI, 98.1% to 99.9%) for this immunoblot assay compared to the consensus result (i.e., results of classical serologic assays and the clinical history). Six of seven indeterminate samples in this study using classical serologic assays tested positive using the INNO-LIA syphilis kit. In another evaluation using 289 seronegative sera, 219 seropositive sera, and 23 sera that were indeterminate based on serologic testing, the INNO-LIA assay had a sensitivity and specificity of 100% (219 of 219) and 99.3% (286 of 288), respectively, and gave a significantly higher number of correct results than either the FTA-ABS (IgG) or TPHA assays.[176]



■ **FIGURE 20-10** Syphilis INNO-LIA immunoblot reaction. (Adapted from INNOGENETICS and references 176, 232.)

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Other immunoblot and Western blot assays have been recently evaluated for the detection of *T. pallidum* antibodies. Two IgG immunoblot assays (Treponema ViraBlot test kit IgG [Viralab, Inc., Oceanside, CA] and the *Treponema pallidum* IgG line immunoblot [Genzyme ViroTech, GmbH, Russelsheim, Germany]) and one IgG Western blot assay (*T. pallidum* IgG Marblot strip test system [MarDx Diagnostics, Inc., Calsbad, CA]) were compared with the RPR, FTA-ABS, and the TPPA test results for 200 samples submitted to a reference laboratory that receives a high percentage of inconclusive FTA-ABS samples from primary screening laboratories.[492] The agreement levels of the Viramed, Virotech, and MarDx assays were 97.0%, 96.4%, and 99.4%, and the agreements of samples inconclusive by FTA-ABS and resolved by TPPA were 91.7%, 83.3%, and 69.4%, respectively. The performance of the two Western blot assays (IgG Western blot assay [*T. pallidum* IgG Marblot strip test system (MarDx Diagnostics, Inc., Carlsbad, CA)] and the INNO-LIA Syphilis) has also been compared with the Murex ICE Syphilis EIA and the FTA-ABS and TPPA tests for the serologic diagnosis of syphilis in 135 patients attending a social hygiene clinic (39 in primary, 20 in secondary, 19 in early latent, and 58 in latent of unknown duration) in Hong Kong.[232] The LIA assay had an overall sensitivity of 94.1% (95% CI, 88.7% to 97%), whereas the MarDx assay was 65.2% (95% CI, 56.8% to 72.7%), but both Western blot assays had a specificity of 100%. The LIA assay had a positive agreement with the consensus results of the other assays (EIA, FTA-ABS, and TPPA) of 98.5% (95% CI, 94.5% to 99.6%), whereas the MarDx assay was significantly lower at 68.2%(95% CI, 59.8% to 75.6%). Therefore, Western blot and

immunoblot assays offer additional accurate treponemal tests that can confirm the currently recommended tests in the syphilis algorithm, but some assays may be better than others at confirming the results of other types of treponemal assays. The ViraBlot immunoblot assay and the INNO-LIA Syphilis Western blot assay currently have the best performance as confirmatory tests. However, the number and quality of immunoblot and Western blot tests will likely expand as our understanding of the nature of the immunologic response to other protein antigens of *T. pallidum* increases.[231,438]

Congenital Syphilis. An excellent review of diagnostic tests for CS in newborns provides an overview of the current use and performance of various tests, including the use of newer EIA, immunoblot and PCR assays.[190] Serologic diagnosis of CS remains difficult because laboratory test results may be indeterminate in the neonatal period due to the presence of maternal antibody (IgG), so that no single test can be used to make the diagnosis. Nontreponemal tests have limited application for diagnosing CS due to the very low sensitivity (4% to 13%) of an infant's serum VRDL/RPR titer being at least fourfold (two or more dilutions) above that of the mother's serum at birth.[443] However, if the VDRL/RPR tests become nonreactive before 6 months of age in an infant who has not received treatment, the diagnosis of CS is excluded.[190] Neurosyphilis may also be confirmed in infants with symptomatic CS by the presence of CSF abnormalities, including pleocytosis, an elevated protein level, and a reactive CSF VDRL.[294]

The detection of specific antitreponemal IgM remains the most sensitive initial test for diagnosis of CS.[348] Unlike IgG antibodies that cross the placenta and enter the fetal circulation, immunoglobulins of the M class do not cross the placenta so that the detection of specific antitreponemal IgM antibodies in a neonate indicates intrauterine infection.[9] Herremans and colleagues[190] summarize the reported performance of different types of IgM assays in newborns at risk or with clinical or asymptomatic CS and healthy newborns. Three different methods are currently used to detect treponemal-specific IgM in neonates in order to diagnose syphilis including: (1) 19S FTA-ABS, (2) IgM immunoblots, and (3) IgM EIAs. Because antibody reactivity to some *T. pallidum* antigens may occur in normal sera, the 19S IgM FTA-ABS test includes fractionation of serum (separation of the maternal IgG fraction for the fetal 19S IgM fraction) to obtain IgM specificity, prior to performing the FTA-ABS test.[217,459] The sensitivity of the 19S FTA-ABS test in clinical CS cases is 72% to 77% with a specificity of 100%. Rosen and Richardson demonstrated good performance with a research assay, but encountered problems when commercial reagents were used.[383] A few at-risk asymptomatic children tested false-positive for IgM using this assay.[383] False-positive reactions occur if antiglobulins, such as

rheumatoid factors or anti-idiotype antibodies, are present in fetal sera. False-negative reaction may also occur due to excessive amounts of IgG antibody blocking the antigenic sites. The 19S FTA-ABS test has, therefore, improved specificity; so it can be used as a confirmatory procedure, but its lower sensitivity compared to the FTA-ABS assay prevents its use as a screening test.

Several IgM EIAs have been evaluated for the diagnosis of CS. The sensitivity (88% to 100%) and specificity (100%) of these assays are high, and positive IgM tests are detected in 3% to 7% of asymptomatic patients at risk for CS.[190] The CAPTIA Syphilis-M EIA (Trinity Biotech) detects only IgM antitreponemal antibodies, and has been cleared by the Food and Drug Administration (FDA) for the diagnosis of CS, and may also be used for the diagnosis of primary syphilis because of its high sensitivity.[241,502] Although few studies have been done comparing commercial IgM EIAs with IgM immunoblots, the later assay may be slightly more sensitive for diagnosing CS. Overall, 21/25 (84%) of infants with a positive IgM immunoblot were also positive by the IgM EIA, and two of the false-negative EIA test occurred in patients with only single antigen bands on the immunoblot.[189] Another study of 97 mother–baby pairs showed that 18 were positive using an IgM immunoblot (7 infants had CS), while the IgM EIA was only positive in 14 infants (5 infants had CS, while 2 were false-positive and had a negative immunoblot).[371] In this study, the nontreponemal test (RPR) was also shown to be inferior to an IgM treponemal test for diagnosing CS.[371]

The 47-kDa protein is one of the major immunogens in CS that stimulate neonatal IgM antibody production that may also be detected in fetal blood.[388] Sanchez and colleagues[388] used Western blot analysis to identify a 47-kDa antigen that was not seen in control patients and was correlated with intrauterine infection. IgM immunoblot reactions are reported as positive in infants when one or more of the 47-, 45-, 30-, and 15/17-kDa reactive bands are present.[190] In CSF, any IgM-reactive band in the 47 and 15.5 ranges are considered positive.[294] The IgM immunoblot is very effective in

confirming CS in symptomatic infants with sensitivity between 83% and 100% and a specificity of 100%.[189,190] In asymptomatic infants at risk for CS, 4% to 22% may have a reactive IgM immunoblot, but the specificity remains high.[190] The sensitivity of immunoblot for detection of IgM antibodies in 14 symptomatic neonates was 92%, whereas 83% of 12 asymptomatic neonates who were later proved to be infected were detected by Western blotting.[293] Twenty-seven of 30 (90%) uninfected infants were correctly characterized.[293] Dobson and colleagues[114] demonstrated reactivity to 47-

and 37-kDa protein antigens in the serum of congenitally infected infants. Although rheumatoid factor could theoretically cause problems in the immunoblot assay, removing rheumatoid factor from the sera did not affect the results.

Sequential testing remains an important part of the laboratory monitoring of CS. If the infant or mother is IgM seroreactive at birth, treatment should be started and follow-up tests arranged for 1, 2, 3, 6, and 12 months after birth to assess the response to treatment. Given appropriate therapy, the serologic tests (VDRL/RPR and IgM) in symptomatic infants decline within 3 months and disappear by 6 months. Treponemal tests are less effective at monitoring the response to therapy because the infant may have persistent maternal IgG until 15 months of age.[190] However, infants with persistently reactive treponemal test at 18 months of age may have CS if the VDRL-RPR is still reactive, and they should be reevaluated.

Molecular Methods. Molecular techniques have increasingly been applied to the direct detection of spirochetes in tissues and fluids. Investigators have concentrated their attention on intrauterine and CNS infection, because serologic techniques are not adequate for these important clinical problems.[172,323] DNA probes have not been sufficiently sensitive, so efforts have been focused on amplification techniques, such as PCR.

T. pallidum has successfully been detected by PCR targeting different gene regions in clinical specimens.[83,172] The *T. pallidum* gene encoding a 47-kDa protein has been used as the target region for most PCR studies, but the DNA polymerase I gene may be a more sensitive target.[255]

The detection of *T. pallidum* by PCR in amniotic fluids, neonatal sera, and CSF has been shown to be a moderately sensitive alternate method (76% to 86%), with a specificity of 100% for diagnosing CS compared with the RIT.[172] Although PCR should be more sensitive than the RIT, the presence of inhibitors in clinical specimens may yield false-negative tests, especially in CSF. Treponemal DNA in spinal fluid appears stable, so negative results are probably not from deterioration of nucleic acids in the specimens.[487] PCR detection of *T. pallidum* has helped confirm a diagnosis of CS in infants born to mothers with syphilis.[294] In this study, spirochetes were detected in the CSF of 17 of 76 infants with no prior antibiotic exposure by rabbit infectivity testing.[294] Most of these infants (16/17) had an abnormal clinical examination and/or abnormal laboratory or radiographs suggestive of CS. Overall, a positive result on IgM immunoblotting or PCR of serum, blood or CSF were the best predictors of CNS infection.

PCR has also been used to detect *T. pallidum* in formalin-fixed, paraffin-embedded tissues. Genest and colleagues[159] developed a PCR assay to detect the 47-kDa treponemal membrane antigen and evaluated the performance of this assay using 49 formalin-fixed, paraffin-embedded placentas (38 from mothers with positive syphilis

serology and 11 with negative serology) against histopathology and Steiner staining. PCR was significantly associated with placental histopathologic results diagnostic of CS and the detection of spirochetes by the Steiner stain, but also detected a few additional patients with negative histology. Behrhof and colleagues[31] developed a semi-nested PCR assay targeting the DNA polymerase I gene and compared this approach with immunohistochemistry and silver staining (Dieterle) for detecting *T. pallidum* in 36 skin biopsy specimens from patient with a clinical and/or serologic diagnosis of syphilis. Twenty skin biopsy specimens contained *T. palladium*: 17 were positive by immunohistochemistry, 14 were PCR-positive and only 9 were detected by the Dieterle stain. PCR was highly dependent on the quality of the tissue specimen and the integrity of the DNA, but was positive in all cases except one when poor quality specimens were excluded, including three cases negative by the other methods.

A few studies have been reported that compared syphilis PCR assays with serology results. Bruisten and colleagues[53] used a basic membrane protein (bmp) gene target in a gel-based nested PCR method, and compared the results against RPR, TPHA, and FTA-ABS results in a cohort of 364 STI clinic patients in the Netherlands. The PCR assay had a correlation of 96% with the other methods but only 12 patients were detected by PCR, 7 patients were detected by serology, and only 3 patients were detected by both PCR and serology. Palmer and colleagues[340] used a gel-based PCR assay to detect the *T. pallidum* 47-kDa integral membrane lipoprotein gene in 98 STI clinic patients in the United Kingdom and compared the results with serology (RPR, TPPA/TPHA, and EIA IgM and IgG). Although the PCR had a sensitivity of 94.7% and specificity of 98.6% for the detection of primary syphilis, the sensitivity decreased to 80% compared with serology for detection of secondary cases most likely because of sampling error or insensitivity of the PCR assay.[340] More recently, investigators have focused on developing reliable real-time PCR assays to detect *T. pallidum* in a variety of clinical specimens. Leslie and colleagues[242] developed a real-time PCR assay targeting the *pol* A gene of *T. pallidum* (TpPCR) with an analytic sensitivity of 1.75 target copies per reaction. TpPCr results were compared with serology for 301 patients and the TpPCR assay showed 95% agreement, with a sensitivity of 80.39% and a specificity of 98.40% but discrepancy occurred for 14 patients (10 had positive serology but negative PCR reactions; 4 had negative serology but positive PCR reactions). Again, the insensitivity of the PCR assay was attributed to incorrect timing or site of sampling. A real-time PCR assay targeting the 47-kDa gene of *T. pallidum* was used to diagnose syphilis in a cohort of 74 patients with and without HIV infection who were at different stages of syphilis (primary [n = 26], secondary [n = 40], and latent) by testing various blood samples, lesion swabs, and/or urine and CSF samples.[158] Overall, the sensitivity of the PCR (regardless of samples type) was 65% during primary and 53% during secondary, but no latent cases were detected. No difference was found in either serology or PCR results among HIV-infected patients.

The performance of a real-time PCR targeting the *polA* gene of *T. pallidum* was recently compared to the diagnosis of syphilis made by the STI clinic (including a DFM result) for 716 patients with primary

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and 133 patients with secondary syphilis.[191] For patients with primary syphilis, the PCR assay had a sensitivity of 72.8%, specificity of 95.5%, and a positive predictive value of 89.2% and a negative predictive value of 95%. However for those with secondary syphilis, the sensitivity of the real-time PCR assay was much lower (43%) compared to skin or mucosal findings plus an RPR titer ≥1:8.[191]

Multiplex real-time PCR assays or microarrays may be the best approach for comprehensive diagnosis of the presence of *T. pallidum* and other pathogens known to cause genital ulcer disease. Suntoke and colleagues[465] developed two duplex real-time PCR assays for detection of *Haemophilus ducreyi* (causative agent of chancroid), *T. pallidum*, and herpes simplex 1 and 2 and used them to detect these pathogens in ulcer swabs taken from 100 people with symptoms in rural Uganda. Of the 100 swabs analyzed from 43 HIV-positive and 57 HIV-negative patients, 71% were positive for one or more STI pathogens by real-time PCR (61% for HSV-2, 5% for *T. pallidum*, 3% for HSV-1, 1% for *H. ducreyi,* and 1% for dual *H. ducreyi*/HSV-2). A real-time PCR test targeting the 47-kDa membrane gene of *T. pallidum* was multiplex with an existing HSV1/2 real-time PCR assay and used to test 692 samples from patients attending genitourinary clinics in Glasgow.[404] Of the 692 samples, 139 were positive for HSV1, 136 for HSV2, 15 for syphilis, and 1 for both syphilis and HSV1. All syphilis PCR-positive cases were confirmed by a second PCR assay, and this method was more sensitive than both DFM and serologic testing for the diagnosis of primary syphilis and unexpected cases of syphilis where HSV was clinically suspected to be the cause of the genital ulcer disease. Multiplex detection of *T. pallidum* and HIV has also been done using a visual DNA microarray-based on gold label silver stain and coupled with multiplex asymmetric PCR.[467] This visual gene detection technique accurately detected HIV and *T. pallidum* in 169 clinical samples compared to EIA and fluorescence quantitative real-time PCR.[467]

Based on the early success of these in-house molecular detection approaches, multiplex detection assays that can simultaneously detect *T. pallidum* and other important STI pathogens will likely be developed (e.g., Luminex microbead-based assay) and commercialized for the reliable, rapid, and efficient diagnosis of syphilis and coinfections in at-risk patients.

Reverse Testing Algorithm. The traditional laboratory based approach to screening patients for syphilis has been to first test with a nontreponemal assay (VDRL or RPR),

and then to secondarily use a treponemal test because of its perceived higher specificity to confirm positive VDRL or RPR results, or to test patients with a negative nontreponemal test where there is a high clinical suspicion of infection. But the perceived "greater" specificity of some treponemal tests is entirely an artifact of their use as confirmatory tests after performance of a nontreponemal test.[180] When the tests are compared head to head as the initial serologic procedure, the VDRL nontreponemal test is actually as specific as or more specific than the treponemal FTA-ABS test.[234]

Due to the commercial availability of highly sensitive automated syphilis EIA IgG and IgM assays, there has recently been an increasing clinical laboratory implementation of a reverse sequence algorithm for syphilis testing that reverse the order of use of nontreponemal and treponemal tests. The clinical advantages of this approach are that test results can be available the same day, and that specific antitreponemal antibodies are detected earlier in the course of the infection thereby enhancing the detection of primary syphilis cases. Testing stops in the new test algorithm if the treponemal EIA test is nonreactive, but a reactive treponemal EIA test leads to testing with a nontreponemal test with titer quantification, and to other tests if the VDRL/RPR is not reactive. All of the new algorithms produce a combination of results (i.e., reactive treponemal EIA but nonreactive VDRL/RPR) that were not produced by the traditional test approach. A recent investigation by the CDC of the use of the newer syphilis screening algorithm investigated highlighted discrepancies in laboratory testing and reporting practices that can further complicate interpretation of the results combinations that may be produced depending on the disease stage and treatment history (Fig. 20-11).[81] The investigators reviewed data on 116,822 specimens tested during 2005 to 2006 at four New York City laboratories using syphilis testing algorithms that started with a treponemal EIA.[81] A total of 6,548 (6%) specimens were reactive to a treponemal EIA test and most (99%) had secondary testing using an RPR test, while further testing was not done on nonreactive samples (94%).[81] Forty-four percent of the 6,548 samples were confirmed positive by the RPR test, and the remaining samples were nonreactive.[81] Patients with a positive result in both tests (EIA + RPR) had either a new or old case of syphilis depending on the RPR titer as follows: (a) RPR titer had not increased fourfold, no further treatment is required, and (b) RPR titer showed a fourfold rise, retreatment is required. Patients with positive EIA results but a negative RPR had early syphilis, old or previously treated syphilis, or a false-positive EIA test. False-positive treponemal EIA tests can be resolved by performing a different treponemal test (TPPA, FTA-ABS) or an immunoblot with a high sensitivity (i.e., INNO-LIA).[81] Although only 69% of the 3,664 samples (EIA + but RPR −) were tested with a second treponemal test, 83% of the 2,528 tested by a second treponemal method were positive.[81] Importantly, these

patients should be staged and treated unless previous syphilis treatment can be documented. The 17% of patients who had discrepant results between the initial reactive EIA and second treponemal test may be considered false-positive EIA results by the laboratory, but these patients should be clinically reviewed to determine whether a third treponemal test should be used to resolve the discrepancy.

Only two studies have evaluated the cost-effectiveness to the newer algorithm approach for syphilis screening. A cohort decision-analysis has been used to estimate the expected costs and effects including follow-ups and overtreatment of a traditional (Nontreponemal-first) versus newer (treponemal-first) syphilis test algorithm in the US healthcare system.[333] For a cohort of 200,000 patients (1,000 current infections and 10,000 previous infections), the net costs were $1.6M (treponemal-first) and $1.4M (nontreponemal-first). The treponemal-first approach resulted in treatment of 118 more cases (986 vs. 868), and a significant increase in the number of patients who were overtreated (964 vs. 38) or had to be followed-up (11,450 vs. 3,756). The estimated cost-effectiveness ratios were $1,671 (Treponemal-first) and $1,621 (Nontreponemal-first) per case treated. A Canadian cost-effectiveness analysis compared the traditional testing approach (Nontreponemal-first) for syphilis screening

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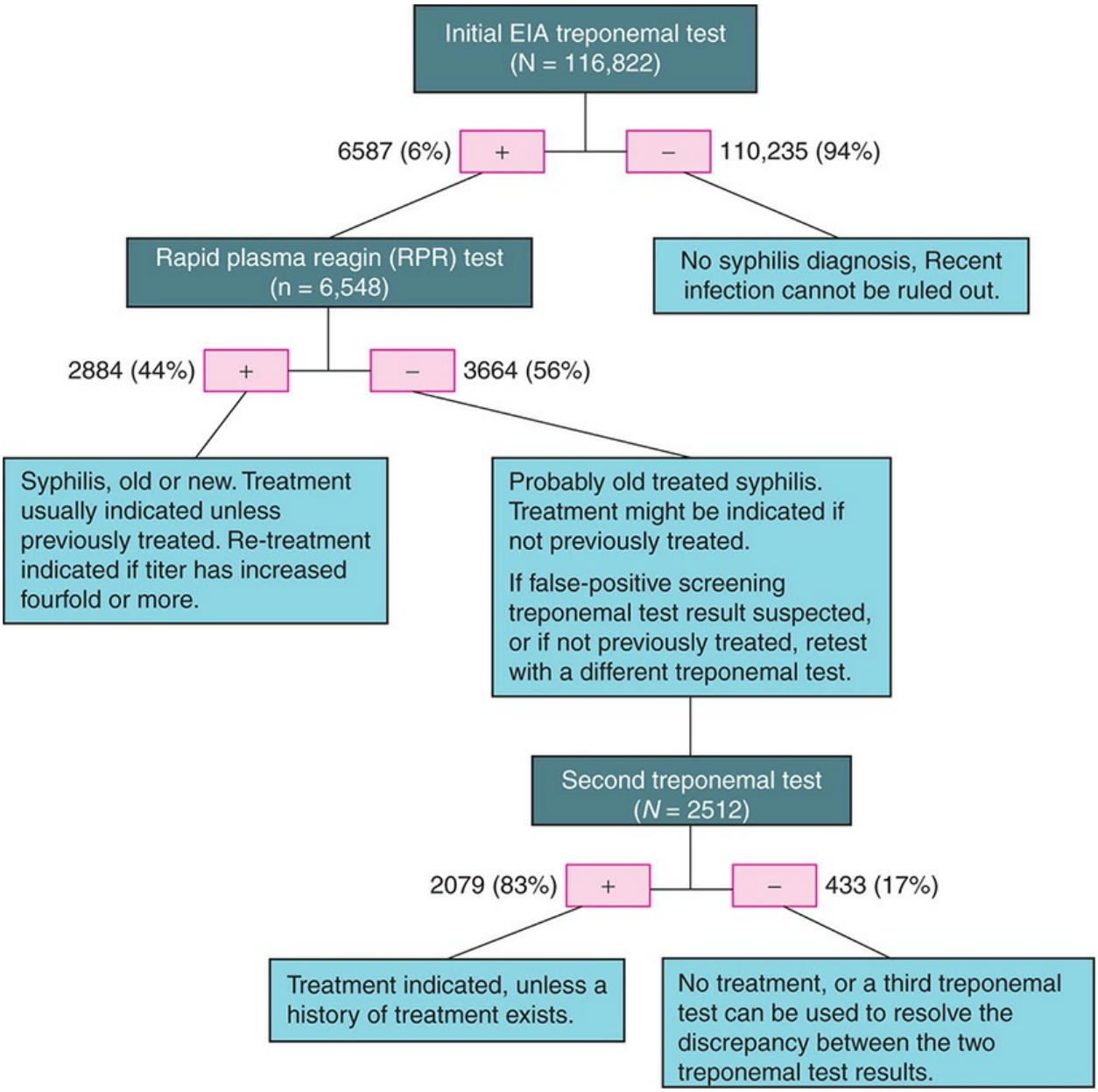
compared to the newer algorithm which uses an initial treponemal EIA and the INNO-LIA syphilis immunoblot (EIA + IL) for confirmatory testing of reactive EIA results.[91] The incremental cost-effectiveness ratio was $461 CDN per additional correct diagnosis (less costly and more effective) using the EIA + IL algorithm compared with RPR + TPPA/FTA-ABS for screening and diagnosis of syphilis.[91]



■ **FIGURE 20-11** Reverse sequence syphilis screening algorithm. (Adapted from reference 81.)

Borrelia

Taxonomy

The genus *Borrelia* is currently made up of 37 species, and several of them are pathogenic for humans and domestic animals (Table 20-9). *Borrelia* spp. are helical bacteria that measure 0.2 to 0.5 µm in diameter and 8 to 20 µm in length with 3 to 10

loose coils.[27] Most have 15 to 30 periplasmic flagella but isolates of *B. burgdorferi* have only 7 to 11 (see Image Plates 20-1C, D, and F). *B. burgdorferi* and other species that have been cultivated in vitro are microaerophilic (Table 20-9). The bacteria stain well with Giemsa, but on Gram's stain these organisms are neither gram-negative nor gram-positive.[27,40] *Borrelia* spp. that are human pathogens are transmitted by insect vectors, either lice or ticks (Table 20-2). The relapsing fever borreliae differ from the Lyme disease borreliae in their arthropod vectors.[27] Soft-bodied ticks in the family *Argasidae* (genus *Ornithodoros*), or lice (*Pediculus humanus*) are the biologic vectors of relapsing fever borreliae, whereas Lyme disease borreliae are restricted to hard-bodied ticks of the family *Ixodidae*. Louse-borne relapsing fever (LBRF) is caused by *B. recurrentis,* TBRF by a variety of species, and Lyme disease by *B. burgdorferi* and related species. Borrelial spirochetes also cause infection in animals, particularly rodents, but some species have yet to be associated with a disease process.[27]

Borrelia spp. have unique genomes consisting of a small linear chromosome (~1,000 kb) and both linear and circular plasmids, which have very low G+C (~30%) content. Genomic data from sequencing the *rrs* (16S rRNA gene) and *flagellin* genes show the phylogenetic classification and taxonomy of relapsing fever *Borrelia* spp. (see Pathosystems Resource Integration Center website at www.patricbrc.org).[156,367] Three main clusters are found in the *Borrelia* genus including: (1) Lyme *Borrelia* complex (agents of Lyme disease), (2) New World TBRF *Borrelia*, and (3) Old World *Borrelia* that includes the louse-borne *B. recurrentis*.

Lyme Disease

Lyme disease was clinically recognized as a multisystem inflammatory disorder of the skin, joints, heart, and CNS in the United States in the 1970s,[451] although there had been a long

preceding history of European investigations that is summarized in Box 20-1.[445,446] Investigators at Yale University reported that an epidemic of arthritis had been occurring in residents of several surrounding Connecticut communities since at least 1972 following the investigation of several cases of unusual arthritis in children.[451] Lyme arthritis was almost always preceded by a very distinctive skin rash, an erythematous papule that developed into a rapidly expanding annular lesion consistent with erythema chronicum migrans (EM),[450] which had been previously described in

Europe, particularly from Scandinavian countries. Although a tick bite was only reported in one of the early Connecticut cases, the nature of the primary skin lesion, and the fact that the initial cases occurred in the summer and early fall suggested an arthropod vector. Several reviews of Lyme disease have recently been published,[142,275,312,444] and the relevant web sites for clinical information have been summarized by Sood.[433,434]

TABLE 20-9 Characteristics of Pathogenic *Borrelia burgdorferi* Sensu Lato Genomospecies

Species	Growth	Vector(s)	Disease	Location
B. burgdorferi sensu stricto genomospecies[a]	Microaerophilic	Ixodidae (Hard-bodied ticks)	Lyme disease	
B. burgdorferi	Microaerophilic	*Ixodes scapularis*	Lyme disease	United States
B. burgdorferi	Microaerophilic	*I. pacificus*	Lyme disease	United States
B. burgdorferi	Microaerophilic	*I. ricinus*	Lyme disease	Europe
B. garinii	Microaerophilic	*I. ricinus, I. persulcatus*	Lyme disease	Eurasia
B. afzelii	Microaerophilic	*I. ricinus, I. persulcatus*	Lyme disease	Eurasia
B. valaisiana	Microaerophilic	*I. ricinus*	Lyme disease	Eurasia
B. lusitaniae	Microaerophilic	*I. ricinus*	Lyme disease	Europe (Portugal), North Africa, Asia
B. bissettii	Microaerophilic	*I. scapularis, I. pacificus*	Lyme disease	United States and Europe
B. spielmanii	Microaerophilic	*I. ricinus*	Lyme disease	Europe
B. japonica	Microaerophilic	*I. ovatus*	Lyme disease	Japan
B. tanukii	Microaerophilic	*I. tanukii, I. ovatus*	Lyme disease	Japan
B. turdae	Microaerophilic	*I. tardus*	Lyme disease	Japan
B. sinica	Microaerophilic	*I. ovatus*	Lyme disease	China
B. andersonii	Microaerophilic	*I. dentatus*	Lyme disease	United States
B. carolinensis	Microaerophilic	*I. minor*	Unknown	United States
B. californiensis	Microaerophilic	Unknown	Unknown	United States

[a] *B. burdorferi, B. garinii,* and *B. afzelii* are the most common agents of Lyme borreliosis.
Adapted from references 354, 441.

BOX 20-1

European Investigations of Lyme Disease

1921–1923	Afzelius in Sweden and Lipschütz in Austria described erythema chronicum migrans. Later, the chronic atrophic skin lesion described by Herxheimer in 1902 was recognized as part of the same process.
1944	Bannwarth described a chronic radiculitis, sometimes preceded by erythema, accompanied by chronic lymphocytic meningitis, and cranial or peripheral neuritis. Later, the disease was associated with ticks.
	Lennhoff described the presence of spirochetes in the lesion of erythema

1948	chronicum migrans.
1951	Hollström described the successful treatment of erythema chronicum migrans with penicillin.

Borrelia burgdorferi *sensu lato*

B. burgdorferi is the longest and narrowest of the borreliae. Molecular analysis showed that this spirochete is related to other *Borrelia* species,[391] but shares almost no homology with species of *Treponema* and *Leptospira.*[203,210] Several genospecies are recognized as definite human pathogens within the *B. burgdorferi* complex (*B. burgdorferi* sensu lato): *B. burgdorferi* sensu stricto is global in distribution, while the other pathogenic species *B. garinii, B. afzelii,* and rarely *B. spielmanii* have also been isolated in Europe,[110,441] Asia,[181] and other parts of the world.[25] *B. garinii* is more commonly isolated from CSF,[331] and appears to be associated with neuroborreliosis,[117] whereas *B. afzelii* is most often associated with arthritis and chronic skin disease—acrodermatitis chronica atrophicans (ACA).[117,446,460,480] A significant number of *I. ricinus* ticks in Europe are infected with more than one member of the *B. burgdorferi* complex.[369,370,435] Strains found

in the United States are relatively homogeneous and conform to the definition of *B. burgdorferi* sensu stricto.[257] The immunologic homogeneity of most American strains was demonstrated by analysis of borreliacidal antibodies in vitro.[260] Among strains of *B. burgdorferi* sensu stricto, however, some investigators have noted greater variation in strains from the United States than in those from Europe.[11,12] A potentially significant finding for studies of genetic homogeneity is the documentation of greater variation in *B. burgdorferi* when amplification products from skin lesions were compared to isolated spirochetes from the same lesions, suggesting that current culture techniques may select for certain genetic types.[258]

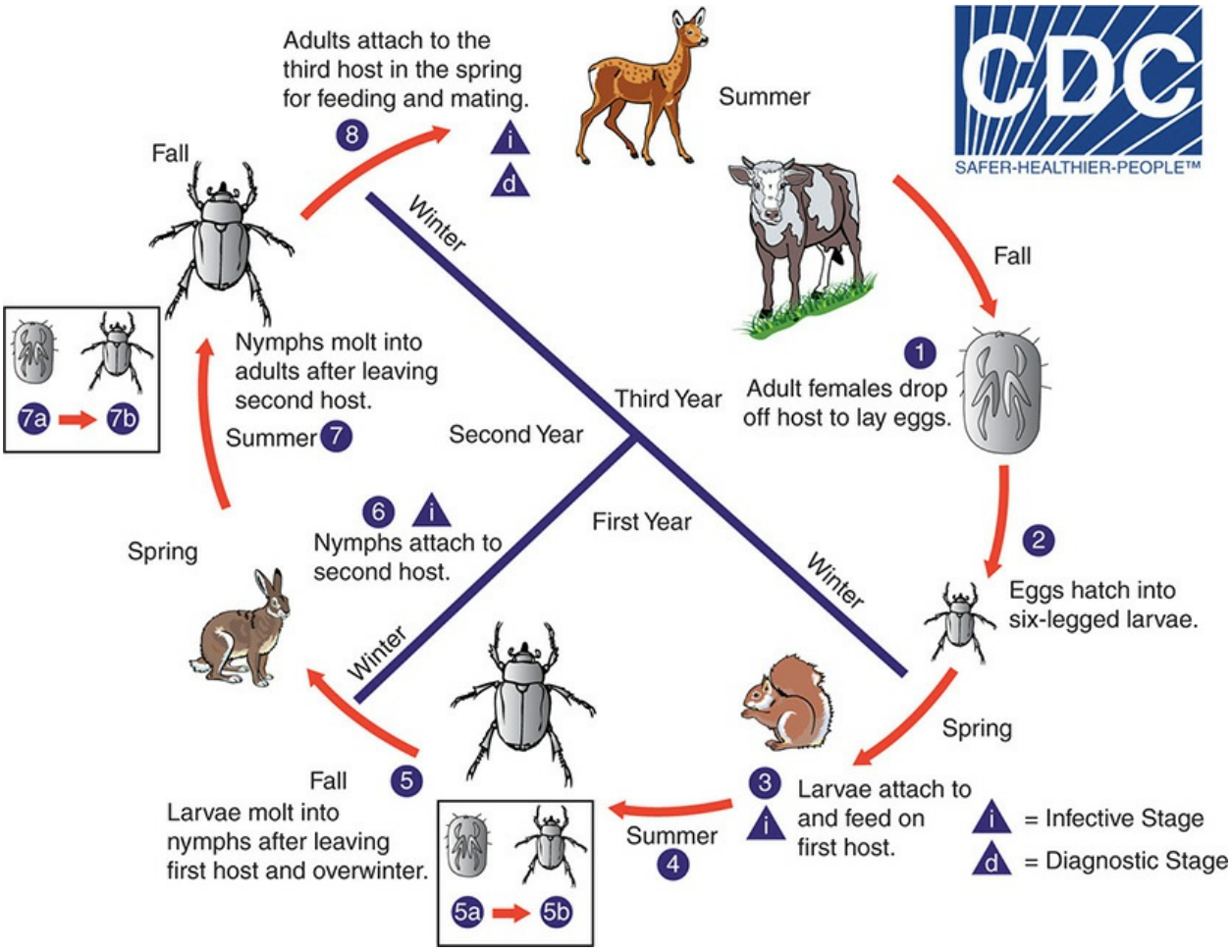
An increasing number of other genomic species have been documented within the *B. burgdorferi* sensu lato complex,[354,441] but the extent of their association with human disease is not yet clear. Evidence of involvement in human disease exists for *B. lusitaniae* and *B. valaisiana*, which have been shown to cause a few cases of Lyme borreliosis (LB) in Europe, Asia, and North Africa.[102,164] In addition, a human disease

that resembles Lyme disease has been associated with a noncultivable spirochete, named *B. lonestari,* so named because it is found in *Amblyomma americanum,* the "lone star tick."[206,396] This species may be responsible for many of the cases of Lyme disease found in the southeastern United States.[19,163,484]

Life Cycle. *B. burgdorferi* is transmitted from infected Ixodes ticks to humans, but the life cycle also involves several other animal species (Fig. 20-12).[200] The tick most often acquires the *Borrelia* spirochetes when feeding on an infected host during its larval stage.[354] The cycle of both *B. burgdorferi*[442] and *Babesia microti*[436] in nature is maintained by a number of small rodents, notably the white-footed mouse (*Peromyscus leucopus*) in North America, and the wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicolis*), and bank vole (*Clethrionomys glareolus)* in Europe,[12,354] all of which maintains an asymptomatic parasitemia or spirochetemia. Birds have also been identified on both sides of the Atlantic as being reservoirs for *B. burgdorferi* sensu lato.[354] White-tailed deer are important for the survival of the tick. Although deer are not directly involved in transmission of the infection, reduction of deer populations diminishes the number of vector ticks, after which the incidence of infection decreases.[200,442] Although interaction of ticks with mice and deer usually occurs in rural areas, Lyme disease may also be acquired in cities in endemic areas.[58]

Ixodid ticks develop in three distinct stages over a life span of 2 years. Ticks feed in late summer as larvae and the next year as nymphs (early spring) and adults (late summer).[354,355] A blood meal is required for the molts from larva to nymph and nymph to adult, as well as for oviposition (laying eggs) by the adult female. The most effective tick stage for transmission is the nymph, which is prevalent during the late spring and early summer, when most cases of human disease occur. Transmission of nymphs is also dependent on their small size, which allows them to escape detection

for longer times than adult ticks. The larval and nymphal stages are so small that many patients do not recall having been bitten. In order to transmit the infection, however, a tick has to have fed on an infected rodent. *B. burgdorferi* is not transmitted vertically from parent to offspring, so the larval stages of ticks that have not fed on an infected host are not infected and cannot transmit the infection. Because adult male ticks rarely feed on humans since they do not require a blood meal, transmission of infection via this route is unlikely to occur.



■ **FIGURE 20-12** Life cycle of *Ixodes* ticks, United States.

Pathogenesis. The causative spirochete for Lyme disease was discovered in 1982,[60] and described as a new species and named as *Borrelia burgdorferi* in 1984 after the main investigator.[61,211,391] Burgdorfer and colleagues[60] isolated a spirochete from the implicated tick vector, demonstrated that the spirochetes produced cutaneous lesions in rabbits, and that the serum of patients with Lyme arthritis contained antibodies to the spirochete. Steere and colleagues[448]were then able to isolate this spirochete from blood, skin lesions, or CSF of infected patients as well as from ticks. Shortly thereafter, spirochetes were isolated from the lesions of EM and those with Bannwarth's syndrome (i.e., chronic lymphocytic meningitis and polyradiculitis) in Europe.[32,361]

Lyme disease, however, has most likely been around for a much longer period. Retrospective examination of *Ixodes ricinus* ticks that had been preserved in Hungarian and Austrian museums in 1884 and 1888, respectively, has documented the presence of DNA from a member of the *B. burgdorferi* complex.[287] Similarly, *OspA*

sequences from *B. burgdorferi* have been demonstrated by molecular techniques in white-footed mice that had been trapped in Massachusetts in 1894.[280] American and European foci of Lyme disease have, therefore, been independently present for at least a 100 years.

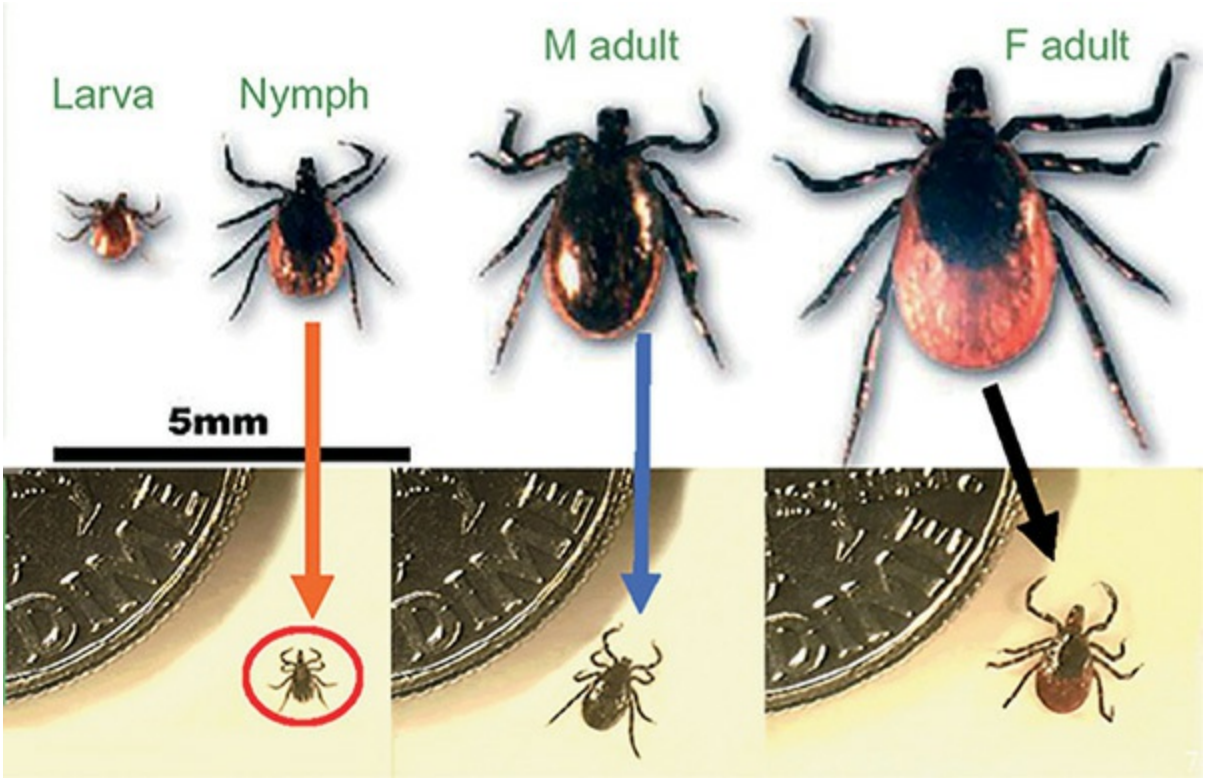
Complete sequencing of the genome of *B. burgdorferi* has provided insight into the pathogenesis of Lyme disease.[154] A large part of the genome encodes lipoproteins, of which the outer surface proteins are the most studied. Outer surface proteins A and C are particularly important in the biology of Lyme disease. The outer membrane antigenic composition changes dramatically when infected Ixodes ticks engorge. Animal studies have shown that OspA and OspB have an important role in persistence of the spirochete within the tick.[313,337,504] *OspA* is expressed predominantly in the midgut of ticks, where it is responsible for adherence to epithelium via a tick receptor, designated as the tick receptor for OspA (TROSPA).[338] High levels of TROSPA enable the spirochete to persist in the tick gut between blood meals. After the tick takes a blood meal, the spirochetes migrate through the hemocele to the salivary glands. In the process the expression of *OspA* is downregulated and there is a dramatic increase in the amount of OspC produced.[337,339] Although cell-mediated and humoral immunity is mounted against multiple antigens, the predominant early antibody response is directed against OspC. Increased expression of the *salp* 15 gene encoding for a 15-kDa feeding-induced salivary gland protein protects the spirochete from antibody-mediated killing.[394] Salp15 inhibits activation of T-lymphocytes by binding to the CD4 co-receptor,[14] inhibits complement-mediated killing of *B. burgdorferi*,[395]and facilitates binding to OspC. OspC is also an important determinant of clinical disease as certain variants are only found in ticks, while others have been associated with limited or localized disease or produce more severe, disseminated infections.[410,411] Lin and colleagues[252] found that the predominant isolates of *B. burgdorferi* sensu stricto in the southeastern United States contain OspC variants that are not associated with invasive disease, which may explain the relative paucity of well-documented cases of classical Lyme disease in this region.

Epidemiology. The distribution of Lyme disease worldwide correlates with the vector range of the Ixodes ticks in various geographic locations. *Ixodes scapularis*, *I. ricinus,* and *I. persulcatus* are the most important vectors of LB worldwide.[200,354,435] In the Northeast and North Central regions of the United States, the vector is usually *I. scapularis* (Image Plate 20-1E), whereas *I. pacificus* carries the spirochetes in the Northwest.[449] These species share many characteristics and are also known as black-legged ticks or deer ticks. There has been controversy over the nomenclature of the black-legged ticks found in the eastern United States, but *I. dammini* is considered by most investigators to be synonymous with *I. scapularis*.[330,436] Eastern deer ticks (*I.*

scapularis and other ticks within the *I. ricinus* complex) may be multiply infected with *B. burgdorferi* and other pathogens including *Babesia microti* and the more recently recognized agent of human granulocytic anaplasmosis (HGA),*Anaplasma phagocytophilum.* As a result, these other tick-transmitted infections share a similar distribution with LB.[58] Not surprisingly, coinfection with various combinations of *B. microti, A. phagocytophilum,* and *B. burgdorferi* also occurs at rates as high as 39% of cases.[13] In the eastern United States, babesiosis and LB most commonly occur together and account for ~80% of all diagnosed coinfections.[13] In Connecticut and Wisconsin, coinfection with HGA and LB occurs much less commonly in only 3% to 15% of patients.[13] In Europe, *I. ricinus* is the most common vector, whereas *I. persulcatus* is the main arthropod host in Eurasia and parts of Asia.[66,99,354,435] A clinical illness that resembles Lyme arthritis has been reported in Australia,[386] but despite ticks (*I. holocyclus* and *I. cornuatus*) being present which cause paralysis in dogs and other mammals,[432] none of the Ixodes tick species that carry *B. burgdorferi* sensu lato complex have been identified despite extensive testing of ticks by culture and PCR.[386]

Other environmental factors also influence the development of human disease in a particular geographic region. Although an enzootic cycle of *B. burgdorferi* infection in *I. spinipalpus* ticks and wood rats exists in northern Colorado, Lyme disease is nonendemic in this area.[289] Remoteness of this area may result in a lack of contact between humans and this species of ticks as a likely explanation for the absence of human infection. Lyme disease is also present in the Pacific Northwest,[52] where the vector is *I. pacificus*, but cases occur much less often than in North Central and Northeastern states. Difference in the life cycle of *Ixodes* ticks into pertinent animal hosts offers one possible explanation. In the central and eastern state, the white-footed mouse acts as the main animal reservoir in which the spirochetes readily grow and are transmitted to hungry ticks. In contrast, ticks in the Pacific states feed on lizards, in which the

borreliae multiply poorly, limiting the intensity of infection in the tick populations.[52] Lizards also produce an unidentified borreliacidal chemical that may limit growth and transmission from this spirochete reservoir. A similar situation exists in the southeastern states where *I. scapularis* ticks also feed preferentially on lizards, rather than mice, which may also contribute to the infrequency of classical Lyme disease in this region.



■ **FIGURE 20-13** Ixodid tick stages (used with permission from www.permatreat.com).

In Europe as well, the presence of particular rodents within a geographic area increases the transmission of Lyme neuroborreliosis. The presence of edible dormice (*Glis glis*) has been shown to amplify the transmission of spirochetes in central Europe.[284]Black-striped mice also appear to be an important rodent reservoir in central Europe, and account for an abundance of spirochete-infected *I. ricinus* ticks, while lizards are not infected.[286] Nymphal *Ixodes* ticks may also feed on rats in urban areas, so that the presence of infected rats may increase the risk of Lyme disease for city dwellers and visitors alike.[285] The intensity of transmission of spirochetes at particular central European sites appears to correlate directly with the presence of rodent populations and inversely with that of lizards.

Lyme disease is the most commonly reported vector-borne infection in the United States (Fig. 20-13). In endemic areas, the probability of Lyme disease after a tick bite ranges from about 0.012 to 0.05.[266] The frequency of tick infection in some areas of the United States is as high as 75%,[177] and the density of spirochetes in adult ticks varies from several hundred to thousands in published field studies.[26,27,80,99] Careful epidemiologic study of several endemic areas has documented the magnitude of the problem. On Fire Island, New York, symptomatic disease developed in 0.7% to 1.2% of summer residents.[178] Antibodies developed in 4 (3.1%) of 129 persons studied

serologically at the beginning and end of the summer, but only 2 of the persons were symptomatic. In Great Island, Massachusetts, Lyme disease developed in approximately 3% of the population each year, and the disease developed in 16% of the population over the 4-year period of study.[457] In retrospect, the earliest case occurred in 1962. The clinical to subclinical ratio was estimated to be 1:1.

As in syphilis, *B. burgdorferi* may be transmitted from mother to infant,[74,415,416,501] but the full effects of congenital infection are not yet known. Although no cases of Lyme disease have been linked to blood transfusion[501], *B. burdorferi* causes spirochetemia[167,490], and organisms survive for long periods of time in stored red blood cells. Patients being treated for Lyme disease should therefore not donate blood. Specific eligibility criteria for blood donation for patients who have completed a course of antibiotic treatment for Lyme disease or another serious infection are provided at www.redcrossblood.org/donating-blood/eligibility-requirements.

Surveillance case definitions in the United States include both clinical and laboratory criteria for reporting Lyme disease.[20] A case of Lyme disease is defined by having the following clinical criteria: (1) physician-diagnosed EM of ≥5 cm in diameter, or (2) at least one objective late manifestation (i.e., musculoskeletal, cardiovascular, or neurologic) with laboratory confirmation of infection with *B. burdorferi*.[20] Laboratory confirmation requires the following: (1) isolation of *B. burdorferi* from a clinical specimen, or (2) demonstration of diagnostic levels of IgM or IgG antibodies to *B. burdorferi* in serum or CSF.[20]

A total of 248,074 cases of Lyme disease were reported to the CDC as part of the National Notifiable Disease Surveillance System between 1992 and 2006.[20] During this 15-year period, the annual number of cases more than doubled from 9,908 in 1992 to a total of 19,931 cases in 2006 (Fig. 20-14). Although cases have been reported in almost every state, the geographic distribution of cases is highly concentrated (93%) in the Northeast and the North Central regions, while a much smaller number of cases occur on the West Coast (Fig. 20-15). Incidence was highest among children aged 5 to 14 years, and 53% of all Lyme disease cases occurred among males. Most patient presenting with EM had illness onset in June and July, compared with only 37%of patients with arthritis.

Lyme disease is also the most commonly reported vector-borne disease in Europe.[440] Recent estimates show that LB shows a gradient of increasing incidence from west to east with the highest incidences in central Europe (e.g., Slovenia, 155/100,000) and the lowest in the United Kingdom (0.7/100,000) and Ireland (0.6/100,000). A gradient of decreasing incidence also is found from south to north in Scandinavia and north to south in Italy, Spain, and Greece.[253] LB has also been reported around the entire northern hemisphere, including the former Soviet

republics.[109]

Based on experimental data, *Ixodes* ticks must remain attached for at least 24 hours to transmit the spirochetes effectively.[356] During the first 12 hours after attachment the body of the tick remains flat, but after feeding for 24 hours the posterior body of the nymph begins to extend, and approximately 5% of infected ticks transmit spirochetes at this time. After 48 hours of feeding, the posterior body is fully distended and appears opalescent; approximately 50% of infected ticks transmit spirochetes at this time. After 4 days of feeding, almost all infected ticks will transmit spirochetes, at which point the ticks are opaque and so bloated that the body is as thick as it is wide.[288] Attempts to culture spirochetes from skin immediately after removal of an infected tick in a hyperendemic area were successful in only 2 of 48 patients, on both of whom the tick had been attached for approximately 24 hours.[33]

Visualization of the degree of tick engorgement is a subjective assessment. A more objective measurement of tick

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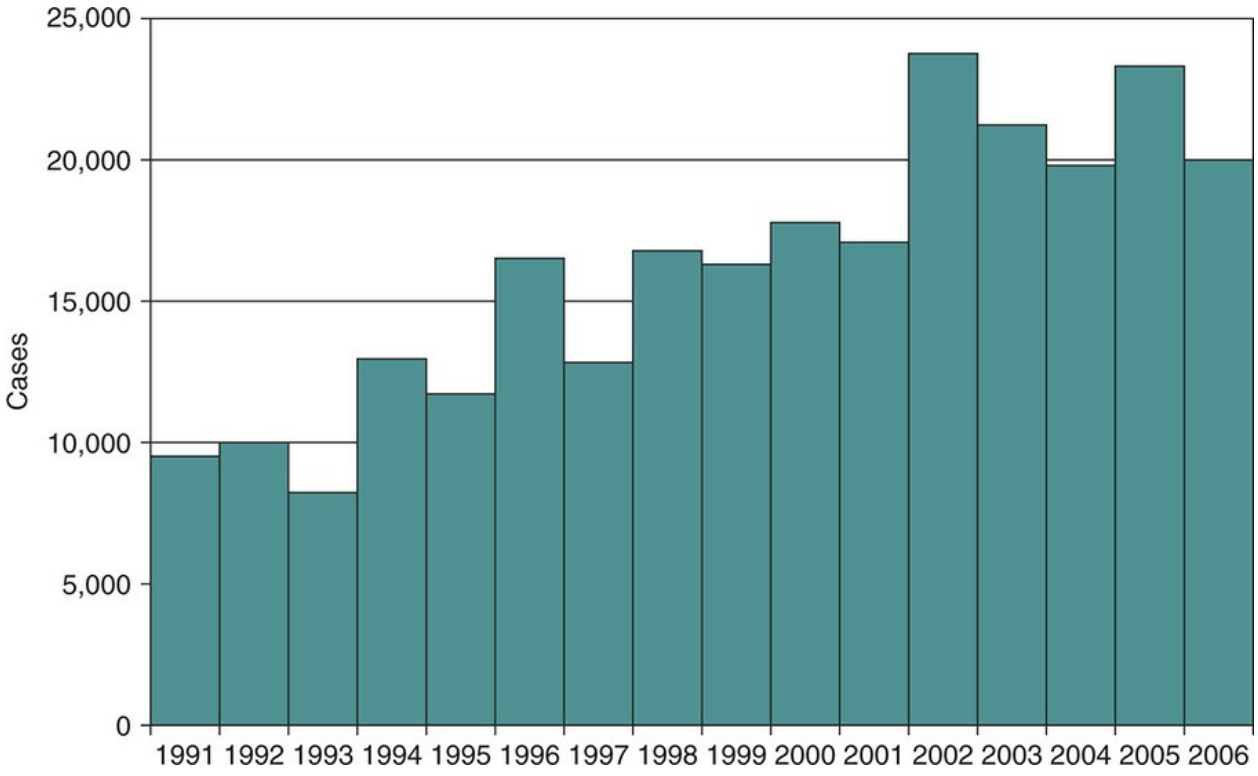
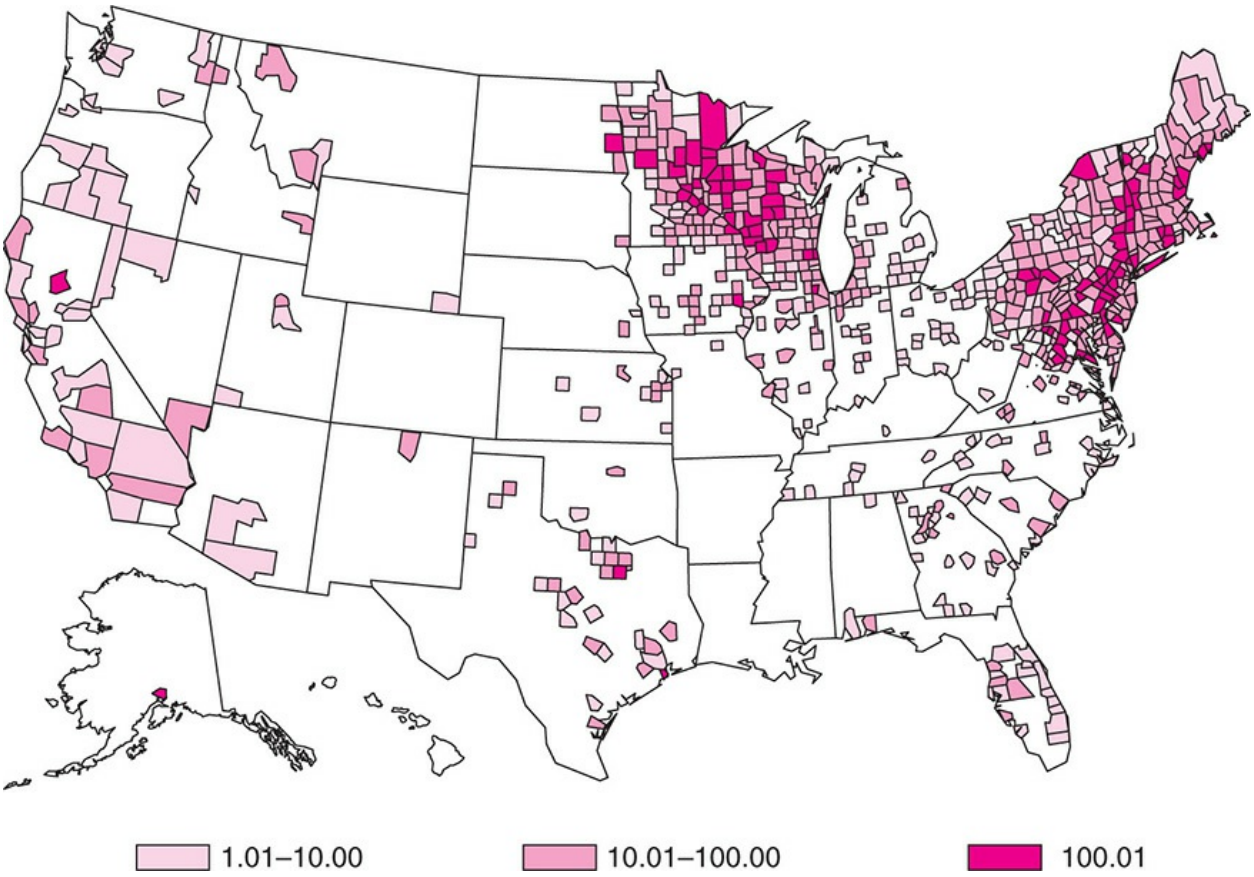
engorgement has been proposed as the "scutal index."[137,356] This index is based on the fact that the body of a nymph or adult female will swell as the blood meal is taken, but the rigid scutum will remain unchanged. The ratio of the scutum to the body is therefore a rough indication of the degree of engorgement. The requirement for prolonged feeding before transmission comes from several factors: the spirochetes must pass from the midgut into the hemocele and thence into the salivary glands, and the spirochetes must downregulate the production of *OspA* and upregulate the production of *OspC*.[337,339]



■ **FIGURE 20-14** Number of reported Lyme disease cases, United States (1991–2006). (Adapted from references 80 and 457.)



■ **FIGURE 20-15** Distribution of Lyme disease, United States (2006). (Adapted from references 80 and 457.)

The incubation period from inoculation via a tick bite to the onset of clinical symptoms is usually 1 to 2 weeks but has a wide variability, and may be much shorter (days) or much longer up to months or years. Unless patients present with early localized infection and a classic skin lesion, Lyme disease may be difficult to clinically diagnose because many of the latter symptoms are nonspecific and can occur with other diseases.

Prevention. Avoidance of exposure to the arthropod vectors is the best available method to prevent a tick-borne infection.[501] However, tick exposure is unavoidable in endemic Lyme areas because most people contract the infection in the wooded yards of their homes. Other recommended measures to reduce the risk of infection include wearing long-sleeved shirts and long pants and using insect repellents but these have not been documented

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to be effective in controlled studies. Routine checking of the entire body surface after being outside and prompt removal of embedded ticks is also recommended. Ticks should be immediately removed intact if possible using fine-tipped tweezers firmly grasping the tick as close to the skin as possible and then carefully pulling upwards. Failure to remove the mouthparts of the tick is not disastrous because the spirochetes are in its salivary glands. Use of a hot match, nail polish, petroleum jelly or other instruments of torture is not recommended. After removal of the tick, clean the area with an antiseptic (http://www.cdc.gov/ncidod/dvbid/lyme/prevent.htm).

Routine use of antibiotic prophylaxis to prevent Lyme disease after a recognized tick bite is not recommended.[501] Des Vignes and colleagues[111] investigated the transmission of *B. burgdorferi* from infected ticks removed from patients in an endemic area. The removed ticks were fed on mice that were evaluated subsequently for infection. It was estimated that the overall transmission rate was 4.6% from these infected ticks. Magid and colleagues[266] performed a decision-analysis to evaluate the outcomes, costs, and cost-effectiveness of three alternative strategies to treat Lyme disease in patients bitten by Ixodes ticks in endemic areas. Empiric treatment of patients was not found to be warranted when the risk of developing Lyme disease was less than 0.01.[266] Prolonged attachment of a feeding tick in an endemic region increases the likelihood of transmission, and empiric treatment is indicated when the probability of *B. burgdorferi* infection is 0.036 or higher.[266] Although retrieval of the tick may allow for examination and confirmation as *Ixodes*, the detection of *B. burgdorferi* by culture or molecular methods does not necessarily correlate with the development of Lyme disease. Although the duration of tick attachment and degree of engorgement as measured by the scutal index were shown to be predictors of *B. burgdorferi* transmission,[137,356] PCR evaluation of the tick did not predict transmission.

A single dose of doxycycline given within 72 hours of the bite of *I. scapularis* is as effective as longer courses of antibiotics in preventing the subsequent development of Lyme disease.[307] The efficacy of treatment compared to placebo was 87%. A single dose of doxycycline may be offered to adults (200 mg) and children >8 years of age (4 mg/kg up to 200 mg) if the following criteria are met: (1) the tick can be reliably identified as an adult or nymphal *I. scapularis* tick that is estimated to have been attached for ≥36 hours based on the degree of engorgement of the tick with blood or of certainty about the time of exposure to the tick, (2) prophylaxis can be started within 72 hours of the time that the tick was removed, (3) ecologic information indicates that the local rate of infection of these ticks with *B. burgdorferi* is ≥20% (i.e., parts of New England, parts of the mid-Atlantic States, and in part of Minnesota and Wisconsin), and (4) doxycycline treatment is not contraindicated.[501] The choice to prescribe prophylaxis should not be empirically based because drug side effects,

however mild, were more common in patients who received a single dose of doxycycline than in those who were given placebo.

A recombinant vaccine against Lyme disease, based on the outer surface protein A (OspA) of *B. burgdorferi* called LYMErix was developed by GlaxoSmithKline and approved for use by the US FDA in 1998. Two controlled trials using LYMErix vaccine involving more than 100,000 people, showed that the vaccine conferred immunity to *Borrelia* in 49% to 68% of the patients in the first year after two doses of the vaccine, and increased to 76% to 92% effectiveness after a third dose was given in the second year.[419,455] A regular booster injection would be required to maintain efficacy. OspA was chosen as the immunizing antigen, because the borreliacidal antibodies that would be elicited could kill the spirochetes in the salivary gland of the tick and/or at the bite site before disseminated infection had occurred. A national review panel recommended that vaccine use be limited to individuals between the ages of 15 and 70 years who were exposed to areas of high or moderate risk for considerable period of times. Although side effects of the vaccine appeared minimal in the original reports, reports of autoimmune side effects in vaccine recipients surfaced that prompted an investigation by the FDA and the US CDC. No connection was found between the vaccine and these health problems. The commercial viability of LYMErix was negated after the publicized fears of vaccine side effects, and vaccine manufacture was discontinued in February 2002. Currently, the only available Lyme vaccine is for dogs (Novibac Lyme, Intervet/Schering-Plough Animal Health), and is based on both OspA and OspC antigens. New human vaccines are also being research using outer surface protein C (OspC) and glycolipoprotein as immunizing agents.[395]

Clinical Disease. Infection with *B. burgdorferi* sensu stricto, *B. afzelii,* or *B. garinii* produces a multisystem disease with a wide range of specific and nonspecific symptoms. Although the general progression of disease is similar and can be divided into three clinical stages in North America and Europe, the clinical manifestations of each stage may be altered according to the geographic location of the patient and the type of *Borrelia* spp. (Table 20-10). The first two disease stages occur within weeks or months after *Borrelia* infection, while the third or late stage occurs several months or years later. Some degree of overlap may also occur between these stages of Lyme disease. The clinical manifestations of Lyme disease in European patients have distinctive features not experienced as frequently or not at all compared with those in the United States,[335,442] particularly with respect to the development of CNS disease and chronic skin manifestations. This is attributed to infection in Europe primarily with *B. garinii*, which is highly neurotropic. Conversely, widespread disseminated infection appears to be more common in the United States than in Europe. As discussed above, clinical differences have been noted between patients infected in New York by *B. burgdorferi* sensu stricto and Slovenian patients infected by *B. afzelii*. Several detailed

reviews of the clinical manifestations of LB have been previously published.[306,415,416,446,447,501]

As outlined below, the clinical diagnosis of Lyme disease in the absence of pathognomonic skin lesions may be difficult due to the vague nature of many patients' complaints, particularly during later stages of the illness or in the post-Lyme disease phase (musculoskeletal pains, malaise, headache, fatigue, nonspecific neurologic symptoms including sleep disturbances). At one university Lyme disease clinic, 38% of children were found to be overdiagnosed and 8% were underdiagnosed.[141] One-fourth of the patients who had been diagnosed correctly were subsequently treated incorrectly. In a study of diagnostic tests at a prepaid healthcare

plan in California, only 19% of laboratory tests were ordered because the physician suspected Lyme disease.[250] Fully 60%of the tests were ordered as part of a battery of tests for patients with vague complaints. In an area where Lyme disease is uncommon, such indiscriminate test ordering only compounds the inherent limitations of diagnostic LB tests outlined in the next section, and contributes to the problem of false-positive diagnoses.

TABLE 20-10 Differences Between European and American Lyme Neuroborreliosis (LNB)

Clinical Feature	American LNB	European LNB
Causative *Borrelia* subspecies	*B. burgdorferi* sensu stricto	Mostly *B. garinii*, occasionally *B. afzelii*
LNB as a percentage of all Lyme cases	<10%	>35%
Multiple erythema migrans lesions	Common	Uncommon
Painful radiculitis	Rare (<10%)	Common (>50%)
"Aseptic" meningitis presentation	Majority	Minority
Cranial nerve involvement	VII, very rarely others	Usually VII, but can include others
Associated chronic skin manifestation	Never	Not rare

(lymphocytoma or ACA)	Never	Not rare
Associated with Lyme arthritis	Common	Almost never
Chronic encephalomyeloradiculitis	Very rare (<0.1% of LNB)	More frequent, but unusual (<3% of LNB)
Intrathecal antibody production	Minority of cases	Common (>50%)

ACA, acrodermatitis chronic atrophicans.
Adapted from reference 442.

■ **FIGURE 20-16** Erythema migrans (EM)—Lyme disease. (Courtesy of www.microbewiki.kenyon.edu/Borrelia.)

Early Disease (Stage I). The classical clinical sign of early localized infection with *B. burgdorferi* is a circular, outwardly spreading rash called erythema chronicum migrans (i.e., EM).[105,416,454] This classical lesion develops at the site of the tick bite so that EM may be found in multiple anatomic locations and to varying extents. EM is estimated to occur in most infected patients. Flu-like symptoms such as headache,

muscle soreness, fever, and malaise may also be present.[140,416] In New Jersey, EM developed in 93% of the patients and approximately half had systemic symptoms, including regional lymphadenopathy.[45] Similarly, 90% of infected children in Connecticut displayed the skin rash.[160]

The rash is typically red, and may be warm, but is usually painless. Classically, EM is described as a "bull's-eye" rash because the innermost portion is dark red and indurated, the outermost portion is red, but the portion in the middle clears (Fig. 20-16). Peripheral edema with central clearing of the lesion has been emphasized, but homogeneous or central redness was more characteristic in a large series of microbiologically confirmed cases.[425] Central clearing was more common in Slovenian patients infected with *B. afzelii* than in New Yorkers infected with *B. burgdorferi* sensu stricto.[460] The spirochete is most easily cultivated from EM during early infection, or it may be visualized in as many as 40% of biopsy specimens by silver impregnation staining.

Early Disseminated Infection (Stage II). The second stage of Lyme disease results from dissemination of the spirochete throughout the body.[447] EM may develop at body sites other than the original tick-bite site. European patients may also present with lymphocytoma cutis, a discrete purplish lump that usually develops on the face, ear lobe, nipple, or scrotum.[440] Severe acute asymmetric, oligoarticular joint inflammation[453] and neuroborreliosis manifestations are common symptoms of early disseminated infection in untreated patients.[334] Bowen and colleagues[44] documented arthritis in 26% of their patients, meningitis in 10%, and cranial nerve palsies in 8%. The meningitis may appear purulent. Patients with neuroborreliosis may also have symptoms of meningoradiculoneuritis (Bannwarth's syndrome) and mild encephalitis, although altered mental status is the only symptom reported in some cases of early CNS infection.[332,334] In addition, disseminated *Borrelia* infection may also cause an acute

hepatitis, an acute myocarditis with associated atrioventricular block and infection of the eye.

Late Persistent Infection (Stage III). The chronic phase of Lyme disease is characterized in North America primarily by chronic arthritis and less commonly persistent nervous system symptoms.[442] In contrast, European patients infected with *B. afzelli* or *B. garinii* have chronic skin lesions (ACA associated with *B. afzelli* infection), chronic neurologic symptoms, and chronic arthritis (Table 20-11).[440,442] The recurrent episodes of arthritis diminish in frequency and severity each year, but

chronic synovitis and permanent disability develop in some patients.

TABLE 20-11 Clinical Manifestations and Recommended Diagnostic Approach for the Diagnosis of Lyme Borreliosis in Routine Clinical Practice

	Primary Diagnostic Testing	Supporting Diagnostic Testing	Supporting Clinical Findings
Erythema migrans			
Diagnosis on the basis of history and visual inspection of the skin lesion[a]	Laboratory testing not needed or recommended if the lesion is typical. Acute- and convalescent-phase serologic testing[b] is recommended for patients with atypical lesions.[c]	Culture or PCR of a skin biopsy specimen	Tick bite at site; regional lymphadenopathy in North American patients
Lymphocytoma (a rare manifestation)			
Painless bluish-red nodule or plaque, more frequent in children (especially on ear) than in adults	Serologic testing[b] usually positive at time of presentation; if negative, test convalescent-phase sera (2–6 weeks later)	Culture or PCR of a skin biopsy specimen	Tick bite at site; recent or concomitant erythema migrans
Lyme neuroborreliosis			
Adults: meningoradiculitis, meningitis, and peripheral facial palsy; rarely encephalitis, myelitis, very rarely cerebral vasculitis Children: meningitis and peripheral facial palsy	CSF pleocytosis and synthesis of intrathecal antibodies to *Borrelia burgdorferi* sensu lato[d] Serologic testing[b] usually positive at time of presentation; if negative, rest convalescent-phase sera (2–6 weeks later)	Detection of Lyme *Borrelia* by culture or PCR of cerebrospinal fluid Intrathecal synthesis of total IgM, IgG, IgA	Recent or concomitant erythema migrans
Cardiac Lyme borreliosis (a rare manifestation)			
Acute onset of atrioventricular conduction disturbances, rhythm disturbances, and sometimes myocarditis or pericarditis. Other diagnoses should be excluded	Serologic testing[b] usually positive at time of presentation; if negative, rest convalescent-phase sera (2–6 weeks later)	None recommended	Recent or concomitant erythema migrans, neurologic disorders, or both
Ocular manifestations (rare)			
Conjunctivitis, uveitis, papillitis, episcleritis, keratitis	Serologic testing[b]	Detection of *B. burgdorferi* sensu lato by culture or PCR from ocular fluid	Concomitant or previous other well-defined Lyme borreliosis manifestations
Lyme arthritis			
Recurrent episodes or persistent joint swelling in one or more large joints. Other diagnoses should be excluded	Serologic testing[b] As a rule, high concentrations of specific serum IgG antibodies present	Synovial fluid analysis Detection of *B. burgdorferi* sensu lato by PCR of synovial fluid or tissue	Previous other well-defined Lyme borreliosis manifestations
Acrodermatitis chronic atrophicans			
Long-standing red or bluish-red lesions, usually on the exterior surfaces of extremities; initial doughy swelling; lesions eventually become atrophic	Serologic testing[b] As a rule, high concentrations of specific serum IgG antibodies present	Histology Culture of PCR of a skin biopsy specimen	Previous other well-defined Lyme borreliosis manifestations

[a]If less than 5 cm in diameter, a history of tick bite, a delay in appearance after the tick bite of at least 2 days, and an expanding rash at the site of the tick bite are needed.
[b]Two-tier serologic testing is recommended, but newer first tier and immunoblot assays are increasingly incorporating the same peptides or recombinant immunodominant antigens of *Borrelia burgdorferi* sensu lato—whether doing the second tier immunoblot still increases overall specificity of serologic testing is less clear.
[c]As a rule, initial and follow-up samples have to be tested in parallel to avoid misinterpreting changes caused by inter-assay variation.
[d]In early cases, intrathecally produced specific antibodies might still be absent.
Adapted from reference 442.

Treatment. Prompt antimicrobial therapy in the initial stages of Lyme disease is usually effective in controlling symptoms and preventing progression of disease. Practice guidelines have been provided by the Infectious Diseases Society of America.[250] In the later stages of illness, it is less clear that antimicrobial therapy is of use.[222,501]

Antibody Response, Serologic Profile, and Immunity. *B. burgdorferi* sensu lato expresses various surface proteins in response to its environment.[200] Recent microarray gene expression profiling studies show that the organism differentially expresses various genes only within the mammalian host, and that different antigens are expressed at different LB disease stages.[200,327] In addition, antigenic composition is also different between different *B. burgdorferi* sensu lato species.[7] The complex antigenic composition of the organisms, therefore, makes the selection of particular antigens for human LB diagnostic serologic assays challenging, and the choice of antigen effects the performance of LB serology tests in patients presenting at different stages of the infection.[7,499] Strong IgM and IgG responses are developed within a few days after infection to the flagellar protein flagellin (41-kDa), or FlaB, and the flagellar outer sheath protein FlaA, as some of the immunodominant antigens in early LB.[4,5] However, these antigens are highly cross-reactive with other bacterial antigens and epitopes in mammalian tissue[261] so that tests utilizing these proteins lack specificity. Another immunodominant antigen found in early infection is the plasmid-encoded OspC protein (21- to 25-kDa). This antigen is heterogenous among *B. burgdorferi* sensu lato species,[5,495] but conserved epitope regions within this protein have allowed development of a synthetic peptide that contains the conserved C-terminal 10 amino acid Osp C (pepC10).[282,336] BmpA protein is also immunogenic (39-kDa), and encoded by a gene located within the *bmp* cluster (BmpA to -D genes).[55] Due to the heterogeneity of the BmpA protein sequences of different *B. burgdorferi* sensu lato species, this antigen has limited diagnostic utility.[55] A surface-exposed lipoprotein encoded by the linear plasmid lp-28-1 of *B. burgdorferi* B31 designated as Vmp-like sequence expressed protein (VlsE) (34- to 35-kDa) is conserved among *B. burgdorferi* sensu lato species and is highly immunogenic, which has made it viable as a broadly reactive immunodominant antigen for diagnosis of LB.[237,251] Finally, there are several other antigens that are expressed in the mammalian host that are being used diagnostically, including P35/BBK32 (47-kDa), P37, and P66 antigens.[147,270] These antigens are present in the ticks during a blood meal,[147] and are also present during the early stages of human infection as well as highly specific immunodominant antigens.[270]

Detection of IgM versus IgG antibodies to one or more *B. burgdorferi* sensu lato

antigens depends on the duration of infection, the manifestations of LB, and the type and source of antigen used. Using Western IgM immunoblots, the number of antigens detected and the strength of the immune response according to band intensity are the most robust in specimens from symptomatic patients with EM, those with multiple EMs, or those with EM for more than 2 weeks.[7] Patients with early Lyme neuroborreliosis also have an expanded and enhanced immunologic response.[5] In contrast, asymptomatic patients with a solitary EM that has been present for less than 2 weeks have a weak and less expanded immunologic response. Antibodies may be absent early, but are usually present after several weeks.

A response to OspA tends to occur late, but may be detected in low titer or complexed to spirochetes early in the course of infection if special techniques are used.[397] A response to the OspC protein or the less specific flagellin antigen occurs more commonly in early sera.[269] For maximum sensitivity in early Lyme disease, the use of an IgM assay has been recommended, either by immunofluorescence[297] or immunoblot[4] techniques. Specific IgM titers may remain elevated throughout the course of illness and even years after treatment, so the presence of IgM cannot be used to establish acute infection.[194] Collection of a second specimen 8 to 14 days after the baseline sample has been recommended for documentation of seroconversion in early Lyme disease. If IgM alone is detected a month or more after infection, the IgM result is probably a false-positive.

Most patients with *B. burgdorferi* infection have an effective immune response that is protective to reinfection with a similar strain. Although apparently rare, there have been well-documented examples of reinfection with a new strain of *B. burgdorferi* (as determined by molecular analysis of sequential isolates) in patients who have been treated early for EM.[326] It is not clear whether reinfections occur after untreated natural disease.

Symptomatic infection appears not to occur in wildlife, but disease may develop in domestic animals.[148,267] Kornblatt and colleagues[228] have described Lyme arthritis in dogs. Duray has described the histopathology of Lyme disease.[122]

Laboratory Diagnosis. Several excellent detailed reviews have been published on the laboratory diagnosis of Lyme disease.[7,372,494] Serologic tests that document the presence of specific antibodies that are directed toward one or more *Borrelia* antigens continue to be the primary method used for LB diagnosis, although it may also be diagnosed by culture of the spirochete, and by demonstration of the spirochetes in tissue using immunologic or molecular techniques. The comparative overall efficacy of various laboratory techniques in the diagnosis of Lyme diseases in 47 patients with EM is summarized in Box 20-2.

BOX 20-2

Laboratory Investigation of 47 Adult Patients With Erythema Migrans

Laboratory Procedure	Diagnostic Yield (%)
Quantitative PCR of skin biopsy	80.9
Two-stage serologic testing of convalescent specimens	66.0
Conventional nested PCR	63.8
Skin culture	51.1
Blood culture	44.7
Serologic testing of acute specimens	40.4

Adapted from reference 325.

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Recommendations for diagnostic methods that were provided by a consensus group in 1997[478] were recently updated,[501,233] but have not changed appreciably. Patient should only be tested for Lyme diseases where the pretest probability based on their exposure history and clinical symptoms ranges between 0.2 and 0.8.[478] It may be difficult, however, to determine the pretest probability in an individual patient because of the nonspecific nature of their symptoms (i.e., fatigue, arthralgias, headache). The indiscriminate use of LB diagnostic tests in unselected patients with a low pretest probability will yield more false-positive results than true cases.[7] LB testing of patients presenting with classical EM is not required since empiric treatment alone has been shown to be more cost-effective due to the low sensitivity of LB antibody assays at this early stage of infection.[316] Even after more than three decades of experience, inappropriate ordering of LB diagnostic tests and inaccurate test results resulting in inappropriate antimicrobial therapy remains a problem.[3,7,150,305,347,364]

Direct Detection. This section refers to a variety of laboratory tests that detect and identify the spirochete, *B. burgdorferi* sensu lato, in biologic specimens such as the tick vectors, host reservoirs, animals that have been experimentally inoculated, and tissues and body fluid samples from patients with LB. Currently, there are four different approaches that have been used for direct detection of *B. burgdorferi* infection, including microscopic examination, detection of specific antigens, in vitro cultivation of the organism, and molecular analysis for *B. burgdorferi*-specific nucleic

acids. Of these, both direct microscopic detection and antigen-capture tests have limited clinical utility in the clinical laboratory, so only a brief description is provided.

Morphologic Detection. Rare numbers of spirochetes are unevenly distributed in clinical samples making the microscopic visualization of *B. burgdorferi* in tissues too insensitive for most stages of the disease.[7,106,123] Spirochete morphology is also variable and may be difficult to distinguish from host tissues in histologic sections.[123] Although silver impregnation methods (Warthin–Starry, Steiner, Dieterle)[106] stain *B. burgdorferi*, and are used to detect the organism in tissue, this staining method is highly nonspecific and may detect any type of bacterium. Specific antisera must, therefore, be used to confirm the spirochete identification in clinical samples. Microscopic examination is mainly used for detection of *B. burgdorferi* in tick vectors during field surveys or surveillance studies.

Antigen Detection Assays. Two types of antigen detection assays are commercially available including the antigen-capture enzyme-linked immunosorbent assay (ELISA), and the reverse Western blot. Although these assays are easily performed in the clinical laboratory, they should not be used for the laboratory diagnosis of active or suspected LB owing to their poor performance and reproducibility.[7,97,223] Klempner and colleagues[223] performed Lyme urine antigen testing on 10 healthy controls and found discrepant test results on aliquots of the same specimen in eight and consistently false-positive results in the other two. Similar problems have been encountered using an antigen-capture assay to detect *B. burdorferi* antigens in CSF samples from patients with suspected LB.[96]

BOX 20-3

Barbour–Stoenner–Kelly Medium for Culture of *Borrelia burgdorferi* (BSK II Medium)

1. Clean all glassware with detergents, rinse thoroughly in glass-distilled water, and autoclave.
2. To 900 mL of glass-distilled water add 100 mL of 10× concentrate of CMRL 1066 medium without glutamine.
3. Add to the 10× CMRL 1066 in the following order:
   5 g neopeptone (Difco Laboratories, Detroit, MI)
   60 g bovine serum albumin, fraction V (Miles Laboratories, Elkhart, IN; No. 81-003)
   2 g yeastolate (Difco)
   6 g *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES; Sigma Chemical Co., St. Louis, MO)

5 g glucose
0.7 g sodium citrate
0.8 g sodium pyruvate
0.4 g *N*-acetylglucosamine (Sigma)
2.2 g sodium bicarbonate

4. Adjust pH of medium at 20°C–25°C to 7.6 with 1 N NaOH.
5. Add 200 mL of 7% gelatin (Difco) that has been dissolved in boiling water.
6. Sterilize by filtration with air pressure (0.2 fm nitrocellulose, Millipore Corp., Bedford, MA). Store the medium at 4°C.
7. Before use, add unheated rabbit serum ("trace hemolyzed," Pel-Freez Biologicals, Inc., Rogers, AR) to a final concentration of 6%.
8. Dispense to glass or polystyrene tubes or bottles. Fill containers to 50%–90% capacity and cap tightly.

From reference 26.

Culture. Cultivation of *B. burdorferi* was the first detection method used, and it remains the gold standard for laboratory diagnosis of LB. *B. burgdorferi* has been isolated in a cell-free modification of Kelly's medium for spirochetes that was developed by Barbour[26] (Table 20-11 and Box 20-3),

although various modifications to that medium have been made over time.[357] Current media preparations such as Barbour–Stoenner–Kelly II (BSK II) (Box 20-3) demonstrate better growth and recovery of the spirochete at lower inocula in a shorter time. The process is relatively easy when infected ticks are triturated and cultured (see Image Plate 20-1E). Subculture from broth to agar results in the formation of spirochetal colonies with varying morphology.

Culture is most effective in early Lyme disease and is rarely positive in later stages. *B. burgdorferi* sensu lato can be cultured from body fluids (i.e., CSF, blood, synovial fluid) and tissue biopsies (i.e., skin lesions, including EM, ACA, and borrelial lymphocytoma, and rarely cardiac tissue) of patients with LB.[7] Even at the primary stage, however, spirochetes were isolated from skin lesions only in 51% and from blood in 45% of 47 adult patients with a clinical diagnosis of Lyme disease.[325] *B. burgdorferi* sensu stricto cannot usually be recovered from skin cultures of EM lesions in patients receiving antibiotic therapy. The yield appears to be higher when the lesions are large and multiple, as in the secondary disseminated phase of the disease.[298]Citrated blood should be cultured, and tubes are incubated at 34°C to 37°C

in the dark. After incubation for 2 to 3 weeks, cultures are examined by darkfield microscopy or by fluorescence microscopy using either the fluorochrome dye acridine orange or a specific fluorescence-labeled antibody.[372] The identity of any spiral organisms detected must be confirmed as *B. burgdorferi* sensu lato by demonstration of reactivity with specific monoclonal reagents or by detection of specific DNA sequences using PCR.

Molecular Detection. An extensive review of molecular amplification methods in the diagnosis of Lyme disease was provided by Schmidt,[392] Dumler,[119] and more recently by Aguero–Rosenfeld.[3,7] PCR-based methods have mainly been used for the diagnosis of LB, either conventional PCR, nested PCR, quantitative PCR, competitive PCR, or increasingly real-time PCR. The most commonly used targets in PCR are *B. burgdorferi* chromosome genes such as *rrs*, *fla* B, *rec* A, and *p66*, and plasmid-derived *osp* A.[3,392] The performance of *B. burgdorferi* sensu lato PCR-based assays is reliant on the selection of the gene target and primer set for amplification. Molecular detection assays for Lyme disease have not been widely implemented by clinical laboratories due to their variable performance for different clinical specimens, their diminished reliability because of potential sample contamination, their lower sensitivity after administration of antibiotic therapy, and the difficulties with clinical interpretation of a positive result. Because the number of spirochetes in infected tissues or body fluids of patients is low, collection and transport and prompt preparation of DNA from clinical samples are critical for providing consistent and reliable PCR results. *B. burdorferi* amplification tests are also susceptible to false-positive results from cross-contamination or from nucleic acid sequences shared with other organisms.[300,392] Thoroughly validated amplification tests for *B. burgdorferi* are only available through referral of specific clinical specimens (i.e., blood and CSF) to a reference laboratory.

Amplification techniques have demonstrated greater sensitivity than culture or morphologic detection in the skin of EM lesions,[375] in peripheral blood, and in joint fluid.[333] The detection of *B. burgdorferi* DNA by PCR in EM lesions ranges from 36% to 88%.[7] Skin biopsies taken from EM patients during a phase III vaccine trial in the United States were positive in 64% of the patients.[455] In Europe, *B. burgdorferi* DNA was detected in 54% to 100% of skin biopsy samples from patients with ACA depending on the target sequences used for detection.[7] In general, the sensitivity of PCR for detection of *B. burgdorferi* sensu lato DNA in blood, plasma, or serum samples from patients with EM and early disseminated disease is low, and negligible in patients with post-Lyme disease syndromes. Borrelial DNA was documented prospectively in the serum of 14 of 76 patients (18.4%) with EM,[167] and the number of clinical symptoms was the strongest independent predictor of spirochetemia. It appears that the spirochetemia in patients with acute Lyme disease is both low level

and intermittent.[490] However, none of 78 patients with post-Lyme disease syndromes, even in the 39 patients with a positive IgG Western immunoblot, had spirochete DNA detected in blood specimens by PCR.[222] Detection of *B. burgdorferi* DNA by PCR in synovial fluid has a good sensitivity for diagnosis of Lyme arthritis.[7,318,362] A total of 75 (85%) of patients with Lyme arthritis had a positive synovial fluid *B. burgdorferi* PCR in one US study, and of the 73 patients who were untreated or incompletely treated with antibiotics 95% had a positive PCR.[318] This study also demonstrated wide variability in the sensitivity of the PCR test depending on the primer set used.

Patients with a wide range of neurologic symptoms in the United States and Europe have had their CSF specimens tested using *B. burgdorferi*-specific PCR.[119,238,317,318,331] Despite documentation that spirochetal DNA can be found in the CSF of some patients with neurologic symptoms,[262] the interpretation of a positive CSF PCR result can, however, be difficult because of the lack of a standardized definition to support the clinical diagnosis of Lyme neuroborreliosis. Examination of CSF fluid of patients with EM documented borrelial DNA in 8 of 12 patients, 4 of whom had no neurologic abnormalities or symptoms. The sensitivity of PCR in CSF in patients with symptomatic neurologic disease has also been low.[238] The sensitivity of PCR in CSF was 38%in early and 25% in late neuroborreliosis, respectively, in one study in the United States, and PCR results were affected by the administration of prior antibiotic therapy.[317] Similar studies in Europe have shown that the CSF PCR was positive only in LB patients with pleocytosis in the CSF.[331] Results of molecular detection in urine have also been disappointing.[119] Recommendations for increasing the yield of PCR testing in urine have been developed by Bergmann and colleagues.[34]

Real-time PCR assays have been validated with a variety of clinical specimens.[17,259,398] The major advantage of real-time PCR assays is the ability to quantify the number of spirochetes in clinical specimens. A real-time assay was used to detect *rec*A DNA in 80% of 50 patients with EM in the United States, and the number of spirochetes ranged from 10 to 11,000 (mean = 2,462) in a 2-mm skin biopsy sample.[259] Real-time PCR has also been used to quantify the number of spirochetes in synovial fluid and synovial membrane biopsies in patients with Lyme arthropathy.[398]

Although PCR is highly sensitive for detection of *B. burgdorferi* DNA in skin biopsy samples from patients with EM, amplification tests do not add significant value to the early diagnosis of Lyme disease because testing is rarely necessary as

the skin lesion is pathognomonic. Diagnosis of disseminated LB may be aided by PCR

analysis of synovial fluid or tissue in patients with Lyme arthritis, and less commonly by CSF PCR in patients suspected of having neuroborreliosis. Detection of borrelial DNA in CSF despite a low sensitivity may be helpful because of the difficulty in interpretation of antibodies in the central nervous system. The specificity of a molecular test, however, can be increased by an initial evaluation of the patient for the presence of borrelial specific antibodies, which should be uniformly present in serum in the later stages of disease; such screening would raise the pretest probability of specimens tested and the performance of the assay.

Serology. Because methods for the direct detection of *B. burgdorferi* sensu lato in clinical specimens lacks sensitivity and are not feasible for clinical laboratory use, antibody detection methods remain the primary test method to confirm a clinical diagnosis of LB. Development of accurate and reliable serologic assays for diagnosis of LB has been difficult due to the number of antigens that are differentially expressed in the tick and the mammalian host (see Antibody Response, Serologic Profile, and Immunity section). Antigenic differences among the *B. burgdorferi* sensu lato species also mean that test kits also have to be modified depending on the geographic location where the infection is acquired (i.e., the United States or Europe).[7] Finally, serologic tests have had limited validation in patients where a clinical diagnosis of LB has been confirmed by culture as the 'gold standard' method, so that serology also has several diagnostic limitations that will be outlined.

Immunofluorescent-antibody assays. Early serologic assays included direct immunofluorescent-antibody assays (IFAs), which used cultured spirochetes fixed onto glass slides, followed by dilution of a patient's serum, absorption of nonspecific antibodies, the addition of fluorescein isothiocyanate-labeled antibodies, and finally, the detection of the presence of antibodies by fluorescence microscopy.[272] Positive titers of 1:256 or 1:128 for IgG and IgM, respectively, are considered indicative of LB. Because of the subjectivity of interpretation of fluorescence microscopy, this assay was modified to use antigens attached to a membrane (FIAX) with the degree of fluorescence detected by an automated system. Although IFA,[272] quantitative fluorescence immunoassay,[350] and a fluoroimmunoassay[184] have been used, they were replaced by EIAs owing to the improved performance and efficiency due to automation of the latter.

Enzyme immunoassays. Serologic testing of peripheral blood should be done on all patients meeting the criteria for suspected LB. The CDC recommends a two-tiered testing approach to diagnose active disease and previous infections as outlined in Figure 20-17.[78] A highly sensitive EIA or less commonly an IFA should be used as the initial test followed by a Western immunoblot. Negative sera need not be studied further but convalescent sera (drawn 4 weeks after the first) should be collected if clinically indicated. If a patient with suspected early LB has a negative serology, serology should be repeated by testing of paired acute- and convalescent-phase serum samples. All

specimens positive or equivocal according to the initial EIA or IFA test should be confirmed with a standardized Western immunoblot. For patients presenting in the early phase of illness (i.e., first 4 weeks of disease onset), both IgM and IgG Western immunoblots should be done. A positive IgM test result by itself should not be used in determining active disease in persons with more long-standing illness due to the high likelihood of a false-positive result in such patients.[78] A strong IgG Western immunoblot reaction to specific *B. burgdorferi* antigens should be found in serum samples from patients with disseminated or late-stage Lyme disease (see Immunoblots section).[78] Examination of cerebrospinal spinal fluid for intrathecally produced antibodies should also be performed for the diagnosis of Lyme neuroborreliosis although patients in Europe have higher positivity rates than those in the United States. This discrepancy may be due to the difference in *B. burgdorferi* sensu lato species causing LB on these continents. Positive

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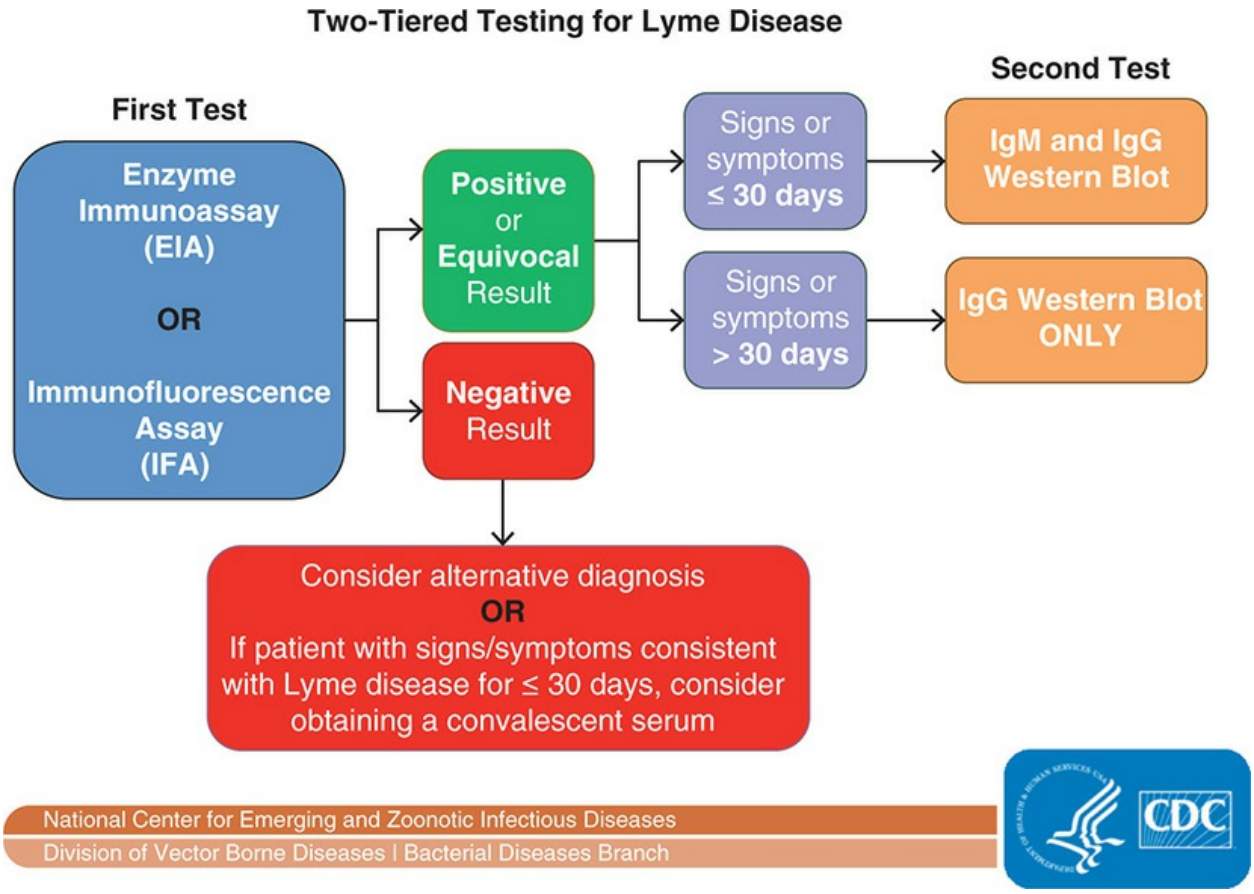
intrathecal CSF antibody production is measured by the CSF/serum index of *B. burgdorferi* sensu lato antibodies, and the presence of higher titers of antibody in CSF relative to peripheral blood (i.e., antibody index = CSF/serum optical density ratios of >1.3) along with CSF pleocytosis, documents infection of the nervous system.[440,496,501,512] However, given the many difficulties encountered with serologic analysis of peripheral blood, these approaches should be reserved for experienced reference laboratories that have documented the accuracy of their tests.[73]



■ **FIGURE 20-17** Diagnostic testing algorithm for Lyme disease.

In the United States, there are more than 70 serologic assays that have FDA approval for diagnosis of Lyme disease, but few if any of these tests have not been standardized against a panel of well-characterized sera. A similar situation exists in Europe where immunoassays have been modified to better detect *B. burgdorferi* sensu stricto, *B. afazelii,* and *B. garinii*. Overall detection rates for serum antibodies using these assays are not optimal for early disease (i.e., 20% to 50% of cases detected) and the early dissemination stages of the disease (i.e., 70% to 90% of cases detected). However, by late-stage disease, nearly all patients with Lyme disease will test positive.[7,494] Considerable variability has been demonstrated between assays due to their different antigenic composition and in particular, in the reliable detection of IgM antibodies.[5] Most of the earlier commercially available LB EIA assays use antigen mixtures comprised of whole-cell sonicates of *B. burgdorferi* sensu lato for the detection most frequently of IgG and IgM antibodies. Due to the lack of antigen standardization, there have been serious problems with reproducibility and with accuracy of EIA assays, compounding the difficulties of clinical diagnosis. In a proficiency testing program, as many as 21% of participants failed to recognize the

presence of antibody at a titer of 512 or higher.[24] With lower concentrations of antibody, as many as 55% of participants failed to recognize the serum as positive. Conversely, the false-positive rate was up to 7% with a polyvalent conjugate and as high as 27% with an IgG conjugate. Reproducibility of results when the same serum was submitted as a challenge at different times was also suboptimal. These proficiency testing problems are further emphasized by the report from a Lyme disease referral center, where 45% of patients who had been referred with an incorrect diagnosis of Lyme disease had had positive serologic tests for *B. burgdorferi* that could not be confirmed in the reference laboratory.[456] Furthermore, in a single laboratory only 16.3% of positive results using an EIA could be confirmed with an immunoblot.[101]

Specificity of *B. burgdorferi* EIA assays may also be suboptimal, particularly in patients with other infections, exposures to infectious disease agents, or underlying inflammatory or immunologic diseases. Cross-reactions with a variety of infectious agents have been found in sera from patients in whom Lyme disease is suspected. Sera of patients with other borrelial infections, treponemal infections, HIV, Epstein–Barr virus, and rickettsial infections may react in assays for *B. burgdorferi*.[268,308,366]Reports on cross-reaction with *Leptospira* antisera are conflicting. Although cross-reactions develop with specific treponemal antigens, the VDRL test is not positive in patients with Lyme disease. The nonspecific reactions have been reported to be concentrated in the IgG2 class of antibodies in several instances.[414] Kaell and colleagues[212] have also demonstrated increased antibodies to *B. burgdorferi* in 13 of 30 patients (43%) with nonspirochetal endocarditis most likely due to cross-reactivity. The specificity of Lyme serology in this population was only 60%. When immunoblots were performed, only one patient had a pattern of reactivity that suggested prior exposure to the spirochete. Lyme serology may also be false-positive in patients with other multisystem immunologic diseases such as systemic lupus erythematosis.[491]

Lyme disease serologic results must therefore by interpreted in the context of the history of tick exposure risk and the initial and evolving constellation of clinical symptoms and manifestations. Positive EIA serology results are most often straightforward when the patient presents with pathognomonic skin lesions, but may be misleading when the clinical presentation is atypical or the symptoms of the patient are vague. Steere[445] has demonstrated situations wherein there was serologic evidence of exposure of *B. burgdorferi*, but the LB was no longer active and the patient's symptoms were due to another cause. Prior vaccination with the Lyme disease OspA outer surface protein vaccine may also give a positive result using an EIA assay that detects this antigen, but the Western blot will not have a sufficient number of bands to confirm acute infection (see Immunoblots section).[6]

In order to increase the sensitivity for early disease (stages I and II) and the general

specificity of serologic assays, more recently developed serologic assays for Lyme disease have used either a purified, recombinant, or synthetic peptide antigen for detection of IgM, IgG, or polyvalent antibodies to *B. burgdorferi*. However, as reviewed in detail by Aguero-Rosenfeld,[3,7] a single antigen has not demonstrated sufficient performance to enable replacement of the two-tiered testing algorithm. Several immunoassays using recombinant and peptide antigens have been evaluated, but few studies have yet been done comparing these newer EIA tests to the two-tiered testing algorithm.[46,47] Table 20-12 provides a summary of the performance of selected recombinant and peptide antigens evaluated for detection of a serologic response to *B. burgdorferi* during the various LB disease stages both in the United States and Europe. Recombinant antigens used so far have included the outer surface proteins, particularly OspC,[270,282,336,342] the internal portion of flagellin (P41) as well as FlaA,[213,270,343] BBK32,[186] DbpA,[187] and VlsE.[18,271,360] Recombinant OspC assays have improved performance in diagnosing patients with early LB in the United States when testing IgM antibodies,[336] but this has not been the case for European patients.[342] Recombinant VlsE assays also have a comparable sensitivity to the OspC recombinant antigen assays during early disease, but have improved sensitivity for diagnosing neuroborreliosis or late-stage disease.[18,271] Evaluation of recombinant DbpA and BBK32 antigens in Europe shows a high sensitivity for detection of IgG antibodies in patients with neuroborreliosis or late stages disease, and although the BBK32 antigen also showed a high sensitivity (74% to 100%) for detecting antibody at any disease stage including EM, this epitope detects IgM antibody.[186,187]

Peptide-based immunoassays have included two peptides pepC10 (OspC region) and C6 (VlsE invariable region or IR6) that are highly conserved among different *B. burgdorferi* sensu lato species that have been shown to be immunodominant protein antigens.[18,47,360,452] A major advantage of using these well studied peptides is that they are

less cross-reactive than the full-length antigens. The pepC10 peptide preferentially binds IgM antibodies and although the sensitivity in patients with EM or early neuroborreliosis is similar to its parent antigen OspC, the reported specificities are much higher for the peptide assay.[18] A major limitation of pep10 peptide is that it does not detect antibodies in patients with late LB.[282] C6 peptide assays have high sensitivity in all stages of LB, and a much higher sensitivity has been found than recombinant VlsE assays in patients with EM.[271] Bacon and colleagues,[18] however, have demonstrated that the recombinant VlsE assay was better than either a C6

peptide or two-tiered testing approach for detecting IgG antibodies in patients with acute neuroborreliosis. All of the patients were diagnosed using the VlsE assay compared to only 9/15 (69%) for IgG antibodies to C6 and 13/15(87%) using two-tier testing. Burbelo and colleagues have also recently compared the performance of a C6-peptide immunoassay to the use of a synthetic protein, designated VOVO, consisting of a repeated antigenic peptide sequence, VlsE-OspC-VlsE-OspC for diagnosing Lyme disease. Analysis of an independent set of serum samples (n = 139) showed that the VOVO luciferase immunoprecipitation system test had similar performance to the C6 peptide assay.[59] Peptides from another surface protein BBK07 have recently been developed and used to evaluate the reactivity of sera from both humans and dogs with Lyme disease.[94] Although EIA studies of the surface protein BBK07 peptides have an overall lower sensitivity than either the pepC10 or C6 peptides for the detection of early Lyme disease in humans, a subset of serum samples were reactive to BBK07 peptides even though they failed to react with VlsE or OspC peptides. BBK07 may be an additional serodiagnostic marker that could be used as a supplemental test for Lyme disease.

TABLE 20-12 Selected Recombinant and Peptide Antigens Used for Detection of IgM, IgG, or Polyvalent Antibodies to *B. burgdorferi*

Recombinant Antigen of Peptide	Location	Range % Positive in the Indicated LB Disease Stage									References
		EM			*Neurologic*			*Late*			
		IgM	IgG	Polyv	IgM	IgG	Polyv	IgM	IgG	Polyv	
OspC	United States	41–73	35–43								100, 102, 183, 184, 241
	Europe	35–44	5–35		47–53	6–33		7–53	3–60		144, 196, 244, 275
pepC10	United States	40–53			53			9			15
	Europe	36	5		45	8		0	0		196
P41 internal fragment	United States	43–53	35–52								183, 184
	Europe				49	34–60					119, 144, 273
VlsE	United States	19–40	44–63	63	73	100	100	39	97	87	15, 161, 185
Invariable region 6 (IR-6, C6 peptide)	United States										
	Acute		45–74			60–95			94–100		15, 171, 255
	Convalescent		70–90			64			83–98		122, 169
	Europe		87								
FlaA (37 kDa)	United States	45–68	15								104
	Europe	27			58	74		37	79		246
DbpA	Europe	9	17			100			93–98		122, 123
BBK32	Europe	13	74–100			100			96–100		122, 123, 160
P66	United States	80	24		50	57		35–63	75–100		228

Polyv, polyvalent enzyme immunoassay.
Adapted from reference 7.

Increasingly, the use of a combination of recombinant or peptide antigens in serologic immunoassays is being evaluated in the hopes of improving the detection of Lyme disease in the earlier stages. Bacon and colleagues compared the use of recombinant VlsE for detection of both IgG and IgM antibodies, the pepC10 peptide for detection of IgM antibodies, and the C6 peptide for detection of IgG antibodies compared to the two-tier approach. The best performance was found using the C6 IgG and pepC10 IgM assays in testing 280 sera from patients with LB (78% were detected vs. 68% using a two-tier approach), particularly in patients with EM (63% were detected compared to only 38% using a two-tier approach). Porwancher and colleagues[360] recently studied the use of a multiplex microsphere immunoassay for simultaneously measuring recombinant VslE-IgG and pepC10-IgM antibodies and compared its performance with Western immunoblotting in the same sample from 82 patients with early disease, 47 with stage I or II disease, 34 patients following antibiotic treatment, and a large number of negative controls.

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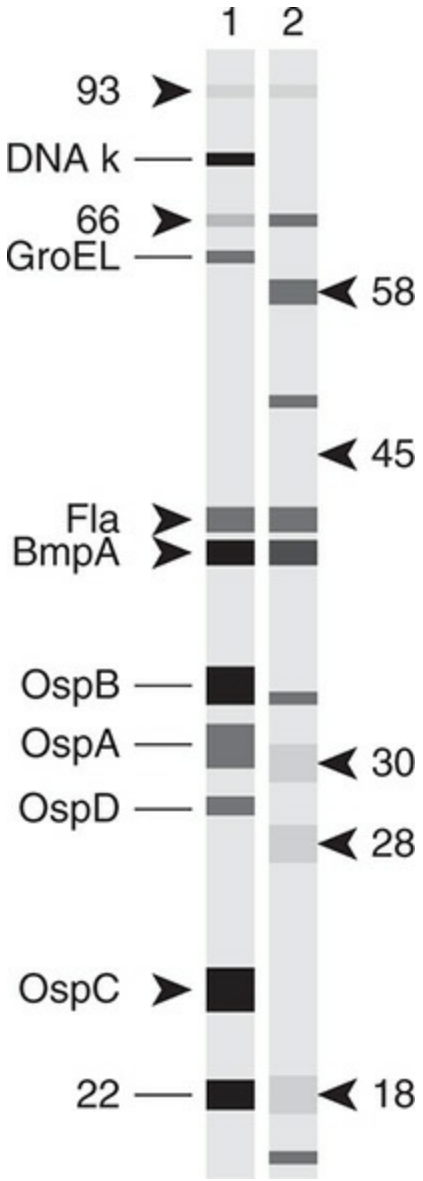
Compared with Western blotting, the multiplex assay was equally specific but much more sensitive as a diagnostic test for Lyme disease.[360] More combination studies need to be done using standardized serum panels and larger numbers of patients to determine the optimal serologic approach to diagnosing Lyme disease.



■ **FIGURE 20-18** Lyme disease confirmatory immunoblot. Positive Western Blot. Strip (1) outlines the borrelial antigens that antibodies are developed against in Lyme disease patients. Strip (2) highlights the borrelial antigens that a patient with Lyme disease is reactive against, providing confirmation of this diagnosis according to the CDC immunoblot interpretive criteria. (Courtesy of the Centers for Disease Control and Prevention, USA.)

Immunoblots. In order to improve the specificity of LB serology similar in approach to that used for HIV serologic diagnosis, it is recommended that all positive ELISA assays be confirmed by a Western immunoblot (Fig. 20-18). Dressler and colleagues[118] developed the criteria for positive immunoblots in a retrospective study. The best discrimination was obtained if detection of 2 of the 8 most common IgM

bands (18, 21, 28, 37, 41, 45, 58, and 93 kDa) was required for positivity in early disease and detection of at least 5 of the 10 most common IgG bands (18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa) was required for diagnosis after the first weeks of infection. Application of these criteria to more than 300 patients in a prospective study yielded a sensitivity of 32% and a specificity of 100% for the IgM test. The sensitivity and specificity of the IgG test were 83% and 95%, respectively. Slightly different criteria have been suggested by Engstrom and colleagues.[135] Their criteria for positivity of IgM immunoblots include recognition of at least two of the following proteins: 24-(OspC), 39-, and 41- (flagellin) kDa. The sensitivities of the IgG and IgM immunoblots in 55 patients with documented EM were 58.5% and 54.6%, respectively, at the time of diagnosis and 100% after an additional 8 to 12 days. Ma and associates[265] have emphasized the importance of antibody to the 39-kDa antigen for specific diagnosis.

The CDC and Association of State and Territorial Public Health Laboratory Directors currently recommend the IgM criteria of Engstrom and the IgG criteria of Dressler. By using these criteria, Aguero-Rosenfeld and associates[6] observed positive IgM immunoblots at the time of initial study in 43% of a group of 46 patients with culture-positive EM and in 84% of the patients 8 to 14 days later. Although antibodies to *B. burgdorferi* developed in 89% of patients, IgG immunoblots were positive by the recommended criteria in only 22% of convalescent sera. After 1 year, 38% of the IgM immunoblots were still positive. IgM antibodies reactive with 39-, 58-, 60-, 66-, and 93-kDa antigens were most often seen in the first month after diagnosis, and these investigators suggest that the presence of these bands may be helpful in suggesting recent infection in patients from endemic areas.

Hilton and colleagues[192] suggest that two additional antigens be added to the criteria for IgG positivity: 31-kDa (OspA) and 34-kDa (OspB) antigens. Of 136 patients evaluated for Lyme disease, 4 (8%) would have been considered positive only if these two antigens were included in the criteria. These 4 patients had EM or arthritis and lived in endemic zones. It is obvious that the criteria for interpretation of immunoblots are still evolving.

Fine-tuning of the criteria for positive serologic tests will undoubtedly continue, but confirmation of an indeterminate or questionable result with an enzyme immunoassay should be sought by performing an immunoblot in an experienced laboratory. Wormser and colleagues[500] have noted, however, that the confirmatory aspects of the immunoblots are reduced because the two assays are not completely independent.

Relapsing Fever

Relapsing fever is a distinctive clinical disease that was probably recognized by Herodotus in ancient Greece. There are two forms: (1) epidemic LBRF and (2) endemic TBRF. *Borrelia* that cause relapsing fever vary their outer surface antigens, resulting in repeated exposure of the host immune system to a new spirochetemia, which causes fever in the host.[436] The epidemiologic, ecologic, and clinical characteristics are compared and contrasted in Table 20-13. Several pertinent reviews are available,[125,54,145,168]and in several parts of the world, TBRF is emerging as an important vector-borne disease.[374,400,401]

Epidemiology

LBRF is caused by infection with *Borrelia recurrentis*. The spirochetes are transmitted to humans by contact with the hemolymph of an infected human body louse (*Pediculus humanus*).[54] The louse is crushed by scratching which releases the insect's hemolymph and inoculates the host's skin or mucous membranes. The human body louse inhabits humans only, and *B. recurrentis* is not transmitted vertically to succeeding generations of lice. The organism must be maintained by passage from louse to a human host and then back to another louse. The louse remains infected for its entire life. LBRF is a disease fostered by poverty, crowding, and poor sanitation, as is louse-borne typhus. Epidemic disease thus

occurs only under conditions of extreme deprivation, and outbreaks have been reported in Africa, the Middle East, and Asia, but not recently in the New World. For the first half of the last century, epidemics occurred approximately every 20 years. In recent times, Ethiopia has been the only major geographic locus of infection.[464] Travelers to the developing world occasionally import LBRF into the United States.

TABLE 20-13 Characteristics of Relapsing Fever

Characteristics	Louse-Borne	Tick-Borne
Epidemiology	Epidemic	Usually endemic
Etiologic agent	*B. recurrentis*	Various; *B. hermsii*, *B. turicatae*, *B. parkeri* in USA
Vector	*Pediculus humanus* spp.	Various; *Ornithodoros hermsii*, *O. turicatae*, *O.*

		parkeri in USA
Mean duration of primary attack	5.5 days	3.1 days
Mean duration (range) of asymptomatic interval	9.25 days (3–27)	6.8 days (1–63)
Mean number (range) of relapses	3 (0–13)	1 (0–3+)
Mean duration of relapse	1.9 days	2.5 days

Adapted from 54, 125, 145, 168.

TBRF is distributed worldwide. In North America, it is endemic in the western United States, southern British Columbia, and the plateau regions of Mexico.[124,125,126] Many soft-bodied ticks of the family *Argasidae* (*Ornithodoros* species) carry distinctive borreliae. Although most cases of TBRF occur after tick bites, borreliae have also been transmitted by intravenous drug use, blood transfusion, and laboratory worker accidents, including the bite from an infected monkey.[125] In the United States, taxonomists have attempted to use the same name for a tick and its associated spirochete. Thus, *O. hermsii* is the vector for *B. hermsii, O. turicata* is the vector of *B. turicatae, and O. parkeri* is the vector of *B. parkeri*.[145] *O. turicata* is much larger than *O. hermsi*, and has conspicuous conical mammillae and dorsal projections on the distal segment of the front legs. *Ornithodorsos* ticks have a similar life cycle but inhabit different ecologic niches and hosts. Their life cycle includes egg, single larva, and several successive nymphs before becoming adults. All stages are obligate feeders may transmit spirochetes when they quickly obtain a blood meal within 15 to 90 minutes of attachment. Ticks feed at night when their natural or incidental hosts are sleeping. Female soft-ticks lay clutches of eggs after each blood meal which dramatically prolongs their longevity compared to hard-ticks.

The epidemiologic characteristics of TBRF depend on the habits of the local vector. *O. hermsii* is the most common vector and *B. hermsii* the most common *Borrelia* in California, the Pacific Northwest, and Canada.[125,126] *O. hermsii* lives in the remains of dead trees and parasitizes ground squirrels, tree squirrels, and chipmunks living near freshwater lakes, which often carry rustic tourist cabins. *O. parkeri* inhabits caves and burrows of ground squirrels and prairie dogs in the western United States. *B. parkeri* is a relatively infrequent pathogen of humans because of the inaccessibility of its vector. *O. turicata* inhabits caves and animal burrows in the western United States, Mexico, and South America, but it has also been found in closer association with

humans under the foundations of houses in Texas. *O. parkeri* is found in burrows, rodent nests, and caves in arid regions and grasslands throughout much of the western United States. So far, transmission of relapsing fever after the bite of *O. parkeri* has been rare.[125] The tick-borne borreliae have a solid ecologic niche with virtually no possibility of eradication. The spirochete can be passed from generation to generation of ticks without intervention of a vertebrate host. *O. turicata* has been reported to survive in a starving state for 5 years, and survival of borreliae in ticks without loss of infectivity has been reported for up to 12 years.[126]

Most TBRF is endemic, afflicting the unfortunate person who inhabits or visits an endemic area where *Ornithodoros* ticks reside, and becomes an incidental host. Under the right conditions, however, epidemic disease can occur. In 1968, there was an outbreak of TBRF among 42 boy scouts and scoutmasters who were camping near Spokane, Washington.[469] Clinical disease developed in 1 of the 42 persons at risk. In 9 patients an exact incubation period could be calculated because they were only at risk for one night (mean, 6.9 days; range, 3 to 9). The attack rate was higher in those who slept in abandoned cabins than in those who slept in tents, a trend that was also noted by Horton and Blaser in Colorado.[199] The authors noted poetic justice in the concentration of disease among scoutmasters and older scouts, who appropriated the cabins for themselves, leaving the younger scouts to "rough it."

In areas that have no indigenous relapsing fever spirochetes, importation of disease from endemic regions provides a diagnostic challenge.[93] Tick-borne infection is a more likely infection than louse-borne infection for tourists visiting Africa. Parasitologists should keep this diagnostic possibility in mind when examining smears collected for the detection of malaria parasites.

Clinical Disease

The clinical presentation of the two types of relapsing fever is similar. The mean incubation period of TBRF is 7 days (range, 4 to >18 days).[125] Differences in frequency and timing

of relapses are noted in Table 20-13. *Ornithodoros* ticks feed on humans very inconspicuously and for short periods. Most patients, therefore, do not remember a tick bite. The clinical manifestations of TBRF were well described by Dworkin and colleagues[124] in a relatively large number of cases acquired in the northwestern US and southwestern Canada. The onset of disease is typically abrupt, with a high fever, usually near 40°C (104°F), shaking chills, delirium, severe muscle aches, and pains in bones and joints. Hepatosplenomegaly and tenderness may be present, and the patient

may be jaundiced. Neurologic complications, including lymphocytic meningitis and facial palsy, resemble those of *B. burgdorferi* infection.[65] Unusual manifestations of the infection include iritis, uveitis, iridocyclitis, cranial nerve palsy and other focal neurologic deficits, rupture of the spleen, myocarditis, and acute respiratory distress syndrome (ARDS).[124,125] However, ARDS has been more commonly described in more recently reported cases of TBRF.[72] The initial episode of TBRF lasts 3 days (range, 12 hours to 17 days), and typically it terminates with a crisis.[168] In general, LBRF last longer on average 5.5 days (range, 4 to 10 days). Fatalities are very uncommon in TBRF although untreated LBRF has a higher mortality (5%).[168] An outbreak of louse-borne relapsing fever in Ethiopian troops after the cessation of civil war provided an opportunity to review the clinical features.[43] The case fatality rate was 3.6%, and 1.8% for patients who had recrudescent disease. The average time between the initial episode and the first relapse is 7 days for TBRF and 9 days for LBRF.[168] When relapses occur, each cycle tends to be less severe than the preceding one but the patient usually has malaise and feels unwell despite the absence of fever in between episodes. A rash may develop during the initial attack, but it does not usually appear in relapses. Although penicillin is effective against borreliae, tetracycline or erythromycin are more effective at eliminating the spirochetes. Jarisch–Herxheimer (JH) reactions may occur during treatment; in an Ethiopian epidemic of LBRF, 43% of patients experienced this complication. Use of low-dose penicillin leads to more frequent relapses but fewer JH reactions than with tetracycline or larger doses of penicillin.[405] Administration of acetaminophen and hydrocortisone modified the changes in vital signs during the JH reaction, but did not prevent the dramatic rigor that occurs.[62] Transient increases in tumor necrosis factor and interleukin-6 and -8 correlate temporally with the reaction,[314] although not all investigators have been able to detect elevations of these cytokines. Control of the vector is also essential for the control of epidemic louse-borne infection.[464]

Laboratory Diagnosis

Detection and Isolation. Laboratory confirmation of relapsing fever is made by the detection or isolation of spirochetes in the blood of the patient during the febrile illness (Image Plate 20-1C). The diagnosis is often made in the hematology laboratory, because the clinical presentation can be enigmatic for physicians unaccustomed to the disease. Horton and Blaser[199] report a case in which the spirochetes were missed by an automated differential scanner, but were noted by an astute technologist who reviewed the smear. In contrast to other spirochetes the relapsing fever borreliae are well stained by acid aniline dyes, such as Wright's or Giemsa's stains. The borrelial spirochetes are thin, undulant, or overtly spiral organisms that are most visible when

they are located between red blood cells (Image Plate 20-1C).[40] Felsenfeld[145] advocates staining with Wright's stain followed by application of a 1% solution of crystal violet for 10 to 30 seconds.

The sensitivity of detecting borreliae by staining peripheral smears during a febrile episode is estimated at 70%. However, it is unlikely that spirochetes will be detected when the patient is in remission during the afebrile intervals between attacks, although borreliae are still present in the body. Thick and thin films should be made as for malaria, because in some cases spirochetes will be detected only by examination of the thick film.[40] A monoclonal antibody for the identification of *B. hermsii* in peripheral blood smears is available but not widely used.[399]

Alternatively, quantitative buffy coat analysis may be used, and is much more sensitive (100×) than thick smear examination. Goldschmid and Mahomed[165] used a microhematocrit centrifugation technique to concentrate the borreliae in and above the buffy coat. Although the buffy coat procedure has not been widely used, its utility has been confirmed in a study from West Africa that included isolation of *B. crocidurae.*[481] Sciotto and colleagues[403] used the fluorescent acridine orange technique to demonstrate borreliae in peripheral smears (Image Plate 20-1D). The fluorescence procedure greatly enhances the visibility of the spirochetes.

Relapsing fever borreliae can also be cultured from the blood of an infected patient in a modification[26] of Kelly's medium for borreliae,[219] but isolation is not a practical diagnostic test in most clinical laboratories because cultures need to be examined for spirochetes using darkfield microscopy for 2 to 6 weeks after inoculation.

Serology. Confirmation of TBRF using serology is demonstrated with a fourfold rise in antibody titer between an acute and convalescent serum samples or with a single convalescent serum sample that is reactive. However, only a few reference laboratories perform serology for relapsing fever diagnosis because the borreliae need to be cultivated for use in an indirect IFA, and to produce large amounts of whole-cell lysate for use in the ELISA assays. An IFA titer of 1:128 to 1:256 or higher is considered positive. Results of ELISA tests are often reported as an absorbance value (i.e., 0.85) after testing at one dilution. Samples that screen positive by either the IFA or ELISA assays should be confirmed with immunoblot to determine the pattern of reactivity to other antigens specific for relapsing fever borreliae. Because of cross-reactivity with the flagellin protein, patients may have false-positive serology tests for relapsing fever, if they also have reactive tests for other species of spirochetes. Approximately 5% of patients will have a positive VDRL test. Patients with relapsing fever may also have reactive Lyme disease serology.[124]

In order to improve sensitivity and specificity of serology tests, an immunoreactive protein, glycerophosphoryl diester phosphodiesterase (GlpQ), has been identified in

borreliae causing relapsing fever, but not those that cause Lyme disease or syphilis.[359,402] Although the GlpQ recombinant protein is not widely available, it has recently been used in an immunoblot to confirm TBRF due to *B. hermsii* infection in a patient exposed in the mountains near Los Angeles County, California, USA.[401]

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Molecular Methods. Tick analysis has been performed using amplification techniques for borrelial nucleic acid and subsequent nucleic acid sequence analysis for identification and characterization. Molecular methods were used to identify and characterize *B. hermsii* found in six ticks in Los Angeles County subsequent to the confirmation of human infection in the above exposed patient.[401] Previously described methods for PCR and DNA sequence analysis were used to characterize several genes including 16S rRNA, flagellar B protein (*fla*B), gyrase B (*gyr*B), *glp*Q, and variable tick protein (*vtp*). Similar techniques have, however, not been widely used to diagnose human disease.

Leptospira

Taxonomy *Leptospira*, together with the genera *Leptonema* and *Turneria*, is a member of the family *Leptospiraceae*. *Leptospira* are motile, obligately aerobic helical rods with characteristic hooked ends that measure 0.1 mm in diameter and 6 to 12 mm in length. They are gram-negative and are only faintly stained by aniline dyes. Darkfield microscopy must be used to visualize unstained *Leptospira* (Fig. 20-18). Originally there were only two recognized species of *Leptospira* that could be separated on the basis of biochemical tests: *L. interrogans* sensu lato which contained all of the human pathogenic species, and *L. biflexa* sensu lato which contained all of the saprophytic environmental species. Leptospires were then further divided into serovars using agglutination after cross-absorption with homologous antisera directed at the surface-exposed epitopes of LPS antigens.[113] *L. biflexa* sensu lato contains more than 60 different serovars, while *L. interrogans* sensu lato contains more than 260 different serovars. A single serovar might be found in several species. For epidemiologic classification purposes, antigenically related *Leptospira* serovars and species are also collected into serogroups. The pathogenic type strain is *L. interrogans* sv. Icterohaemorrhagiae,[209] and the clinical disease is leptospirosis. Several reviews, some from the older literature, reflect the state of knowledge in many areas.[1,131,143,182,183,225,243,290]

Studies of the DNA relatedness among serovars of *Leptospira*[505] have identified

genomospecies, and the number worldwide will continue to expand as unique species are discovered.[1,243,244] The currently recognized genomospecies and the corresponding serogroups of *Leptospira* are detailed in Table 20-14. Recently, the taxonomy of *Leptospira* was changed when it was decided to give the status of species to previously described genomospecies 1, 3, 4, and 5. Thirteen pathogenic species of *Leptospira* are now recognized, including *L. interrogans*, *L. alexanderi*, *L. alstonii* (genomospecies 1), *L. borgpetersenii*, *L. inadai*, *L. fainei*, *L. kirchneri*, *L. licerasiae*, *L. noguchi*, *L. santarosai*, *L. terpstrae* (genomospecies 3), *L. weilii*, and *L. wolffii*, which contain more than 260 serovars. Saprophytic species of *Leptospira* include *L. biflexa*, *L. meyeri*, *L. yanagawae* (genomospecies 5), *L. kmetyi*, *L. vantheilii* (genomospecies 4), and *L. wolbachii*, which contain more than 60 serovars.

Whole-genome sequences have now been published for at least six leptospiral strains including two serovars (Lai and Copenhagen) of *L. interrogans*, two strains of *L. borgpetersenii* serovar Hardjo, and two strains of *L. biflexa* serovar Patoc.[57,309,353,373]These analyses reveal some unique feature of *Leptospira* genetics, and are allowing different virulence genes to be identified in pathogenic compared to saprophytic strains. The genome is comprised of two circular chromosomes, and there is a high degree of homology between pathogenic serovars despite differences in host specificity.[309]

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Comparison of the two pathogenic and one saprophytic species has identified 2,052 genes which make up the core leptospiral genome, suggesting a common origin for all pathogenic and saprophytic *Leptospira*.[1] Comparison of the *L. interrogans* and *L. borgpetersenii* genomes also show a high degree of plasticity and large-scale genome rearrangement with a much higher number of pseudogenes, insertion sequences, and genetic remnants than in the saprophytic strain.[57] *L. interrogans* also has a much higher number of genes (~500) that encode proteins of unknown function, which supports the notion that *Leptospira* have unique virulence factors that have not been identified in other spirochetes or bacteria. *Leptospira* have more than 70 genes with a putative regulatory role which is much higher than that found in other spirochetes, some that allow the organism to survive for extended periods in the host and external environment.[309] In particular, three toxin–antitoxin systems may control gene regulation during times of nutritional stress.[515] The loss of gene function in *L. borgpetersenii* may have occurred because the saprophytic *Leptospira* serovars do not survive in the environment and have a stricter host-to-host transmission cycle.[57]

TABLE 20-14 Genomospecies and Serogroups of *Leptospira*

Genomospecies	Serogroup
L. interrogans	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin
L. noguchii	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona
L. santarosai	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri
L. meyeri	Ranarum, Semaranga, Sejroe, Mini, Javanica
L. wolbachii[a]	Codice
L. biflexa[a]	Semaranga, Andamana
L. fainei	Hurstbridge
L. borgpetersenii	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
L. kirschneri	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae
L. weilii	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
L. inadia	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica
L. parva[a]	Turneria
L. alexanderi	Manhao, Hebdomadis, Javanica, Mini

[a] No pathogenic serogroups associated with this species to date.
Adapted from reference 113.

Transmission of *Leptospira* to humans occurs through direct or indirect contact with the urine of an infected animal, or with contaminated water or soil. Leptospires usually enter the body through small cuts of abrasion, or by contact with mucous membranes such as the conjunctiva or through wet skin. During the initial bacteremic phase of the illness, spirochetes circulate in the bloodstream for up to 7 days. Once the host generates an antibody response, the organisms are cleared from the bloodstream. However, in severe cases, *Leptospira* may rapidly penetrate and disseminate in the body, establishing persistent colonization in the renal tubules. The pathogenic mechanisms that cause leptospiral host disease and host tissue damage have not been elucidated, although leptospires likely migrate through intercellular junctions. Leptospiral toxin(s) or toxic cellular components are likely involved in the development of symptoms. Damage to the endothelium of small blood vessels leads to localized organ ischemia, which causes renal tubular necrosis, pulmonary damage, hepatocellular damage, and inflammation of the meninges, myocardium, muscles, and placenta.[103,104,169,406,511]

Leptospirosis

Leptospirosis is a systemic disease of humans and animals. Although commonly designated as Weil's syndrome in the older literature, many other names have also been attached to the illness (i.e., canicola fever, nanukayami fever, 7-day fever, Rat Catcher's Yellows, Fort Bragg fever, black jaundice, and pretibial fever).[243] Adolf Weil first reported a case of "icterohemorrhagic fever" in 1886 where the patient had acute infection with splenomegaly, jaundice, and nephritis.[131] *Leptospira* spirochetes were initially observed from postmortem renal tissue sections in 1907. Leptospirosis as a cause of febrile illness has been misdiagnosed worldwide, but global attention has recently established that this zoonosis is a neglected problem that has a major health impact in resource-limited countries in tropical areas.

Epidemiology. Leptospirosis is a spirochetal zoonosis that is globally reemerging due to urban epidemics in poor communities of industrialized and resource-limited nations. *Leptospira* are distributed worldwide and infect many types of domestic and wild animals. Humans become incidental, "dead-end" hosts because transmission from humans to animals or to other humans does not occur. Most human cases occur in tropical countries because the spirochetes are better able to survive in warm, humid conditions. Leptospirosis is, therefore, a seasonal disease in temperate climates, with cases occurring in the summer or fall.

Domestic rats infected with pathogenic strains of *Leptospira* have been classically associated with this disease, but domestic animals, mainly dogs, cattle, and pigs, may be infected like humans as incidental hosts. Foxes, raccoons, skunks, shrews, and hedgehogs are among the many types of wild animals worldwide that may also carry

the leptospires. Animal hosts are usually not symptomatic and antibody does not develop in them, despite overwhelming infection. Several different serovars of *Leptospira* may be carried by a single animal genus, however, and a single serovar may be associated with more than one animal host. Dairy cattle may become infected with serovars Pomona and Hardjo, pigs with Bratislava, Tarassovi, or Pomona, and dogs with Canicola.[243]

A key factor to the persistence and epidemiology of leptospirosis is the renal carrier state of diseased animals. Leptospires have a propensity to colonize the surface of renal proximal tubular epithelial cells. Spirochetes are excreted into the urine of the host in high concentration, either continuously or intermittently, surviving best in alkaline urine.

Leptospirosis has divergent epidemiologic patterns for the poor in rural and urban areas in resource-limited countries, compared to the more affluent people in these same countries. Leptospirosis worldwide is acquired through environmental exposure with contaminated water or soil, either due to occupation, day-to-day activities, or recreation. Leptospirosis due to occupational disease has been concentrated among workers in sewers, abattoirs, and agriculture, particularly subsistence farmers in rice and sugarcane fields in resource-limited countries.[243] Epidemiologic factors that were associated with leptospirosis in Hawaii included catchment of rainwater for household use, contact with cattle and cattle urine, or handling of animal tissues.[216] Since the 1970s, only a small number of cases have occurred in the United States because of occupational contact.

More recently, leptospirosis has increasingly been recognized as an urban problem worldwide. The dramatic migration of poor rural people to urban slum communities and the increased number of urban homeless people create ecologic conditions for rodent-borne transmission. Urban leptospirosis was initially recognized in US inner-city homeless populations. Annual outbreaks of leptospirosis are also reported in poor urban communities in Brazil, India, and others parts of the world during the same seasonal monsoon period of heavy rainfall.

Leptospirosis is also a risk for affluent people who pursue recreational activities in endemic areas, particularly those who participate in backcountry and extreme sports. Imported disease must therefore be considered in travelers presenting with a febrile illness.[479] Leptospirosis has been reported in association with kayaking.[418] Epidemic disease occurred during a triathlon in Illinois, where it was associated with brief exposure to the water in a pond,[205] and among

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white water rafters in Costa Rica.[76]Participants in an Eco-Challenge race in Borneo

also contracted acute leptospirosis during the race due to contact with the Segama River during kayaking, spelunking, and swimming.[82]

Epidemic disease may also occur from flooding or another common waterborne source. A large outbreak of 'hemorrhagic fever' followed flooding in rural Nicaragua in 1995 when 2,259 residents developed a febrile illness and 15 died of severe pulmonary hemorrhage.[474] In Brazil, more than 10,000 cases of severe leptospirosis are annually reported due to rainfall-associated urban epidemics in poor urban slums.[380] Several large outbreaks have also occurred in India after monsoons or cyclones.[407,408] In Italy, an outbreak occurred due to a leptospire-infected hedgehog being trapped in a water reservoir.[64] Several waterborne outbreaks have also occurred in the United States.[507] A waterborne outbreak of leptospirosis also occurred in Illinois that was traced to a contaminated pond; a drought was hypothesized to have increased the risk of exposure to infected animal excreta.[205]

A small outbreak due to exposure to contaminated soil also occurred in a suburb of St. Louis, Missouri.[144] *Leptospira interrogans* sv. icterohaemorrhagiae was isolated from rats and house pets and from soil in the yards of homes. Some of the dogs from whom they isolated leptospires had been immunized with commercially available anti-*Leptospira* vaccines.

Clinical Disease. Leptospirosis in humans varies in severity based on a number of host factors and the infecting *Leptospira* serovar. Classical leptospirosis is a biphasic disease, which consists of an initial bacteremic phase that lasts for a week, and a secondary immune phase characterized by antibody production, dissemination of spirochetes to tissues, particularly the renal tubules, and excretion of leptospires in the urine. After an asymptomatic interval of 1 to 3 days, the immune phase of the infection develops. Leptospires are rapidly cleared from the blood and CSF, and an inflammatory process develops. A CSF pleocytosis will develop in 90% of patients during the second week of illness, but only half of these patients have symptoms of aseptic meningitis.

Leptospirosis starts abruptly with fever, headache, myalgia, malaise, conjunctival suffusion, and occasionally a transient rash. Patients may present with skin rashes that are macular, maculopapular, urticarial, or hemorrhagic. A distinctive pretibial rash accompanied an epidemic of *L. interrogans* sv. Autumnalis infection and was named Fort Bragg fever. This pretibial rash has accompanied infections with other serovars. Although the symptoms are incapacitating in the initial stage, most patients recover without developing the complications that are seen in the more severe forms of the disease. Severe disease manifestations may occur with infections due to any of the pathogenic serovars, but milder disease is usually caused by Hardjo and others.[1] Despite recovery from the disease symptoms, patients may remain weak, fatigued,

and depressed for weeks to months after the initial illness.

Infection with serovars icterohaemorrhagiae, Copenhageni, Lai, and others are usually associated with severe disease.[1] Although most patients with leptospirosis are asymptomatic or develop mild disease, there is a major burden of illness worldwide due to the severe disease forms, Weil's disease (jaundice, acute renal failure, and hemorrhage) and severe pulmonary hemorrhage syndrome (SPHS). Patients with SPHS have a high mortality rate (>50%), while over 10% of patients with Weil's disease also die. Older adults (>30 to 40 years of age) are more likely to die.[103,104] Several studies have also demonstrated that acute renal failure, respiratory failure, hypotension, arrhythmias, and altered mental status are independent prognostic markers for mortality.[121]

Leptospirosis initially causes a unique nonoliguric hypokalemic form of acute renal insufficiency that if diagnosed early can be treated with volume repletion and potassium replacement.[406] SPHS due to leptospirosis infection usually manifests as ARDS, pulmonary infiltrates, and hemoptysis that may not be evident until intubation.[169] The leptospiral burden in the lungs of patients with SPHS is extremely high even though few spirochetes have been observed at autopsy, suggesting that this is an immune-mediated condition.

Pregnant patients with leptospirosis, whether they have mild or severe forms of the disease, are at risk for intrauterine infection and fetal death. Congenital transmission of leptospirosis from mother to infant has not been reported.

Patients with severe leptospirosis often require intensive care including ventilator support and dialysis to survive. Because the Jarisch–Herxheimer reaction has been reported after treatment, caution should be used in prescribing antibiotics to patients with self-limited mild disease.[133] The rationale for treating patients with severe disease with antibiotics has, however, been reviewed.[488] Clinical trials in Thailand have also demonstrated equivalent efficacy of treatment with ceftriaxone, cefotaxime, and doxycycline compared with penicillin therapy.[466]

Humoral immunity to leptospirosis mediated by specific antibody production occurs in both humans and animals. LPS is the major antigen recognized by convalescent sera, and immunity is restricted following naturally occurring infection to serovars with antigenically related LPS.[2] Recent studies have also shown that mice require intact TLR2 and TLR4 activation pathways to control a lethal infection.[88]

Laboratory Diagnosis. Diagnosis of leptospirosis must be confirmed by the laboratory because of the wide range of clinical manifestations of this disease. Although culture allows a definitive diagnosis, it is rarely performed because it is slow and not widely available. Leptospirosis may also be demonstrated by detection of the organisms in specimens, or by the detection of antigens or nucleic acid, but serology

remains the main diagnostic method. Monoclonal antibodies have been used to detect leptospiral antigen in urine in specimens from Thailand, but there are no commercial antigen tests so this will not be discussed further.[387]

Direct Detection. *Leptospira* may be detected by microscopic examination of blood, CSF, or urine, but spirochetes in blood can only be detected during the early bacteremic phase of the illness. Centrifugation of specimens at low speed of oxalated or heparinized blood to remove cellular elements and then at high speed to concentrate *Leptospira* has been recommended. Darkfield microscopy examination may be attempted but a high leptospire concentration (10^4/mL) must be present for spirochetes to be visualized.[486] Nonspecificity is also a problem using darkfield examination because fibrils or extrusions from red blood cells are misinterpreted

as spirochetes. Although examination of CSF and urine are somewhat less likely to contain artifacts that may produce a false-positive result, similar caveats apply.[426] Silver staining using the Warthin–Starry stain has been widely used for histologic examination of tissues. Immunohistochemical staining can also be performed at a reference laboratory (i.e., CDC).[511]

Culture. *Leptospira* can be isolated from blood, peritoneal dialysate, and CSF during the first week of illness. During the immune stage, the organisms disappear from the blood and CSF, but *Leptospira* are excreted in the urine for up to 1 month (Image Plate 20-1G). The most commonly used commercial culture media are Fletcher's semisolid medium (Difco, BD Diagnostic Systems, sparks, MD) or semisolid bovine serum albumin–Tween 80 (oleic acid) medium (Ellinghausen–McCullough–Johnson–Harris [EMJH, Difco]) that contains 200 µg of 5-fluorouracil. The media may be stored at room temperature for long periods. It is recommended that at least four 10-mL tubes of culture media from two different lots be inoculated with the clinical specimen. For recovery of fastidious serotypes, 0.4% to 1.0% sterile rabbit serum may also be added to the bovine serum albumin–Tween 80 medium. Only very small blood or urine volumes (50 µL) should be inoculated, because larger volumes will introduce interfering substances. Media should be inoculated with one to two drops of specimen per tube. If the procedure cannot be done at the bedside, oxalated or heparinized blood may be sent to the laboratory. A triturated clot may also be inoculated, but citrated blood may be inhibitory. When urine is tested, the pH should be immediately neutralized by sodium bicarbonate, and an undiluted specimen and a 10-fold dilution should be inoculated. When CSF is used, 0.5 to 5.0 mL should be inoculated.

The cultures are incubated in the dark at 28°C to 30°C for 6 to 12 weeks and examined weekly by darkfield microscopy for the presence of spirochetes (Fig. 20-

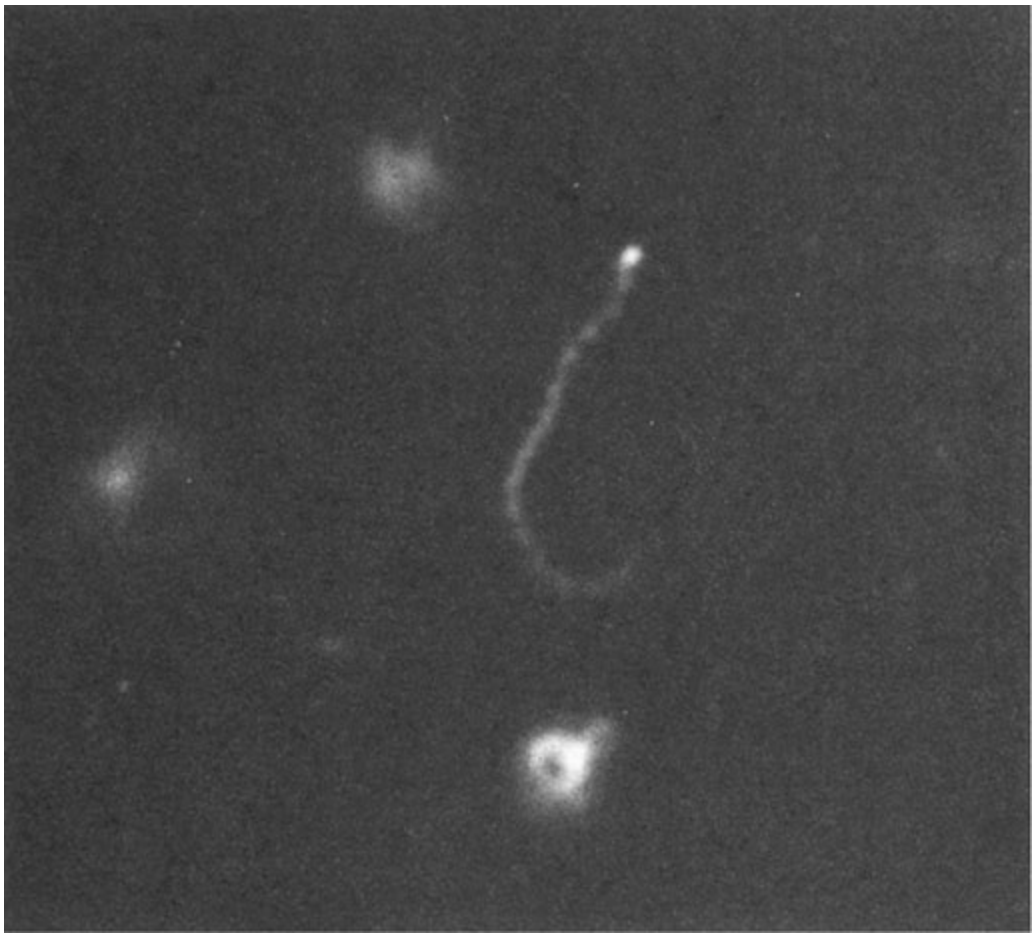
19). Growth is often delayed for several weeks but occurs considerably earlier in EMJH media. *Leptospira* typically grow as a band 0.5 to 1.0 cm below the surface of semisolid media, known as Dinger's ring (Image Plate 20-1H)

Palmer and colleagues[341] demonstrated that commercial blood culture systems had variable ability to maintain viability of leptospires. In general, the combination of an aerobic blood culture and an incubation temperature of 30°C enhanced the viability of leptospires for up to a week in some systems. However, a positive signal from an automated blood culture system is not likely to occur in a patient's sample since the organisms do not grow in conventional media.

Identification of Leptospira recovered in culture must be done using serologic cross-agglutination absorption methods in order to establish a serovar.[113] Isolates should be referred to a reference laboratory that maintains an extensive antisera bank for this purpose. Genomospecies identification must also be done using sequence analysis of 16S rRNA or other genes.[301]

Serology. Leptospirosis is mainly diagnosed by detection of antibody using serologic tests. Patients begin to produce antibody toward the end of the initial 7-day illness.

Microagglutination test. The gold standard serologic test continues to be the microagglutination test (MAT) whereby leptospiral antigen suspensions are reacted with the sera.[473] MAT is performed by mixing diluted patient sera with leptospiral cultures in a 1:1 ratio, incubating the mixture for 2 to 4 hours at 30°C or room temperature, and then using darkfield microscopy to examine them for agglutination. The reaction is positive when the proportion of free leptospires is less than 50% compared to the control suspension. The highest dilution of serum that shows 50% agglutination compared to the control mixture determines the end-point titer. The MAT test, often considered serovar-specific, is, in fact, serogroup-specific. Only certain reference laboratories perform MAT testing because it is a complex test to perform and interpret. Furthermore, the sensitivity of MAT in the acute phase is low, and paired sera must be collected to confirm leptospiral infection. The difficulty of maintaining large numbers of cultures of *Leptospira* is obvious. Formalin-killed leptospira are used as a practical compromise, but they result in somewhat lower sensitivity. A large number of antigens must be tested against the sera; WHO has recommended 19 serovars or 16 serogroups. The serovar that demonstrates the highest titer in MAT is considered to be the infecting serovar (serogroup), but the test has been shown to be an inaccurate predictor in this regard.[245,430] Sera may be screened using commercially available serogroup-specific leptospiral antigens in a macroscopic agglutination test, but positive reactions should be confirmed by the MAT in a reference laboratory.



■ **FIGURE 20-19** Darkfield examination of *Leptospira interrogans* culture. The spiral coils, which are not clearly demonstrated, appear as alternating bright and dark areas. The very bright upper end of the organism may represent the hooked end that many leptospira possess. It is easy for inexperienced observers to confuse cell debris and artifacts with leptospira in clinical specimens. ×1000. (Photomicrograph courtesy of David Miller, DVM, MS.)

A titer of ≥1:100 in the MAT test is usually considered evidence of past infection. The CDC has suggested a titer of >1:200 in a single serum specimen by the MAT test as presumptive evidence of leptospirosis in a patient with a compatible clinical illness, but in endemic areas other experts have suggested a titer of 1:800 or 1:1600 as better cutoffs. Titers may reach extremely high levels and take months or years to decrease, so the height of the titer cannot be used for determination of recent infection.[380,381]

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Other serology tests. Other antibody detection tests have been developed that

enable rapid screening but similar to MAT they have suboptimal sensitivity during the early stages of infection, and some of these assays may also have poor accuracy in areas with endemic leptospirosis.[21,127,243,246,247,428,429,489] A comprehensive discussion of other serologic test evaluations has been published.[243,473] Techniques such as latex agglutination,[413,428] dipstick,[174,409,427,506] enzyme immunoassays,[21,127,246] indirect hemagglutination (IHA),[243,247] and lateral flow assays[413,429] have been reported, but some of these tests have not been extensively evaluated. These assays contain lipopolysaccharide (LPS) antigens from nonpathogenic *Leptospira biflexa* serovar Patoc,[283] and detect IgM and/or IgG antibodies against LPS from all pathogenic serovars due to the cross-reactivity with Patoc LPS. An IHA developed by the CDC is approved by the US FDA. This IHA uses sensitized red blood cells to detect both IgM and IgG antibodies, and initially was shown to have a sensitivity of 92% and a specificity of 95% compared to the MAT.[463] In more recent studies, however, the IHA was positive in only 44% of acute-phase sera taken approximately 5 days after symptom onset.[249]Hull-Jackson and colleagues[201] more recently evaluated a commercial latex agglutination test using a well-characterized sera bank that included 40 patients with leptospirosis, as well as doing a prospective study of consecutive patients admitted to hospital with an acute febrile illness. Although the sensitivity of the latex agglutination test was similar in the two groups of patients, the specificity was considerably less using the well-characterized sera bank (81%) versus during the prospective study (98%). This study demonstrates that newer tests for leptospirosis need to be evaluated and validated in different populations before being implemented for routine use.

Patients with leptospirosis also produce antibodies to several other protein antigens, some of which are present during the acute disease phase.[116,173] Identification of one or more antigens has led to the development of recombinant protein-based diagnostic assays for leptospirosis, but the performance of rLipL41- and rOmoL1-based EIA assays has shown variable sensitivity.[115,151,310] *Leptospira*-immunoglobulin-like (Lig) proteins (i.e., outer membrane proteins) have been shown to be better at diagnosing early disease using an immunoblot assay than either whole *Leptospira*-based assays or recombinant assays.[98] Other antigens that have recently been used in recombinant-based diagnostic assays include Lp29, MPL17, MPL21, and LipL21 in EIAs,[315,329] LipL32 in a dipstick assay,[42] and Lip41 in both a latex agglutination and flow-through immunoassay.[413] Improved, rapid diagnostic tests for leptospirosis are needed in endemic areas that are able to detect acute-phase disease. Identification and use of other immunogenic protein antigens that are conserved among pathogenic leptospires will enable the development and testing of an expanding number of rapid tests for this purpose.

Molecular Detection. Various genes have been the target of leptospiral DNA amplification by PCR, particularly for the diagnosis of acute-phase disease. Although PCR has been used for research, few methods were evaluated for diagnostic use.[50,51,170,292]Merien and colleagues[292] amplified a 331-bp fragment of the *rrs* (16S rRNA) gene of both pathogenic and nonpathogenic leptospires. Two primer sets, G1/G2 for the *sec*Y gene and B64I/64II for the flagellin gene are included in the WHO manual.[498] The G1/G2 primers do not amplify all pathogenic serovars necessitating use of a multiplex assay.[170] These two primer sets have been used clinically to detect leptospiral DNA in the serum, urine, aqueous humor, CSF, and tissues at autopsy of patients with leptospirosis.[243,473] More recently, several targets (i.e., *rrs*, *lig*A and B, *lip*32, *sec*Y, and genomic locus LA0322 in serovar Lai) have been used to develop real-time PCR assay using both hydrolysis (i.e., TaqMan) probes and SYBR green fluorescence.[248,422,458,473]

Rat-Bite Fever (Sodoku)

RBF is a bacterial zoonosis that occurs after being bitten by a rat and inoculated with one of two organisms in the animal's oral flora. This term is used to describe two distinct diseases with different microbial etiologies. Cases in North America are caused by *Streptobacillus moniliformis*, a highly pleomorphic, filamentous, gram-negative, nonmotile, and non–acid-fast rod, while most cases in Asia are caused by *Spirillum minus*, an oral spirochete. This section describes RBF due to the latter, or sodoku the name used for this disease in Asia.

Spirillum minus

S. minus is a gram-negative spirochete that is spiral-shaped and measures 0.2 to 0.5 mm wide and 1.7 to 5 mm long. The bacterium is actively motile by bipolar bundles of flagella and the six spirals that give the organism its coiled appearance. *S. minus* was first described as the cause of RBF in 1916, 30 years after spiral bacteria called "*Spirillum minor*" were found in wet mounts of wild rat blood.[157] Several other names were also given to this organism in the older literature prior to it being named *S. minus* in 1924.[157]

RBF due to *S. minus* is an infection that more commonly occurs in Asia, but rarely occurs in the United States. The frequency of infection relates to the amount of contact between rats, other rodents, and humans. Although overall a rare infection, RBF is still a threat when there is rodent exposure. A few cases have also occurred in children and adolescents in contact with wild field mice, or in people who have gerbils

as pets.[157] Rats are also being kept as pets such that pet shop employees and rodent owners may also be at risk for acquiring RBF.[328] Human-to-human transmission has not been documented. One to four weeks after the bite a systemic illness occurs, characterized by chills, headache, vomiting, and fever. The site of the bite, which had healed, ulcerates to form a chancre-like lesion, and is accompanied by regional lymphadenitis and a blotchy maculopapular rash that may affect the palms and soles. In contrast to the streptobacillary form of RBF, polyarthritis rarely occurs with sodoku. Relapsing fever with a periodicity of every 2 to 3 days may occur for weeks, months, or years in untreated patients. Complications include endocarditis, myocarditis, meningitis, nephritis, hepatitis, and splenomegaly.[157]

Spirillum minus cannot be cultured on artificial media; so little is known about the phenotypic or genotypic characteristics of this organism. Animal inoculation remains the only way to recover the organism, and no specific serologic tests

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are available. False-positive serologic tests for syphilis occur in 50% of patients with RBF. The spirochetes may be visualized in blood, exudates, or tissue using Giemsa's or Wright's stain or darkfield microscopy.

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CHAPTER 21

Mycology

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Species That Produce True Hyphae

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Serologic Diagnosis of Fungal Diseases

Introduction

The diagnosis of a fungal infection requires the cooperative efforts of the clinician, the anatomic pathologist, and the microbiologist. The clinical team is responsible for recognizing the signs and symptoms of fungal infections and for the appropriate collection and transport of specimens to the laboratory. It is important for the clinicians to communicate their clinical considerations to the microbiologist, when certain types of fungal infection are suspected for which special procedures may be needed. For example, when zygomycosis is suspected, then more delicate specimen processing techniques will be used. The surgical pathologist and cytopathologist must be alert to the tissue reactions that suggest a fungal infection and must be able to recognize fungal elements in stained tissue sections. The mycologist is responsible for implementing the laboratory techniques for optimal detection and recovery of fungi in culture; for making an accurate identification; and, if necessary, for performing antifungal susceptibility testing.

Patients at Risk for Fungal Infections

Following are the individuals who are immunosuppressed:

- Patients with advanced HIV infection.
- Organ transplant recipients, particularly during the posttransplantation period of immunosuppression.[201]
- Patients with malignant neoplasms, particularly those with leukemia and lymphoma, or during periods of chemotherapy.
- Patients with a variety of debilitating immunologic and metabolic disorders, including systemic lupus erythematosus (SLE), and other collagen vascular diseases, diabetes mellitus, dysgammaglobulinemia, and alcohol or IV drug abuse.
- Recipients of corticosteroids, cytotoxic agents, prolonged antibiotic therapy, and newer modulators of the immune system (e.g., tumor necrosis—α inhibitors).

Travelers to or inhabitants of regions of the world known to be endemic for fungal infections are also at risk. Participants in activities or occupations that bring them in direct contact with infected animals, humans, and/or contaminated materials are at risk for fungal infections.

General Signs and Symptoms Suggesting Fungal Infection

The early presenting symptoms leading to the suspicion of a fungal infection commonly are atypical, vague, and nonspecific, reducing the chance for making the correct clinical diagnosis. Low-grade fever, night sweats, weight loss, lassitude, easy fatigability, cough, and chest pain are common presenting symptoms. Deep-seated or disseminated fungal diseases may mimic other infections, such as tuberculosis, brucellosis, syphilis, sarcoidosis, and metastatic cancer. Careful examination of the skin and mucous membranes should always be performed, as systemic fungal infections often present with mucocutaneous lesions.

- Ulcerating lesions in the intestine, larynx, pharynx, genitals, and tongue may complicate disseminated histoplasmosis in up to 50% of patients.
- Patients with blastomycosis may initially present with verrucous or pustular lesions of the skin or ulcerating granulomas of mucous membranes, including the larynx,[72] the esophagus, and the oral cavity, including the tongue.[210]
- Coccidioidomycosis in patients with profound immunosuppression may present as papular or macular skin lesions, or as eosinophilic, basilar meningitis.[227]

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The signs, symptoms, and probable agents of pulmonary mycoses are listed in Table 21-1. The signs, symptoms, and probable agents of extrapulmonary mycoses are summarized in Table 21-2.

Nonspecific laboratory findings such as accelerated erythrocyte sedimentation rate, increase in C-reactive protein, elevations in gamma globulin, or low-grade and persistent elevations in the peripheral blood neutrophils and/or monocytes may provide initial clues to the presence of a fungal infection.

Clinical Categorization of Fungal Infections

Fungal infections are often characterized by the location of the infection. These may be referred to as superficial, subcutaneous, or systemic, which is also referred to as deep-seated. Although these broad categorizations remain useful, it is important to recognize that in the context of profound immunosuppression, fungi that are commonly superficial may cause subcutaneous infection and those that are subcutaneous may disseminate. In addition, fungi that are uncommon pathogens or even usual contaminants may cause disease in the absence of an immune response. The following terms allow for better communication between clinicians and mycologists.

The terms *deep-seated* and *systemic* classically refer to a group of fungal infections caused by agents that inherently can be highly virulent, that can invade deeply into tissues and organs, and that have the capability of spreading widely throughout the body. Classically, these terms are often used to refer to infections caused by the dimorphic fungi. These are fungi that exist in the form of mold in the environment (i.e., at room temperature incubation) and exist as yeasts or a yeast-like fungus when in the body. All of the dimorphic fungi, except *Coccidioides,* are thermally dimorphic, which means the yeast or yeast-like form can be demonstrated by incubating the cultured fungus at 35°C to 37°C (i.e., body temperature). Currently, because of the increase in the number of individuals who are immunosuppressed, many fungi, which were formerly considered only as "saprobes" or "contaminants," are now agents of systemic disease. One prime example is the hyaline mold *Penicillium marneffei*, which causes a disseminated reticuloendothelial infection in patients with AIDS in Southeast Asia that resembles histoplasmosis clinically and pathologically.

The term *opportunistic* is now used to describe fungi that may be present in the environment or as part of our commensal microbiota that do not normally cause infection, but are able to infect when conditions change (i.e., the opportunity arises). These are fungi, usually of inherently low or limited virulence. Conditions that favor opportunistic infection include changes in the mucosal microbiota (e.g., antibiotics suppress bacteria and promote candidiasis); immunologic suppression (e.g., invasive zygomycosis in patients with poorly controlled diabetes mellitus); foreign material (e.g., *Candida* infections of indwelling intravascular catheters); and physical (e.g., sporotrichosis following inoculation trauma).

TABLE 21-1 Signs and Symptoms of Pulmonary Mycoses

Type of Infection	Signs and Symptoms
General	A transient influenza-like syndrome or pneumonia that localizes in one lobe or spreads to other lobes. Cough, minimal sputum production, dyspnea, tachypnea, hemoptysis. Chest pain, frequently pleuritic in nature. Rales or rhonchi and a pleural friction rub may be detected on auscultation. Roentgenography of the chest may reveal small pulmonary infiltrates and hilar adenopathy or more diffuse and confluent opacities.
Allergic bronchopulmonary	Symptoms characteristic of asthma: nonproductive cough, wheezing, and tightness in the chest. Episodic bronchospasm. Segmental atelectasis caused by plugging of bronchioles by mucus. Charcot-Leyden crystals and eosinophils in sputum; peripheral blood

	eosinophilia. Cutaneous hypersensitivity reaction to antigens of *Aspergillus* spp. Elevated serum IgE concentration and IgG anti- *Aspergillus* antibodies.
Fungus ball	Growth of fungus colony within a preexisting cavity. Hemoptysis, despite little or no invasion of cavity wall. Dissemination is rare, even in patients receiving corticosteroids.
Invasive	Symptoms of acute pneumonia. Low-grade, undulant fever. Cough may be productive or nonproductive; chest pain usually present. Progressive dyspnea and shortness of breath. Hemoptysis may indicate infarction and parenchymal necrosis. Chest radiography may reveal a diffuse infiltrate emanating from the hilum, finely nodular fibrosis, multifocal abscesses, or cavitation, depending on the fungus species. Clues to specific agents: "Millet seed" fibrosis: histoplasmosis Peripheral coin lesion: coccidioidomycosis Cavitary lesions: histoplasmosis, aspergillosis and other causes of hyalohyphomycosis, or coccidioidomycosis

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TABLE 21-2 Signs, Symptoms, and Probable Agents of Extrapulmonary Mycoses

Type of Infection	Signs and Symptoms
Cutaneous	Superficial scaling lesions—varying in size, shape, and color—of the thorax or back: tinea versicolor secondary to *Malassezia furfur* infection.
	Itching, scaling lesions known as tinea or ringworm: dermatophytosis
	Thickened, crusting, hyperkeratotic, exophytic fungoid affliction known as favus: *Trichophyton tonsurans, T. violaceum,* and *T. schoenleinii*
	Scaling or crusting lesions confined to the moist intertriginous areas of skin suggest yeast infections: *Candida albicans*.
	Primary subcutaneous pustular infection at the site of inoculation, with

	proximal spread and evolution of secondary skin ulcers along the course of the lymphatics: *Sporothrix schenckii* complex
	Nonhealing pustules, ulcers, or draining sinuses: disseminated dimorphic fungal diseases and mycetomas secondary to a variety of fungal agents.
	Purpuric lesions and subcutaneous cysts: phaeohyphomycosis.
	Fungating, discolored, hemorrhagic lesions: chromoblastomycosis.
CNS	Insidious onset of headaches that increase in frequency and severity, accompanied by nausea, irritability, and clumsiness; cryptococcosis. Meningitis and meningoencephalitis: zygomycosis, particularly in diabetics. Brain abscesses: *Cladophialophora bantiana*, other dematiaceous fungi; *Aspergillus* species.
Urinary tract	Pyelitis and pyelonephritis associated with long-term administration of antibiotics, corticosteroids, immunosuppressants, antineoplastic drugs, and prolonged insertion of indwelling catheters for urinary drainage, particularly in elderly women: candidiasis.
	Limited pyuria, lower abdominal pain, frequency of urination: nonbacterial cystitis in middle-aged women: *Candida glabrata*.
Ocular infections	Conjunctivitis, corneal infections, and keratoconjunctivitis: *Fusarium* spp., *Aspergillus* spp., *Cladosporium/Cladophialophora* spp., *Acremonium* spp., *Bipolaris* and others. Intraocular infections, usually following trauma or eye surgery: *Candida albicans*, *Aspergillus* spp., or *Zygomycetes* spp.
Endocarditis	Low-grade fever, cardiac murmurs, positive echocardiogram: *Candida* spp., *Aspergillus* spp., *Paecilomyces* spp. and others.
Sinusitis	Facial pain and cutaneous hyperemia, headache, low-grade fever, radiographic evidence of the filling of a sinus or air-fluid levels: *Aspergillus fumigatus*, *Alternaria* spp., *Pseudallescheria boydii* complex, and others.

Aspergillus species, *Candida* species, and members of the Zygomyces (i.e., agents of mucormycosis) are the three groups of fungi that cause the majority of opportunistic infections. However, as noted earlier, any fungus is a potential pathogen

in the profoundly immunocompromised host. This includes various genera of less commonly encountered hyaline and darkly pigmented (i.e., dematiaceous) molds that formerly were considered to be contaminants.

The general term for invasive infections caused by hyaline molds, which is usually used prior to the identification of the etiologic agent, is *hyalohyphomycosis*, whereas the general term for invasive infections caused by dematiaceous fungi is *phaeohyphomycosis*.[5,6] When the specific etiologic agent is known, then specific terminology of infection may be employed, such as aspergillosis or fusariosis. One could also state that the disease in a particular patient was a hyalohyphomycosis caused by *Pseudallescheria,* for example. The most important point to all this terminology is one of communication. This is because if the type of etiologic agent is not known, then more broad-spectrum antifungal agents will be used, whereas if the specific etiologic agent is known, then the possibility to tailor therapy may exist.

Common Mycologic Terms

A few additional terms warrant definition. The term *mycology* itself is derived from the Greek word *mykes*, a direct counterpart of the Latin word *fungus*, which is in turn thought to be a modification of the Greek word *sponges*, from which our word "sponge" is derived. There remains controversy around members of what has been traditionally referred to as *Zygomycetes*. Taxonomically, these are being

reassigned from the phylum *Zygomycota* to the phylum *Glomeromycota*, which is problematic, since the terms *zygomycosis* and *mucormycosis* are so ingrained in medical practices and in the medical literature for disease caused by these fungi. Clinicians would not know what glomeromycosis would represent if this term was universally used tomorrow. Many have continued to use zygomycosis, until the newer terminology becomes more well recognized. This is acceptable, since the reporting objective of the medical mycologist is to communicate to the physician the cause of disease so that appropriate therapy may be given. Finally, with respect to the *Zygomycetes*, the term *aseptate hyphae* has also become quite ingrained in medical practice and literature, although this is not technically correct, since rare septations may occur. Therefore, the term pauciseptate (i.e., few septations) or mucoraceous hyphae is preferred. This is important, since the failure to recognize zygomycete hyphae, because of the presence of rare septations, could lead to an inappropriate therapeutic decision, since *Zygomycetes* are innately resistant to voriconazole. Formerly, it was common to refer the fungus that demonstrated only asexual reproductive structures by the **anamorph** name, whereas if sexual reproductive structures were present, then the **teleomorph** nomenclature may be used. This, too,

was terribly confusing to our clinical colleagues and could contribute to errors in medicine. Fortunately, with the widespread genomic analysis of most of these organisms, it is now best practice to refer to the fungus by a single name. This was a true contribution of molecular methods to medical mycology. The continued application of molecular techniques should better define the true taxonomic position of fungi of medical importance, and hopefully will not be associated with too much reclassification, which often causes confusion.

Fungi are contained within their own kingdom, fungi, because of the characteristics unique to this group, as well as the lacking of characteristics present in other organisms. Fungi are **eukaryotic** (i.e., they possess a membrane-bound nucleus, as well as organelles, such as endoplasmic reticulum, Golgi apparatus, and mitochondria). They also possess a rigid cell wall composed of chitin (*N*-acetyl-D-glucosamine linked by β1-4 glycoside bonds) and mannans (polymers of glucose in α or β glycoside bonds). These cell wall constituents adsorb several dyes, which provide for the application of special stains by which they can be identified in laboratory mounts and tissue sections.

Fungi that exist predominantly as single cells, especially when in a liquid environment, and reproduce by budding are known as **yeasts**. Those with multiple cells forming a filamentous mycelium, which are more adapted to growing on or into a solid substrate, are known as **molds**. Fungi reproduce through the production of **spores**, which may be either sexually or asexually derived. The spores most commonly encountered in the laboratory are asexually derived and are referred to as **conidia**. There are a variety of different types of spores or conidia. These may be derived directly from the vegetative mycelium, such as **arthrospores, chlamydospores,** and **blastospores**, or special fruiting bodies (**conidiophores**). The morphology, arrangement, and mode of derivation of spores serve as important criteria by which genus and species identifications can sometimes be made. Additional terms used in the laboratory workup of fungi are presented in Box 21-1.

BOX 21-1

Terms Useful in the Examination of Fungi

The fundamental microscopic unit of a mold is the threadlike structure called a *hyphum.* Several hyphae combine to form the matt of growth known as the *mycelium.* Hyphae that are subdivided into individual cells by relatively abundant transverse walls or septae are called *septate;* those with very rare septations (i.e., the *Zygomycetes*) are *pauciseptate.* Sometimes in an individual preparation no septations are seen, and so the hyphae have been described as *aseptate,* when in fact they are *pauciseptate.* Pseudohyphae form from elongation of budding yeast cells (i.e., *blastoconidia*), and

show sausage-like constrictions between segments (Fig. 21-1).

The portion of the mycelium that extends into the substratum of the culture medium, and is responsible for absorbing water and nutrients, is the *vegetative mycelium*; the portion that projects above the substrate is the *aerial mycelium,* also called the *reproductive mycelium,* since special spore or conidia-bearing fruiting bodies (i.e., *conidiophores*) derive from this portion of the mycelium.

The traditional identification and classification of fungi are based primarily on the morphologic differences in reproductive structures and the manner in which spores or conidia are formed from specialized cells called *conidiogenous cells.*

Three general types of reproduction are commonly observed in the fungal species of medical importance, *vegetative* sporulation, *aerial* sporulation, and *sexual* sporulation.

Vegetative Reproduction

Three types of spores or conidia may form directly from the vegetative mycelium: *blastoconidia, chlamydoconidia*, and *arthroconidia*. The term *spore* should technically be reserved for reproductive elements that arise from meiosis (i.e., sexual reproduction), such as ascospores, oospores, or zygospores, whereas those that are derived from mitosis (i.e., asexual reproduction) are termed *conidia.* The term *endospore* is also used for asexually derived structures produced within the spherule of a *Coccidioides* species.

Blastoconidia are the familiar budding forms characteristically produced by yeasts. A bud scar (dysjunctor) often remains at the point at which the conidium becomes detached.

Chlamydoconidia (chlamydospores) are formed from preexistent cells in the hyphae, which become thickened and often enlarged. Although *chlamydoconidia* is the correct terminology, the term *chlamydospores* is also commonly used. Chlamydospores may be found within (*intercalary*), along the side (*sessile*), or at the tip (*terminal*) of the hyphae. This type of conidiation is characteristic of *C. albicans*.

Arthroconidia also are formed from preexisting cells in the hyphae, which become enlarged and thickened. This type of conidiation is characteristic of the mold form of *Coccidioides immitis* and *Geotrichum* species, among others.

Aerial Reproduction

Emanating from the hyphae and extending from the mycelial surface are specialized fruiting bodies that give rise to a variety of conidia. Fruiting bodies may form closed sacs called *sporangia,* within which spores called *sporangiospores* are produced (Note: these are termed sporangio *spores* even though they are asexually derived, which is a deviation from the rule described above). The specialized hyphal segment that supports the sporangium is called the *sporangiophore* (the suffix "phore" [Greek

phoros] means "bearing") (Fig. 21-2). This type of sporulation is characteristic of the *Zygomycetes*. Many other fungi produce elaborate fruiting bodies that give rise to conidia, which is derived from a word meaning dust. The specialized hyphal segment that supports a conidia-bearing fruiting head is called a *conidiophore* (Fig. 21-2).

The conidiophore may produce cells called *metulae,* which in turn produce conidia-producing segments, called *phialides.* This property of branching into metulae and phialides is characteristic of the fingerlike fruiting body of *Penicillium* species. Phialides by definition are conidiogenous cells that produce conidia from a locus inside its apex, which does not increase in width or length during conidiogenesis. This is in contrast to *annellide* formation, in which the tip of the conidiogenous cell cyclically extends and retracts when conidia are formed, leaving a succession of scars or rings. The conidia of *Scopulariopsis* spp. and *Exophiala* spp., for example, are *annelloconidia;* that is, form in basipetal sequence in which the conidiogenous part of each conidium elongates at the time a new conidium is formed, and then contracts to form a *ringlike scar, annulus,* or *collarette* at the truncated base. Conidia may be borne singly, in long chains (*catenulate*), or in tightly bound clusters. *Acropetal* is a term used to describe the process of chain formation in which each new conidium is derived in sequence from the previous one, so that the youngest cell is at the tip (e.g., *Penicillium* spp.). In contrast, *basipetal sporulation,* in which each new conidium forms at the base of the chain, pushing all other conidia in the chain ahead, so that the oldest cell is at the tip (e.g., *Paecilomyces* species).

Tiny one-celled conidia, usually borne either directly from the sides of the hyphae or supported by a hairlike conidiophore, are called *microconidia,* in contrast to the much larger, multicelled *macroconidia,* which assume a variety of sizes and shapes. A multicelled macroconidia that is divided by both transverse and longitudinal septations, giving a mosaic appearance, is a *dictyospore,* more commonly referred to as *muriform* (resembling a stone wall). The term *aleuriospore* refers to a conidium, usually a macroconidium that is attached to the hyphae by a supporting cell that fractures when the conidium is released (e.g., *Microsporum canis*). The detection of single-celled aleurospore produced by the submerged vegetative hyphae is important for the identification of *Aspergillus terreus.*

Sexual Sporulation

Sexual sporulation requires the merging and nuclear recombination of two specialized haploid, fertile cells (i.e., sex cells derived from meiosis). If the reproductive cells formed by fusion of morphologically identical cells, often from the same hyphae (homothallic), the spore is called a *zygospore,* characteristic of the *Zygomycetes*. If the fusing reproductive cells are derived from two different cells, often derived from separate hyphal segments, the resulting spore is called an *oospore* (Fig. 21-3).

The sexual spores of several members of the class *Ascomycetes,* previously mentioned, which are of medical importance, are called *ascospores.* For example, certain strains of *Aspergillus* species, notably of the *A. nidulans* and *A. glaucus* groups, produce large closed, baglike structures called *cleistothecia*, which in turn

contain smaller baglike structures called *asci* (Fig. 21-3). Within each ascus are four ascospores, the product of meiotic division. Medically important fungi, other than *Aspergillus* species, in which sexual sporulation may be observed include *Saccharomyces* spp. and members of the *Pseudallescheria boydii* complex (Fig. 21-3).

The sexual form of a fungus is known as a *teleomorph,* in contrast to the term *anamorph,* which refers to the various asexual reproductive forms or structures produced by an imperfect fungus (such as phialides, annellides, branching chains, etc.). The traditional approach of assigning different anamorphic and teleomorphic names, when both the asexual and sexual states are known is being changed, since it is now possible to definitively assign a fungus a genus and species based on genetic analysis. Therefore, the nomenclature in mycology will, hopefully, be less confusing in the future.

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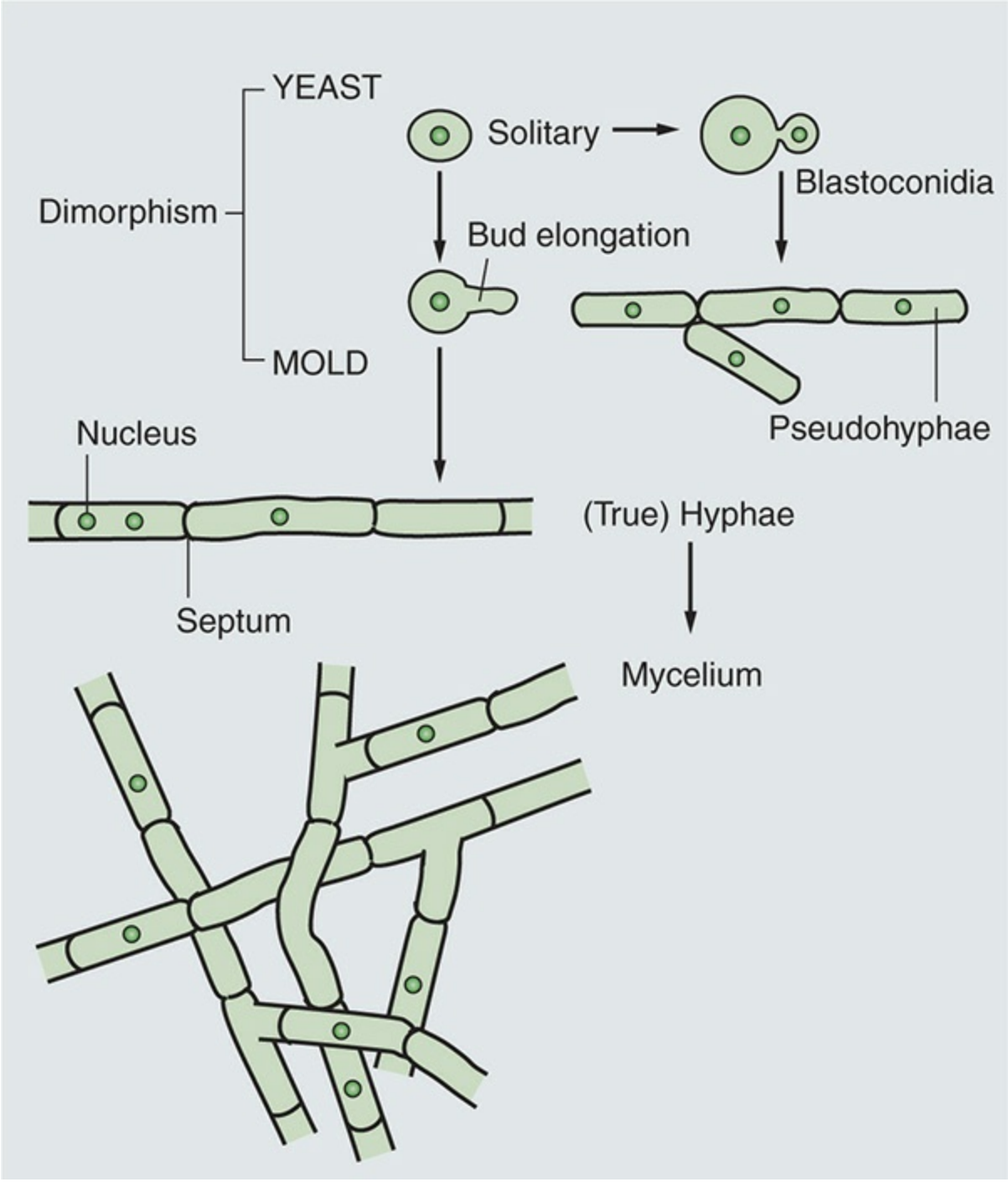
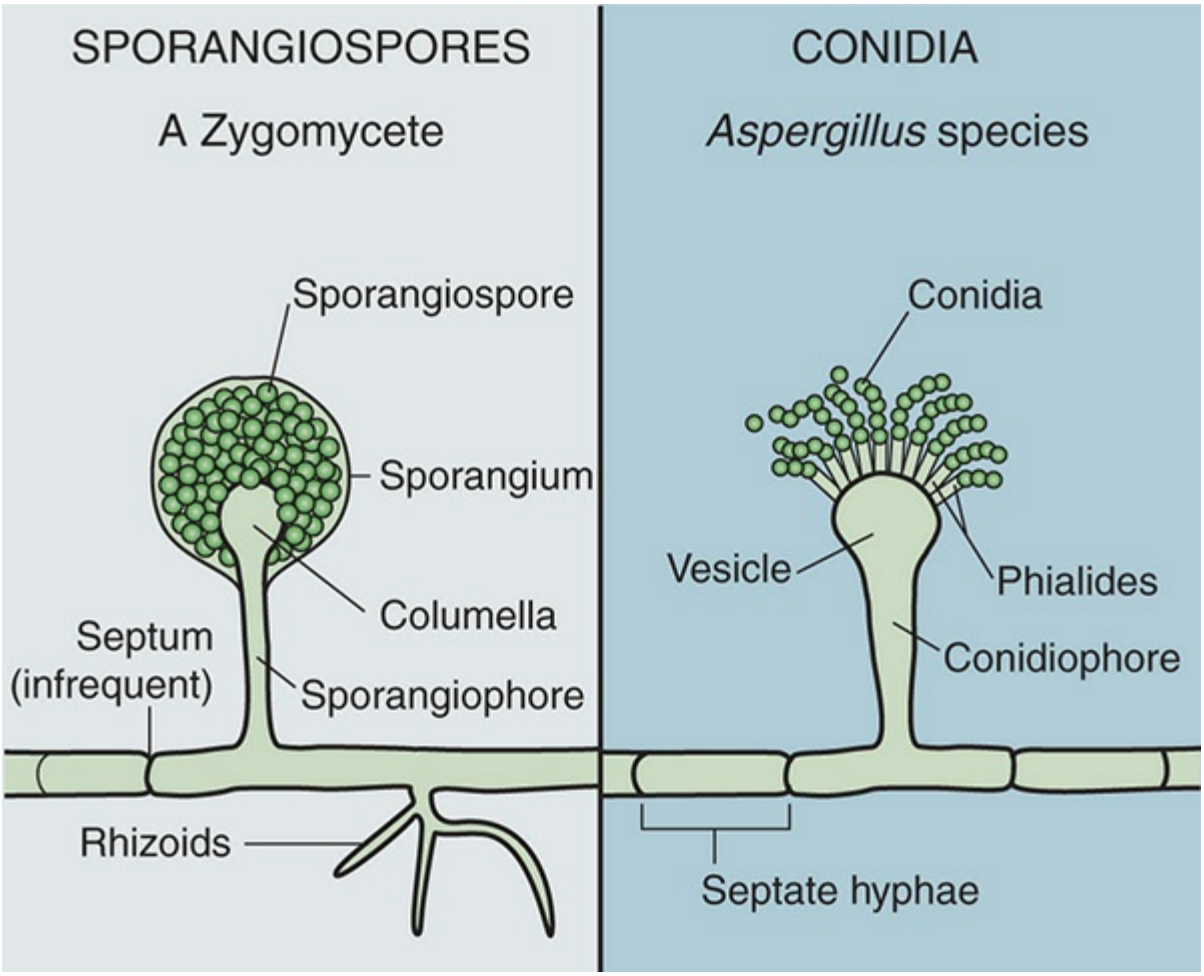


■ **FIGURE 21-1** Sketches illustrating basic fungal structures.

Laboratory Approach to the Diagnosis of Fungal Infections

Once a fungus is recognized in culture, a presumptive-to-definitive identification is made based on a visual examination of the colony morphology, in conjunction with microscopic observations of a direct mount preparation. If only a presumptive identification is possible, consultation with the primary physician may be warranted to determine whether full genus or species identification is needed.



■ **FIGURE 21-2** Sketches illustrating complex asexual reproductive structures of a zygomycete and *Aspergillus*.

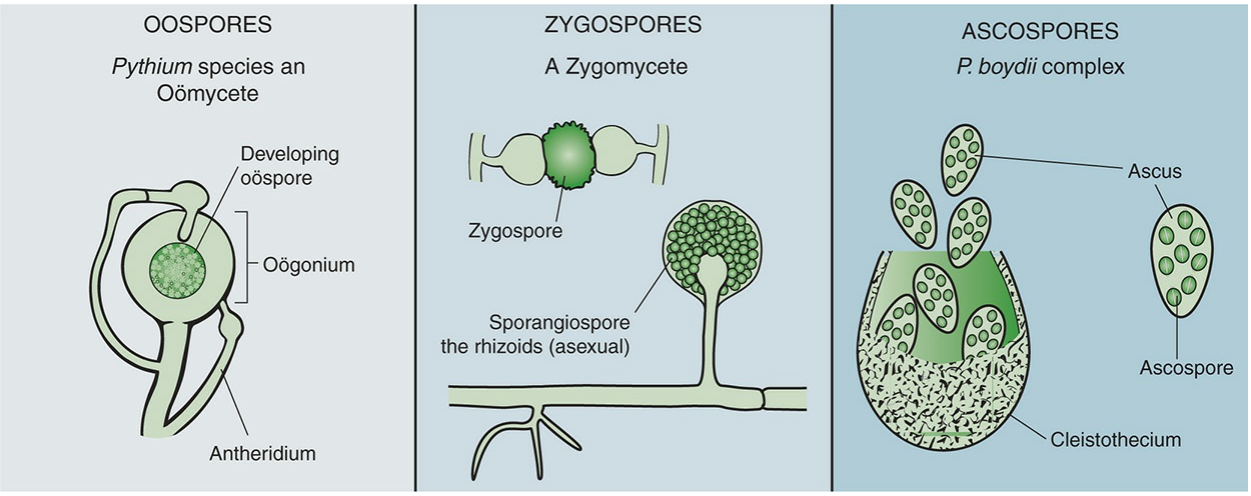
Although most fungal identifications are based primarily on the assessment of colony morphology and microscopic features, key biochemical tests may be required to differentiate closely related genera or species. Nucleic acid probe assays are available for the definitive identification of the dimorphic pathogenic fungi that are commonly encountered in North America. Serologic studies and antigen-detection tests are available for some fungi; these are used in some instances to aid in the diagnosis, assess the status of a previously diagnosed mycosis, or determine the efficacy of

therapy. Guidelines, therefore, will be set forth in this chapter to assist the mycologist in performing these various tasks, expanding from the laboratory observations to include assessments of the clinical parameters and the histopathologic criteria that must be interwoven to make a definitive diagnosis. Additionally, there is CLSI guidance on this topic: *Principles and Procedures for Detection of Fungi in Clinical Specimens—Direct Examination and Culture; Approved Guideline (M54-A).*[49]

The final laboratory identification of a fungal isolate, based on colonial and microscopic features, will be covered later in this chapter. The analytic phase begins with the performance of the direct examination, which may provide the first definitive evidence of a fungal infection. After this, the culture is reviewed for growth. Procedures must also be in place to provide guidelines for the workup of an unknown isolate, which should include the appropriate selection of differential tests, when needed, to make a genus/species identification. The paragraphs that follow detail these steps.

When appropriate, sections will also be included to briefly describe nonculture methods for establishing the identity of a given fungal species, using antibody, antigen, and molecular techniques. Nucleic acid sequencing is an option for difficult-to-identify fungi, particularly those that fail to sporulate. Prior to sending these for sequence-based identification, it is prudent to assure that they are actually thought to be responsible for the disease state of the patient. They are most commonly offered in academic settings and reference laboratories.[116] At this juncture, because of the technical expertise requirements and the cost of instrumentation and supplies, this technology will not be implemented in smaller laboratories, in which only basic practices are performed. However, more recently introduced mass spectrometry–based identification is becoming routine for the cost-effective identification in many laboratories. These methods have been demonstrated to perform well in the identification of yeasts, and protocols for the filamentous fungi are being worked out.

Likely, the most widely used, commercially available system for sequence-based identification of fungi has been the Applied Biosystems (Foster City, CA) MicroSeq D2 large-subunit rDNA fungal sequencing kit. A study of the use of nucleic acid sequencing for the identification of yeasts showed that 98% of 19 different species of *Candida* species seen in the clinical laboratory were correctly identified compared with identification by phenotypic methods.[115] DNA sequencing identified 32 isolates of yeasts belonging to nine genera, including *Trichosporon*, *Cryptococcus,* and others. Approximately 81% were correctly identified, and the primary reason for most incorrect identifications resulted from



■ **FIGURE 21-3** Sketches illustrating the structures of sexual reproduction.

a lack of data entries in the library containing sequences for many yeasts. Many of these issues have been addressed since these early studies. It is important to recognize that the quality of the identification is largely dependent on the quality of the sequence database, since the generation of high-quality DNA sequence is usually no longer a technical challenge. Other options include the use of public databases; although informative, these are not adequately curated, and so individuals who take this approach should review the quality of the sequence, the credentials of the laboratory that submitted the sequence, and use this information in conjunction with phenotypic features, whenever possible. SmartGene (www.smartgene.com) provides fee-for-service access to their curated database, which represents another option.

The identification of filamentous fungi has proven more challenging than the identification of yeasts. In another study performed at the Mayo Clinic to determine the usefulness of sequencing in identifying molds, nucleic acid sequencing identified 234 isolates (67.5%) to the correct genus or species level.[116] Incorrect identifications were primarily due to an incomplete list of sequences in the database. Each laboratory can construct a custom database of sequences or purchase access to curated databases, greatly enhancing the accuracy of performance. This technology offers great promise of simplifying the identification of yeasts and molds recovered from clinical specimens as well as those important to industry and agriculture. The Clinical Laboratory Standards Institute (CLSI) has published *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline (MM18-A),* which is a must for the benchtop of those working in this area. How much DNA sequence–based identification will be used in the routine laboratory remains to be seen, but it will likely remain the means by which fungi are definitively characterized.

MALDI-TOF is a method of mass spectrometry, which has been introduced into

many laboratories in the form of one of two FDA-approved instruments. These both have FDA-approved libraries for the identification of yeasts, but no libraries are yet approved for filamentous fungi. As with bacteria, this approach, for the most part, is fast, accurate, and inexpensive. The traditional approach to yeast identification (i.e., biochemicals, assimilations, and cornmeal agar morphology) is already changing in laboratories that have validated and implemented this technology. Westblade et al. evaluated a collection of 852 medically important yeasts by MALDI-TOF mass spectrometry and reported that 96.6% were identified to the genus level and 96.1% were identified to the species level. DNA sequencing of the D2 region was used as the comparative gold standard.[287] Others have reported similar findings, which demonstrate the usefulness of this technology. Applications for the filamentous fungi are being developed.

Specimen Collection and Transport

There are many preanalytic parameters that are important for the optimal recovery of fungi from clinical specimens. A lapse in any of these may result in a failure or delay of recovery of the pathogenic agent, which, in turn, could result in untoward clinical consequences. Preanalytic parameters of importance begin with only culturing those patients in whom mycoses are suspected. Once this is determined, proper collection of the specimen is paramount. The use of swabs is inappropriate for the detection of infections caused by filamentous fungi due to inadequate sampling and an inability to disassociate the hyphal elements from the human tissues in which they are invading. Next to be considered is suboptimal specimen transport and/or processing, which may result in a loss of viability. Specimens should be delivered to the laboratory promptly. Specimens that are not processed immediately should be held at room temperature. *Cryptococcus neoformans*, *Histoplasma capsulatum,* and *Blastomyces dermatitidis* do not survive well in frozen or iced specimens. Several fungal species, including the above-named species, and *Aspergillus* spp. can be recovered from samples that have been in transit for as long as 16 days if not allowed to dry out; therefore, an attempt should always be made to recover fungi even if processing is delayed.[244]

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Sealed, sterile transport containers should be used for all liquid or moist specimens. Skin scrapings, nail fragments, and hairs can be transported dry in an envelope, Petri dish, or other convenient conveyance. To curtail the growth of commensal bacteria possibly present in nonsterile specimens that may be delayed in processing or mailed to a reference laboratory, penicillin (20 U/mL), streptomycin (100,000 mg/mL), or chloramphenicol (0.2 mg/mL) can be added to the specimen. Directions for the proper packaging and labeling of specimens for shipping and mailing are discussed in Chapter

2.

Physicians, nurses, ward personnel, and laboratory technologists must work together in developing protocols that ensure the proper collection and prompt transport of specimens submitted for fungus culture. The selection of appropriate collection devices and transport containers, affixing labels that include pertinent patient information, and establishing a means of communication for special requests are possibly the most important considerations in ensuring the accurate diagnosis of fungal infections.

General guidelines for the collection and transport of specimens for culture are given in Chapter 2. Guidelines for the collection of various types of specimens are presented in Table 21-3. The criteria by which a specimen is judged unacceptable for culture and rejected should be written in the laboratory procedure manual. This information should also be noted in the test directory for the laboratory and communicated to clinicians. In Box 21-2, select rejection criteria and the actions to be taken for handling unacceptable specimens are presented. These criteria must also be understood by physicians, nurses, and other personnel who are assigned to collect the specimen, as not only will time and money be saved in the mycology laboratory, but an unacceptable delay in establishing the diagnosis can be avoided. Individual criteria must be established within each practice setting to accommodate practice in the local setting. With experience, the individual collecting the specimen can make a preliminary assessment of the material obtained and immediately collect a second specimen, if necessary.

Specimen Processing

Then, following the prompt transport of the specimen and receipt in the laboratory, it is important that specimens are inoculated onto the appropriate primary fungal culture media. Once received in the laboratory, a specimen should be examined as soon as possible. As mentioned, swab specimens are inadequate for the recovery of molds. These, however, are acceptable for the recovery of yeasts from patients with mucosal candidiasis (e.g., thrush or vulvovaginal candidiasis). Whenever possible, aspirated material or tissue biopsies should be submitted, as these are the optimal specimens. Direct wet mounts or smears should be prepared (see below), if appropriate, and a portion of the specimen transferred to appropriate fungal culture media. It is critical that when tissue specimens are submitted that portions of tissue are placed onto and pushed into the agar media to enhance recovery. The appropriate processing of specimens is critical in the ultimate recovery of fungi and should not be neglected or delegated to marginally trained personnel. Further guidelines for the processing and direct examination of clinical specimens are presented in Table 21-4.

TABLE 21-3 Recommendations for Specimen Collection for Fungus Culture

Specimen	Recommended Procedure
Sputum	The first early-morning sample should be collected after rising, but before breakfast. Patients are instructed to vigorously rinse their mouths with water immediately before coughing 1/2–1 oz of sputum into a sterile, screw-capped container. Sputum induction with a heated aerosol saline suspension may be required if an adequate specimen cannot be obtained.
Bronchoscopy	Bronchial brushings, biopsy, or bronchoalveolar lavage fluid should be transported promptly to the laboratory in sterile, sealed containers. Middlebrook 7H11 broth is used in some laboratories as a transport medium because mycobacteria will also be preserved.
Cerebrospinal fluid	As much CSF as possible should be used for the culture of fungi. If processing is to be delayed, samples should be left at room temperature because CSF is an adequate fluid culture medium in which fungal elements can survive until subcultured.
Urine	The first early-morning urine sample is preferred; random samples are acceptable. Specimens should be collected aseptically in sterile, screw-cap containers and sent immediately for processing. If a delay in processing beyond 2 hours is anticipated, the urine sample should be refrigerated at 4°C to inhibit overgrowth by rapidly growing bacteria.
Prostatic secretions	Some deep-seated mycoses, notably blastomycosis and, less commonly, histoplasmosis or coccidioidomycosis, may infect the prostate. The bladder is first emptied, followed by prostatic massage. Secretions should be inoculated directly into appropriate fungal culture media; also, 5–10 mL of urine should be collected in a separate container.
Exudates	The skin over pustular lesions should be disinfected and exudates aspirated using a sterile needle and syringe. The aspirated material should be discharged into a sterile specimen transport container, and the needle-bearing syringe discharged in an appropriate biohazard (i.e., “sharps”) container. The needle-bearing syringe should not be sent to the laboratory, as this represents an occupational hazard. Biopsy of the lesion may be necessary if the aspirate fails to yield fungi.
	First, swab the area of skin to be sampled with 70% alcohol to remove surface bacterial contaminants. Sample the peripheral,

Skin, nails, hair	erythematous, growing margin of typical "ringworm" lesions, scraping with the side of a glass microscope slide or the edge of a scalpel blade. Infected nails should be sampled from beneath the nail plate to obtain softened material from the nail bed. If this is not possible, scrape away the surface of the nail before collecting shavings from the deeper portions. Hairs should be collected from areas of scaling or alopecia, or those that fluoresce when viewed under a Wood's (long-wavelength) ultraviolet lamp.
Tissue biopsies	Tissue biopsies of suspected sites of infection should be transported in a sterile gauze moistened with physiologic, nonbacteriostatic, sterile saline solution in a screw-cap container. The specimen should not be frozen or allowed to dehydrate prior to culture. If the tissue biopsy is not bisected with half sent for histopathologic review, then a second biopsy, adjacent to the first, should be submitted for this purpose.
Blood	Standard blood cultures are sufficient for the recovery of most yeasts. However, a fungal blood culture that utilizes a lysis-centrifugation systems, such as the Isolator (Wampole Laboratories, Cranbury, NJ), is highly recommended, particularly for the recovery of *H. capsulatum*.

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BOX 21-2

Criteria for Specimen Rejection

Situation: Absence of patient identification on the container, or a discrepancy between the information on the request form and the container label.
Action: Return to sender for resolution.

Situation: Sputum specimen with >25 squamous epithelial cells per low-power field (i.e., criteria for rejection of respiratory samples submitted for bacterial culture).
Action: The criteria for rejection of specimens submitted for bacterial culture do not pertain to fungal specimens. Pathogenic fungi may be recovered from such specimens in the presence of oral contamination, if an antibiotic-containing selective culture medium is used, which is recommended.

Situation: A dried-out swab is received or the material collected is insufficient in

volume.
Action: Inform the person submitting the specimen to send a second sample, if possible. As a general criterion, swab specimens are not suitable for the recovery of fungi, with the exception of yeasts from a mucosal surface (e.g., vagina) and should be rejected, except in unusual circumstances. Rather, deep aspiration or biopsy of the affected site may be necessary, depending on the clinical presentation.

Situation: The sample is submitted in an improper container or in an unsuitable condition (evidence of drying, leakage, or lack of sterility).
Action: Notify the individual submitting the specimen that the chance for obtaining relevant results is compromised. If a second specimen cannot be conveniently obtained, process the specimen, but indicate on the final report that the quality of the specimen was compromised and that the result can be interpreted only in light of the clinical presentation.

Situation: A 24-hour sputum or urine specimen for fungal culture is received.
Action: It should be understood in the rejection protocol that 24-hour sputum and urine samples are suboptimal for the recovery and identification of fungi. Chances for contamination with bacteria and environmental molds are high, confusing any results that may follow. Establish in the protocol that first early-morning samples should be obtained on three successive days. As is the case with the culture recovery of mycobacteria, fungi also tend to be shed at intermittent intervals, thus making the chance of recovery much higher if successive samples over 3 days are collected.

Direct Examination

It is highly recommended that a direct microscopic examination be made on most specimens submitted for fungal culture. This may provide the first definitive evidence of a fungal infection for the physician. It may also aid in the selection of appropriate culture media, but in practicality, the inoculation of media is usually performed simultaneously with the preparation of the slides for the direct examination. KOH/calcofluor mounts are likely the most commonly used direct examination preparations, although laboratories without fluorescence microscopy capabilities may perform KOH preparations and traditional microscopy. The advantage of the former is the ease of detection of fungal elements, which fluoresce brightly when stained with calcofluor white. In either preparation, the KOH serves to dissolve human cells, which lack a cell wall.

In settings wherein there is a high prevalence of HIV, the India ink stain remains useful. Although this method of direct examination is not as sensitive as the cryptococcal antigen test, it is rapid, inexpensive, and may be highly specific when

used by an experienced microscopist. The frozen section evaluation of tissues removed during surgeries is also a form of direct examination. All these methods afford the opportunity to provide rapid results to the clinician, which may translate into the more rapid administration of antifungal therapy and better outcomes for the patient.

When fungal elements are recognized in a direct examination, there should be an attempt to further characterize the structures present. Reporting of the fungal elements as "fungal elements" is only marginally useful to the clinician. This should only be reported in instances wherein it is not possible to further characterize the structures present. If one is in this situation, a consultation with a more experienced microscopist should be considered. The microscopist performing the direct examination should try to characterize the fungal elements as budding yeasts, yeasts with pseudohyphae and/or true hyphae, hyaline septate hyphae, dematiaceous hyphae or mucoraceous hyphae (i.e., broad, pauciseptate hyphae). If specialized fungal forms are seen, such as the spherule of *Coccidioides* or the broad-based bud and double-contoured cell walls of *Blastomyces dermatitidis*, then these should be reported, as they provide a definitive diagnosis from the direct specimen.

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TABLE 21-4 Guidelines for Direct Examination and Processing of Specimens Submitted for Fungal Cultures

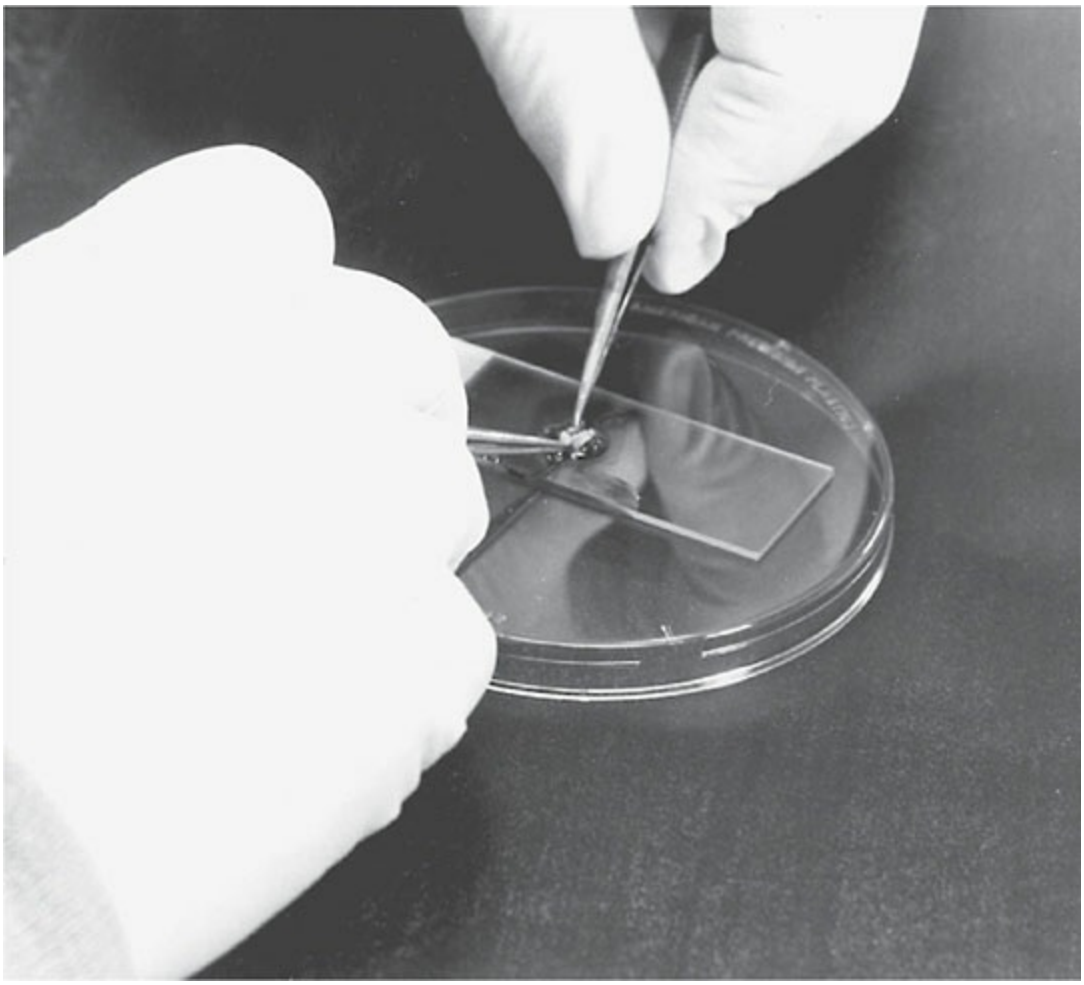
Respiratory	The sputum quality-grading system described in Chapter 2 is not applicable to specimens submitted for fungal cultures. Select the most purulent or blood-flecked parts of the sample. If the sample is highly viscid, it should be homogenized by adding a small amount of crystalline *N*-acetyl-L-cystine to the specimen. NaOH or other digesting agents, used for the processing of specimens for the recovery of mycobacteria, should not be used prior to fungal culture. Prepare a mount of the homogenized sample for direct microscopic examination and inoculate about 0.5 mL to each of the culture media to be used. Because respiratory secretions are often contaminated with bacteria, media containing antibiotics should be used. A combination of a nonselective agar, such as potato flake agar, and an inhibitory agar such as brain-heart infusion with chloramphenicol and cycloheximide are recommended.
	CSF samples may be centrifuged at 1,500–2,000 *g* for 20 minutes and the sediment inoculated onto the surface of noninhibitory culture media, such as potato flake agar. Preferably, if more than 2 mL of fluid is available,

Cerebrospinal fluid	pass fluid through a 0.45-µm membrane filter. Place the filtrate side of the filter paper face down on the surface of appropriate culture medium. The paper should be repositioned to other sites on the medium using sterile forceps on an every-other-day schedule. If only a scant amount of fluid is provided, spot-inoculate the surface of the agar medium directly in three- or four-drop aliquots. The India ink (nigrosin is an acceptable substitute) mounts may be prepared when *Cryptococcus neoformans* is suspected, but performing cryptococcal antigen testing is preferred as a more sensitive alternative. To prepare the mount, either a drop of the centrifuged sediment or a small amount of material from the surface of the membrane is mixed with a drop of India ink on a microscope slide. A coverslip is applied and the mount microscopically examined for the presence of encapsulated, budding yeast forms.
Urine	Centrifuge about 10 mL of urine sample, then inoculate 0.5 mL of sediment to both a noninhibitory agar, such as potato flake agar, and to an inhibitory medium, such as brain-heart infusion agar, containing chloramphenicol and cycloheximide. Direct mounts can be prepared and examined microscopically for yeast or hyphal forms, but this is rarely done in practice.
Skin, nails, hair	Skin scales, nail scrapings, and hairs should be examined after KOH treatment. The KOH preparation is made by emulsifying the specimen in a drop of 10% KOH on a microscope slide. The purpose of the KOH is to clear out any background scales or cell membranes that may be confused with hyphal elements. Clearing can be accelerated by gently heating the mixture over the flame of a Bunsen burner or more commonly on a slide warmer. A coverslip is applied, and the specimen is examined for the presence of narrow, regular hyphae that characteristically break up into arthroconidia. The visualization of hyphae is improved by adding calcofluor white to the potassium hydroxide reagent and examining with a fluorescence microscope fitted with filters of appropriate wavelengths. With hairs, mosaic arrangement of spores may be seen on the surface of the shaft (ectothrix infection) or hyphal fragments and arthroconidia may be seen internally (endothrix infection). Skin scales, nail scrapings, or hairs should be placed directly on the surface of the culture medium, such as brain-heart infusion agar with chloramphenicol and cycloheximide (commercially available as Mycosel or Mycobiotic agars). With a straight inoculating wire, submerge a few of the fragments beneath the agar surface. Examine in the areas of inoculation at frequent intervals for the appearance of surface colonies. Hold cultures for a minimum of 30 days before discarding as negative.

Tissue	When processing tissues for the recovery of fungi, the use of a mortar and pestle or a tissue grinder should be avoided. The hyphal forms can easily be destroyed by grinding, making it difficult to recover viable organisms in culture, particularly if the pauciseptate hyphae of one of the *Zygomycetes* are present. Rather, mince the tissue into 1-mm cubes with sterile scissors or a sharp scalpel blade and place the tiny fragments directly onto the agar, submerging them slightly beneath the surface with an inoculating needle. A 5–10 mL sample of tissue homogenate, bone marrow, or fluid specimen sediment should be placed onto the surface of appropriate culture media. Nonselective culture media, without antibiotics, such as potato flake agar, is adequate, as these specimens are usually sterile and antibiotic inhibition is not necessary.
Blood	Commercial blood culture bottles, designed for the recovery of bacteria from blood are adequate for the recovery of yeasts associated with bloodstream infections (e.g., *Candida* species). The Wampole Isolator Blood Culture System (Alere) is recommended for the detection of *H. capsulatum*. The filamentous fungi rarely grow in blood cultures, with the exception being *Fusarium* species.

Preparation of Mounts From Cultured Isolates

The tease mount, the dig mount, the cellophane tape preparation and the microslide technique are four commonly used methods for the microscopic examination of filamentous fungi (i.e., molds).[153] The tease mount (Figure 21-4) is accomplished by obtaining surface or aerial hyphae and conidiophores with a loop or hook. The dig mount is rarely used, but as the name implies, the loop or hook is used to dig out mycelium that is submerged, so as to demonstrate structures produced in this area, such as aleuroconidia. The cellophane tape preparation (Figure 21-5) uses this type of tape to touch the aerial hyphae and conidiophores, which become adherent to the tape for subsequent microscopic evaluation. Finally, the microslide culture technique (Figure 21-6) is usually reserved for those instances wherein a mold cannot be identified using the former methods, but is considered clinically important. This method involves the cultivation of the mold directly onto glass slides, which affords the assessment



■ **FIGURE 21-4** Tease mount preparation, illustrating dissection of the colony fragment with needles in a drop of lactophenol aniline blue prior to placement of the coverslip. These preparations should be performed in a biological safety cabinet.

of their microscopic morphology with minimal mechanical disruption.

In each instance, a portion of the mold colony is mounted in a drop of lactophenol aniline (cotton) blue stain on a glass slide and examined microscopically. The techniques for performing the tease mount (Box 21-3), the cellophane tape preparation (Box 21-4), and for the microslide culture (Box 21-5) are presented. Additional terms used in the laboratory workup of fungi are presented in Box 21-1.

Selection and Inoculation of Culture Media

The battery of culture media for the recovery of fungi from clinical specimens need not be elaborate. Although the recovery rate may be somewhat enhanced by using a variety of isolation media, considerations of cost, storage, and incubator space and demands on technologist time generally dictate a more conservative approach in most

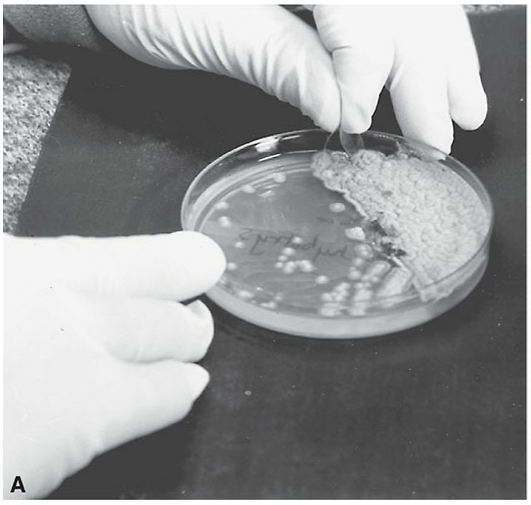
laboratories.

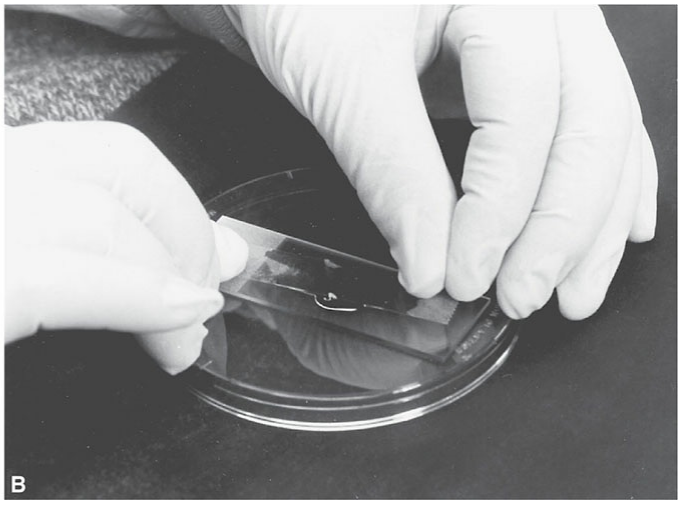
Three general types of culture media are essential to ensure the primary recovery of all clinically significant fungi from clinical specimens. One medium should be nonselective, such as potato dextrose, potato flake or brain-heart infusion agar. This type of media not only will permit the growth of virtually all clinically relevant fungal species, but also will support the growth of some bacteria. The use of Sabouraud's dextrose agar (SDA) as a primary recovery medium is discouraged because it is insufficiently rich to recover certain fastidious pathogenic species, particularly most of the dimorphic fungi. Rather, the use of potato flake agar (PFA), potato dextrose agar (PDA), inhibitory mold agar (IMA), or combination of Sabouraud's dextrose agar with heart infusion (SABHI) agar is recommended.[153] SDA is sufficient for the recovery of dermatophytes from cutaneous samples or yeasts from mucosal cultures. The use of either Mycosel or Mycobiotic agar, which essentially is SDA with cycloheximide and chloramphenicol added, may be considered for the recovery of dermatophytes.

V8 agar or carnation leaf agar are glucose-poor media that are useful for the subculture of fungi recovered on enriched medium to enhance typical sporulation. Czepak's agar has classically been used for the subculture of *Aspergillus* species, if the colony morphology is not typical and deemed important for the identification of an unknown isolate.

For the recovery of the more fastidious dimorphic fungi, such as *Blastomyces dermatitidis* and *H. capsulatum*, an enriched agar base such as IMA, SABHI, or brain–heart infusion (with cycloheximide and chloramphenicol) should be considered. Table 21-5 lists the more commonly used fungal isolation media, the key ingredients in each formula, and the specific applications for which each is designed.

The use of a secondary media that contains antibacterial antibiotics should be used for the culture of fungi from





■ **FIGURE 21-5** **A.** Transparency tape preparation: the sticky side of tape being pressed to the surface of the fungus colony. **B.** Transparency tape preparation: stretching the inoculated tape over a drop of lactophenol aniline blue on the surface of a microscope slide. These preparations should be performed in a biological safety cabinet.

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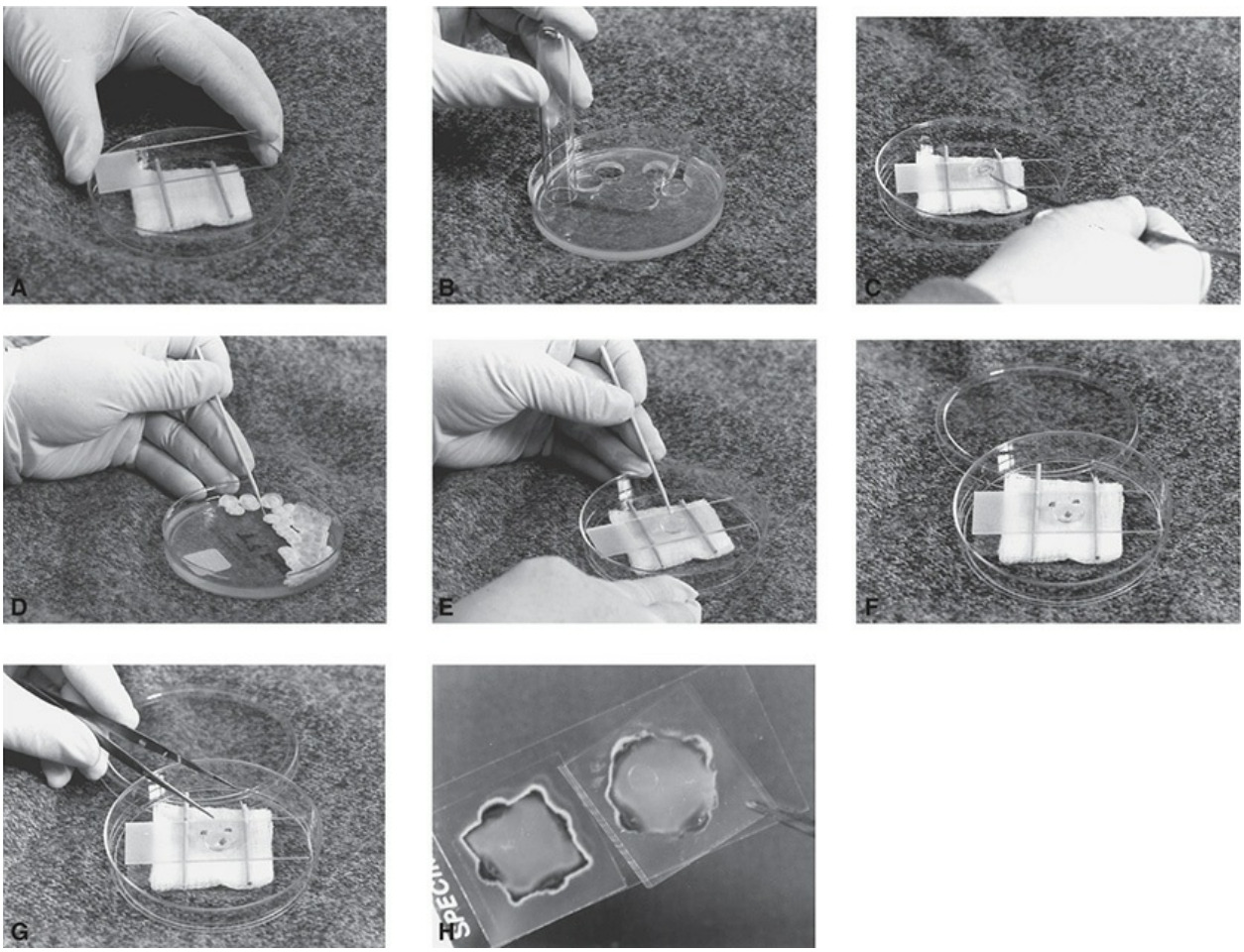

■ **FIGURE 21-6** Series of photographs outlining the preparation of a microculture. See Box 21-5 for details. These preparations should be performed in a biological safety cabinet.

BOX 21-3

Procedure: The Tease Mount

- With a pair of dissecting needles or pointed applicator sticks, dig out a small

portion of the colony to be examined, including portions of the subsurface agar.
- Place the colony fragment on a microscope slide in a drop of lactophenol aniline blue, tease the colony apart with the dissecting needles (Fig. 21-4), and overlay with a coverslip.
- Gentle pressure on the surface of the coverslip with the eraser end of a pencil may help to disperse the mount, particularly if small pieces of agar are present.
- Examine the preparation microscopically, first under the low-power (10×) objective and then under high-power (40×) objective or under oil immersion (100×), if necessary. Oil immersion microscopy is usually not necessary, but is helpful in visualizing annellides.
- Teasing the colony often disrupts the delicate fruiting structures of the filamentous molds, making it difficult in some instances to observe the characteristic spore arrangements or hyphal attachments necessary for a definitive identification. In such cases, a transparency tape mount or microslide culture may be required.

clinical specimens that are from anatomic sites that normally contain bacterial microbiota. The addition of one or more antibiotics, including penicillin (20 U/mL), streptomycin (40 U/mL), gentamicin (5 mg/mL), or chloramphenicol (16 mg/mL) may be used to inhibit the growth of bacteria. IMA is perhaps a poorly named media that actually contains antibacterial antibiotics that inhibit bacterial growth (i.e., it does not inhibit the growth of molds).

Just as bacterial overgrowth may interfere with the recovery of a slowly growing fungal pathogen, so, too, may rapidly growing fungal contaminants overgrow a culture plate and interfere or inhibit the recovery of the more slowly growing fungal pathogen. This third type of media contains cycloheximide (Acti-Dione) in a concentration of 0.5 mg/mL which is added to prevent the overgrowth of certain rapidly growing environmental molds. If a medium that incorporates

BOX 21-4

Procedure: Cellophane Tape Preparation

- The cellophane or transparency tape method of preparing cultures for microscopic examination is often helpful because the conidial arrangements of the more delicate filamentous molds are better preserved.
- Press the sticky side of unfrosted, clear cellophane tape gently but firmly to the surface of the colony, picking up a portion of the aerial mycelium (Fig. 21-5A).

This operation should always be performed under a biologic safety hood. Gloves should always be worn, but still take care not to touch the surface of the colonies and contaminate the gloves.

- The preparation is made by placing a drop of lactophenol aniline blue stain on a microscope slide.
- Stick one end of the tape to the surface of the slide adjacent to the drop of stain.
- Stretch the tape over the stain, gently lowering it so that the mycelium becomes permeated with stain (Fig. 21-5B).
- Pull the tape taught and stick the opposite end to the glass, avoiding as much as possible the trapping of air bubbles.
- Some practice may be needed in removing the sticky surface of the tape since gloves are worn.
- The preparation can now be examined microscopically in the same manner as described above for the tease mount preparation, with the exception that the use of oil immersion is generally less than satisfactory because of interference from the substance of the tape.
- The transparency tape method is inexpensive, rapid, simple to perform, and, with few exceptions, allows one to make an accurate identification.

BOX 21-5

Procedure: The Microslide Culture Technique

The microslide culture technique is recommended in instances wherein neither the tease mount nor the transparency tape preparation establishes an accurate identification, or when permanent slide mounts are desired for further study or for study use by students. Although somewhat tedious to perform, high-quality preparations in which the spore structures and arrangements are beautifully preserved can be made. The technique is as follows:

1. Place a round piece of filter paper or gauze flat on the bottom of a sterile Petri dish. Place a pair of thin glass rods or applicator sticks cut to length to fit on top of the filter paper to serve as supports for a 3-in.× 1-in. glass microscope slide (Fig. 21-6A).
2. Place a block or plug of cornmeal agar or PDA (Fig. 21-6B) on the surface of the microscope slide (Fig. 21-6C). Two blocks, separated by about 1 in., can be placed on the same microscope slide if more than one mount is desired.
3. Inoculate the margins of the agar plug in three or four places with a small portion of the colony to be studied, using a straight inoculating wire or the tip of a dissecting needle (Fig. 21-6D and E).
4. Gently heat a coverslip by passing it quickly through the flame of a Bunsen burner

and immediately place it directly on the surface of the inoculated agar block. Heating the coverslip produces a tight seal between the bottom of the coverslip and the surface of the agar, which is briefly melted by the warm glass.

5. Pipette a small amount of water into the bottom of the Petri dish to saturate the filter paper or the gauze. Place the lid on the Petri dish and incubate the assembly at room temperature (or 30°C) for 3 to 5 days.
6. When growth visually appears to be mature (Fig. 21-6F), the coverslip can be gently lifted from the surface of the agar with a pair of forceps, taking care not to disrupt the mycelium adhering to the bottom of the coverslip (Fig. 21-6G).
7. Place the coverslip on a small drop of lactophenol aniline blue applied to the surface of a second 3-in.× 1-in. glass slide (Fig. 21-6H). The mount can be preserved for future study by rimming the outside margins of the coverslip with mounting fluid or clear fingernail polish. This activity should be performed under a biologic safety hood.
8. After the coverslip has been removed from the agar block (or blocks), the agar block itself can be removed by prying it away from the glass slide with an applicator stick. This operation is performed over a beaker or pan containing 5% phenol decontamination fluid, into which the agar blocks are placed. The mycelium adhering to the surface of the original glass slide after the block is removed can also be stained with lactophenol aniline blue and a coverslip overlaid, serving as a second stained mount. Again, the mount can be preserved for future study by rimming the coverslip with mounting fluid or fingernail polish, as previously described.

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TABLE 21-5 Common Fungal Culture Media and Indications for Use

Culture Media	Essential Ingredients	Indications for Use
Brain-heart infusion agar	Calf brain and beef-heart infusions; proteose peptone, NaCl, disodium phosphate, and agar. (Cycloheximide and chloramphenicol may be added for selective recovery of dermatophytes and dimorphic molds.)	Primary recovery of saprophytic and dimorphic fungi
Inhibitory Mold Agar (IMA)	Tryptone, beef extract, yeast extract, dextrose, starch, dextrin, chloramphenicol, gentamicin, and	Primary for the recovery of fungi from specimens that contain bacterial

	saline buffers	microbiota
Mycosel/Mycobiotic agars	Phytone peptone, dextrose, agar, cycloheximide, chloramphenicol	Primary recovery of dermatophytes and dimorphic fungi
Sabouraud's heart infusion agar (SABHI)	Potato flakes, dextrose, agar	Primary recovery of saprophytic and dimorphic fungi, particularly fastidious strains
Sabouraud's dextrose agar	Dextrose, neopeptone, agar	Although this is a traditionally used medium, it is not optimal. Fungi grow well on the medium, but do not sporulate well due to the high glucose content
Potato flake agar	Potato flakes, dextrose, agar	Primary recovery of saprophytic and dimorphic fungi, particularly fastidious and slow-growing strains

cycloheximide is used in the battery of fungal media, then it is imperative that a nonselective medium must always be used in parallel. Opportunistic pathogenic fungi, such as *Cryptococcus neoformans* and *Aspergillus fumigatus,* are partially or totally inhibited by cycloheximide, and could be missed, if only a cycloheximide-containing medium was used for fungal culture. Cycloheximide-containing media are particularly useful for fungal cultures for dermatophytes and dimorphic fungi. It is particularly useful for dermatophyte cultures, since some specimens, like nails, may contain transient fungal microbiota that would overgrow and obscure the presence of the dermatophyte. Similarly, some of the dimorphic pathogens, like *H. capsulatum*, are slow-growing and could potentially be overgrown by a stray spore of a contaminating species (e.g., *Aspergillus niger*). The presence of cycloheximide would inhibit the growth of the contaminating fungus and allow the pathogen to grow.

Again, whenever cycloheximide is used, it is critical to include a similar medium without cycloheximide in parallel. This is because the disease of the patient may actually be caused by a fungal species that is susceptible to cycloheximide, and if a cycloheximide-containing medium alone were used, then a false-negative culture result

would be given.

Incubation of Fungal Cultures

It is currently recommended that all fungal cultures be incubated at a controlled 30°C. A former practice of incubating a second set of plates at 35°C for the recovery of the yeast forms of dimorphic fungi is not cost-effective nor needed given the molecular methods currently available to confirm dimorphic fungal pathogens. Similarly, the previous practice of attempting to convert fungal isolates suspected of being dimorphic molds to their yeast form has largely been replaced by nucleic acid probe assays or other molecular methods. The probe assay can be performed as soon as colonies are visualized on the primary recovery media, prior to the observation of diagnostic sporulation, in instances wherein disease secondary to these fungi is suspected.

All fungal cultures should be incubated for a minimum of 30 days before discarding as negative, even if plates appear contaminated with bacteria or other fungi. Colonies of *H. capsulatum*, for example, may be observed growing on the surface of colonies of *Candida albicans* or on the top of contaminating molds.

The choice between the use of culture tubes or plates is optional. Some low-volume laboratories, staffed with personnel who may not be as familiar with the handling of fungi, or where incubator and storage space may be at a premium, have opted for the use of tubes. Large culture tubes (150 × 25 mm) with screw-cap lids are recommended. The media should be poured in thick slants to prevent dehydration during the prolonged incubation period. After the medium is inoculated, the cap should not be tightened too much, because the anaerobic environment that will soon result will inhibit fungal growth. The cap should be tightened enough so that it will not fall off, but not so much that it is completely tightened (i.e., it should "jiggle" when lightly shaken). The ease of transport of cultures recovered in tubes is an added advantage. The chief disadvantage is the difficulty in preparing stained mounts for microscopic examination. The use of tubes has significantly declined in recent years, likely because of this latter disadvantage.

Petri dishes, in contrast to tubes, have the advantage of providing a larger surface for growth, resulting in better colony separation and making the cultures easier to examine and subculture. Fungal colonies in mixed culture are easier to separate and work with as individual colonies. Tease mounts or transparency tape preparations are more effectively made from plate cultures, potentially leading to an improved chance for making a more rapid identification. Because plate cultures may become dehydrated during the prolonged incubation period, they may be placed into a sealed, moisturized polyester bag, or the edge of the Petri dish may be sealed with

oxygen-permeable tape. If an incubator is used only for fungal cultures, placing a flat, open pan of water on the bottom shelf serves to provide the humidity needed for optimal recovery. Routine cleaning of the water basin is necessary, however, to control the growth of environmental mold in the water.

Laboratory Approach to the Presumptive Identification of Fungal Isolates

Once the observation of a culture plate reveals the growth of a probable fungus, several characteristics of the colony may be assessed to determine within which major group of fungi the isolate may belong. A guide to the genus and species identification of medically important fungi based on the colony morphology and microscopic features of clinical isolates is presented in Table 21-6, which is based on the observation of primary fungal isolates incubated at 30°C. If the fungal isolate presents a distinct wooly or cottony aerial mycelium, one of several species of molds must be considered. If the mold is rapidly growing, does not display an outer border, and fills the Petri dish within 48 to 72 hours, then one of the *Zygomycetes* must be considered (Display 21-1 at the end of this chapter).

Molds that grow within 3 to 5 days, have a distinct border, and are white or pastel on the surface, often with a white apron of new growth at the periphery, most likely belong to the hyaline, septate group of molds. Keys to the identification of the hyaline septate molds are presented in Display 21-2, and for the identification of molds recovered from skin scrapings, nail shavings, or hairs, are potentially dermatophytes which are discussed further in Display 21-3.

One of the dimorphic species should be considered for molds that are generally slower-growing (7 to 14 days), have a delicate or cobweb aerial mycelium, and are usually white to gray. The dimorphic molds are in the filamentous form when incubated at ambient temperature (25°C to 30°C), and in the yeast form when incubated at body temperature (35°C to 37°C). Upon initial isolation, the colonies of certain dimorphic molds may appear focally yeast-like when incubated at 30°C; these will become more fully filamentous with time, but this feature is important to recognize to avoid misidentifications. Dimorphic molds can be converted, sometimes with difficulty, from the mold to the yeast form by incubating a subculture on enriched agar at 35°C to 37°C, but as mentioned above this is rarely performed given the availability of molecular methods. The approach to the species identification of the dimorphic molds is presented in Display 21-4.

One of the dematiaceous molds should be considered when colonies that develop a dark gray to black mycelium, which is best determined by observing a brown or black reverse of the colony. The genus and species separation of the more rapidly growing

saprophytic species of this group is shown in Display 21-5. The keys to the identification of the slower-growing, pathogenic dematiaceous species, associated with chromoblastomycosis and mycetoma, are shown in Display 21-6.

If the colony has a smooth, creamy, viscous, or pasty appearance, a yeast must be considered. For the genus and species identification of common and less commonly encountered yeasts, including the yeast-like, arthroconidia-producing species that may produce a low aerial mycelium and the "black yeasts," see Display 21-7.

Once the initial observations outlined in Table 21-6 are made, and a fungal isolate has been placed within one of the groups outlined therein, further studies will be required to make a final genus/species identification, using the following microscopic criteria:

- If hyphae are observed, determine the structure of the hyphae. Are they
  - septate or coenocytic (i.e., pauciseptate/aseptate)?
  - branching (if so, at what angles), or not branching?
  - pigmented or not pigmented?
  - even or uneven in width?
  - composed of arthroconidia or pseudohyphae?
- Determine the structure and derivation of fruiting bodies.
  - Visualize the type of conidiation
  - The size, shape, and arrangement of the conidia
- Look for the presence of special diagnostic structures: pycnidia, cleistothecia, Hülle cells.
- If only yeast cells are observed:
  - Note their size, shape, and arrangement.
  - Look for the presence or absence of a capsule.
  - Determine the type of blastoconidiation—are daughter cells single or multiple?
- Further guidelines for the presumptive identification of fungi based on direct microscopic examination of clinical specimens are presented in Table 21-7.

Extent of Laboratory Genus/Species Identification

Table 21-6 can also be used as a guide for the extent to which genera and species identifications are required in most clinical situations. As virtually any filamentous mold recovered in pure culture from normally sterile body sites may represent an opportunistic pathogen, clinical correlations are recommended before the isolate is discarded as insignificant. Species identifications need not go beyond those listed in the algorithm; other isolates not specifically identified should be reported with the appropriate genus name and, if typically performed, species (e.g., *Aspergillus fumigatus* and *Fusarium* species).

Because yeasts are commonly recovered as commensals from skin and respiratory

specimens, the definitive workup should be limited and performed only when clinically indicated. Clinically significant yeast isolates recovered from tissues (particularly if fungal elements are observed in stained tissue sections), normally sterile body fluids and aspirates should be identified to both the genus and species level. This is readily accomplished by commercially available methods. The identification of certain species, *Candida krusei* for example, predicts resistance to select antifungal agents, such as the imidazoles. Isolates recovered from swabs or superficial wounds must be evaluated as to their clinical significance. The criteria by which the fungi included in Table 21-6 can

TABLE 21-6 Identification of Medically Important Fungi, Initial Growth at 30°C

Hyaline Mold Colonies				Dematiaceous Fungi		Yeast Colonies	Yeast-Like Colonies
Growth <3 days Hyphae broad and pauciseptate **Suspect *Zygomycetes*** *Rhizopus* *Mucor* *Rhizomucor* *Lichtheimia* *Syncephalastrum* *Circinella* *Cunninghamella*	Growth 3–5 days Hyphae hyaline and septate **Suspect Agents of Hyalohyphomycosis** **Conidia in Chains:** *Aspergillus* *Penicillium* *Paecilomyces/ Purpureocillium* *Scopulariopsis* **Conidia in Clusters:** *Acremonium* *Fusarium* *Trichoderma* *Gliocladium* **Conidia Borne Singly:** *Pseudallescheria boydii* complex *Chrysosporium* *Sepedonium*	Growth 3–5 days Colonies often granular and pigmented; hyphae septate, hyaline **Suspect Dermatophyte** **Genus *Microsporum*** **Common:** *Microsporum canis* *Microsporum gypseum* Uncommon: *Microsporum audouinii* *Microsporum nanum* **Genus *Trichophyton*** **Common:** *Trichophyton rubrum* *Trichophyton mentagrophytes* *Trichophyton tonsurans* *Trichophyton verrucosum* **Uncommon:** *Trichophyton violaceum* *Trichophyton schoenleinii* **Genus *Epidermophyton:*** *Epidermophyton floccosum*	Growth >5 days Hyphae hyaline and slender. Growth on cyclohexamide agar. Yeast forms when incubated at 35°C, except for *Coccidioides* species **Suspect Dimorphic Fungi:** *Blastomyces dermatitidis* *Coccidioides* spp. *Histoplasma capsulatum* *Sporothrix schenckii* complex *Paracoccidioides brasiliensis*	Growth 3–5 days Dark colony; black reverse; hyphae golden-brown and septate **Suspect Agent of Phaeohyphomycosis** **Conidia Muriform:** *Alternaria* *Ulocladium* *Stemphylium* *Epicoccum* **Conidia Divided by Transverse Septa Only:** *Curvularia* *Bipolaris* (*Drechslera*) *Exserohilum* **Pycnidia Produced** *Phoma* *Chaetomium* **Conidia Borne Singly:** *Scedosporium prolificans*	Growth >5days Dark colony; black reverse; hyphae golden-brown and septate **Suspect Agent of Chromoblastomycosis or Mycetoma** ***Cladosporium*-Type Sporulation:** *Cladophialophora carrionii* *Cladophialophora bantianum* ***Phialophora*-Type Sporulation:** *Phialophora verrucosa* *Pleurostomophora richardsiae* *Exophiala* spp. **Acrotheca-Type Sporulation:** *Fonsecaea pedrosoi* *Fonsecaea compacta*	Growth 2–5 days Smooth, pasty, or mucoid colonies: **Suspect Yeast** **Common:** *Candida albicans* *Candida* species *Cryptococcus neoformans* Other *Cryptococcus* spp. *Rhodotorula* spp. **Uncommon:** *Hansenula anomala* *Malassezia* spp *Saccharomyces cerevisiae* **Black Yeasts:** *Exophiala werneckii* **Yeast forms of dimorphic fungi (35°C incubation)**	Growth 2–5 days Yeast-like colonies with low aerial mycelium **Arthroconidia Produced** **Suspect:** *Geotrichum candidum* *Trichosporon* spp. *Blastoschizomyces capitus*
See Display 21-1 for species-identifying characteristics.	See Display 21-2 for species-identifying characteristics.	See Display 21-3 for species-identifying characteristics.	See Display 21-4 for species-identifying characteristics.	See Display 21-5 for species-identifying characteristics.	See Display 21-6 for species-identifying characteristics.	See Display 21-7 for species-identifying characteristics.	See Display 21-7 for species-identifying characteristics.

TABLE 21-7 Presumptive Identification of Fungi Based on Direct Microscopic Examination of Material from Clinical Specimens

Presumptive Direct Microscopic Observations	Identification
Hyphae relatively small (3–6 μm) and regular in size, dichotomously branching at 45-degree angles with distinct cross-septa	*Aspergillus* spp. or other hyaline molds (e.g., *Fusarium* or *Pseudallescheria*)

Hyphae irregular in size (6–50 μm), ribbonlike, and pauciseptate	*Zygomycetes: Rhizopus, Mucor, Lichtheimia*
Hyphae small (2–3 μm) and regular, some branching, with rectangular arthrospores sometimes seen; found only in skin, nail scrapings, and hair	Dermatophyte group *Microsporum* spp. *Trichophyton* spp. *Epidermophyton* spp.
Hyphae regular in diameter (3–6 μm), parallel walls, irregular branching, septate, dark golden-brown	Dematiaceous fungi
Hyphae, distinct points of constriction simulating link sausages (pseudohyphae), with budding yeast forms (blastospores) often seen	*Candida* spp.
Yeast cells spherical and irregular in size (5–20 μm), classically with a thick polysaccharide capsule (not all cells appear encapsulated), with one or more buds attached by a narrow constriction	*Cryptococcus neoformans* *Cryptococcus* spp.
Small budding yeast, relatively uniform in size (3–5 μm), with a single bud attached by a narrow base, extracellular or within macrophages	*Histoplasma capsulatum, Candida glabrata,* and other small yeasts
Yeast forms, large (8–20 μm), with cells appearing to have a thick, double-contoured wall, with a single bud attached by a broad base	*Blastomyces dermatitidis*
Large, irregularly sized (10–50 μm), thick-walled spherules, many of which contain small (2–4 μm), round endospores	*Coccidioides* spp.

be identified follow. The presentation of more detailed identification criteria for several groups of fungi will follow the order in the columns of Table 21-6, with linkages to Displays 21-1 through 21-7, located at the end of this chapter.

Zygomycetes (Glomeromycetes) and Zygomycosis

Major Genera of Zygomycetes

The fungi belonging to the Phylum Zygomycota have undergone taxonomic reclassification and are now known as Glomerulomycota. Traditionally terminology is retained here, because this is commonly used in medical practice. The fungi in this group are widely distributed environmental inhabitants of soil, dung, and vegetative matter. They are most commonly encountered as bread molds. Humans usually

become infected through the inhalation of airborne spores, although ingestion of contaminated foodstuffs may result in primary gastrointestinal disease. Direct inoculation and contamination of wounds may lead to primary cutaneous infection. Most members of the *Zygomycetes* have a proclivity for hyphal invasion of the walls of blood vessels (i.e., angioinvasion) once a primary infection is established, often resulting in the dissemination of mycotic thrombi and the development of metastatic foci in many organs. Additionally, these fungi may tract along the loose connective tissue surrounding nerves (i.e., the perineurium) as a means of local spread of infection.

The medically important *Zygomycetes* are separated into two orders, *Mucorales* and *Entomophthorales*.[159] It is the fungi in the former category that are most commonly encountered in clinical medicine. The six members of the *Mucorales* that are presented here, as outlined in Display 21-1, are *Rhizopus* spp., *Mucor* spp., *Lichtheimia* (formerly *Absidia*) spp., *Syncephalastrum* spp., *Circinella* spp., and *Cunninghamella* spp. These may be identified by the criteria outlined. *Rhizomucor pusillus, Saksenaea vasiformis, Apophysomyces elegans*, and the entomophthoromycotic agents, *Conidiobolomyces coronatus* and *Basidiobolobus* spp., are rarely encountered in clinical laboratories and will not be presented here.

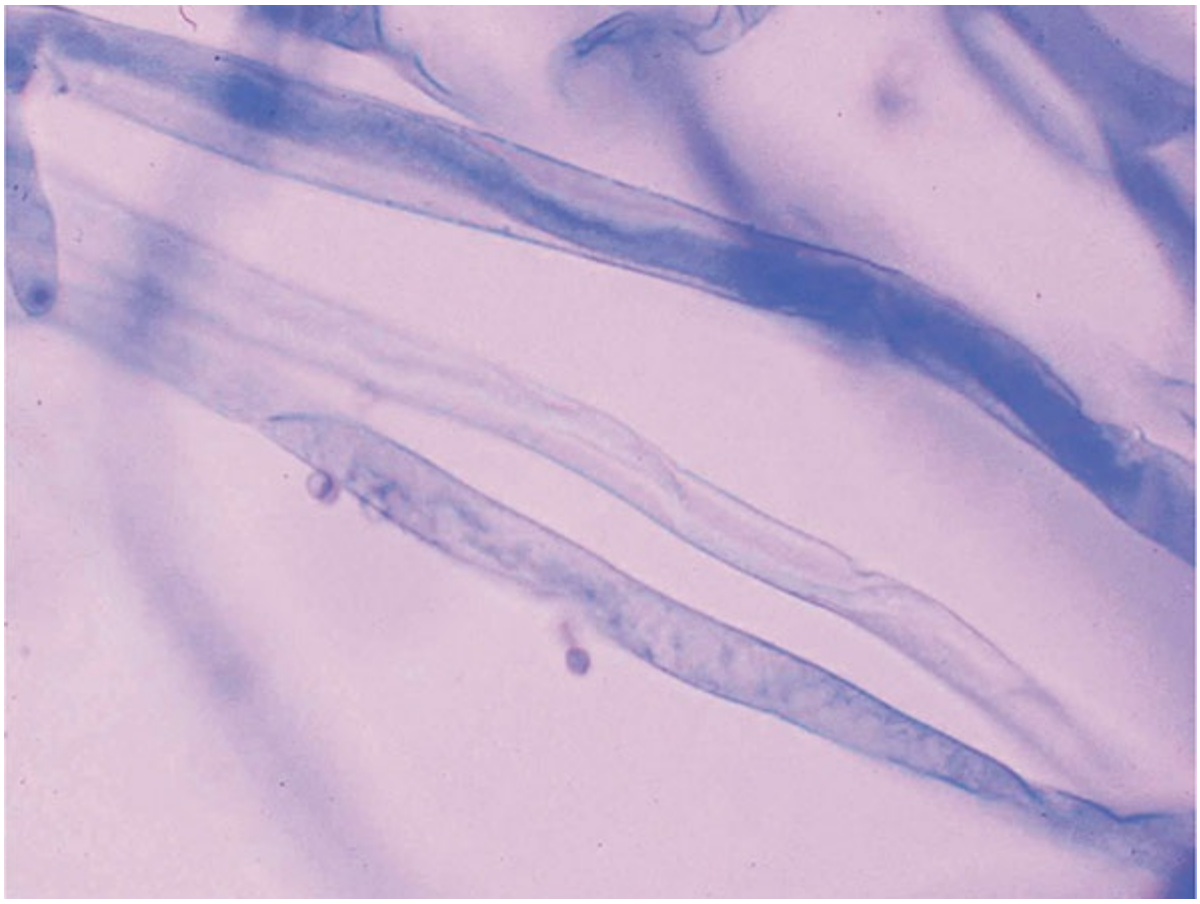
As outlined in Display 21-1, the initial clue to the diagnosis of zygomycosis is the recovery of a rapidly growing colony on primary fungal recovery culture medium, usually within 48 to 72 hours, with the surface of the agar covered with a wooly mycelium that extends from border to border in the Petri dish; these have been referred to as "lid-lifters" because of their rapid growth (Image Plate 21-1A and B). Microscopic examination of a mount made from the colony reveals broad hyphae with irregular width that appears ribbonlike. The hyphae is pauciseptate (i.e., septations are rare) and may be aseptate hyphae in any particular microscopic field (Fig. 21-7). The spores, termed sporangiospores, are produced in saclike structures called sporangia (Fig. 21-8) in the most commonly encounter species (e.g., *Rhizopus* and *Mucor*). Following detection and characterization of the sporangia, the microscopist should search for the presence or absence of

The genus-level identifications, which is all that is medically necessary, can then be made following the criteria outlined in Display 21-1; the descriptions and figure cross-references are presented below.

***Rhizopus* Species.** The key to the identification of *Rhizopus* species is the production of and location of distinct

rootlike structures called **rhizoids.** Rhizoids that are characteristically derived from the hyphae immediately at the base of the sporangiophores are called "**nodal**" (Fig. 21-

10). The sporangiophores of *Rhizopus* species may measure up to 1,000 µm terminate in a concave columella that extends into the saclike sporangium. The umbrella-like collapse of the post-mature sporangium is better illustrated in the close-up view in Figure 21-11. This collapse, which is often observed as a secondary feature in older cultures, is particularly helpful in making a preliminary identification when rhizoids are poorly developed.

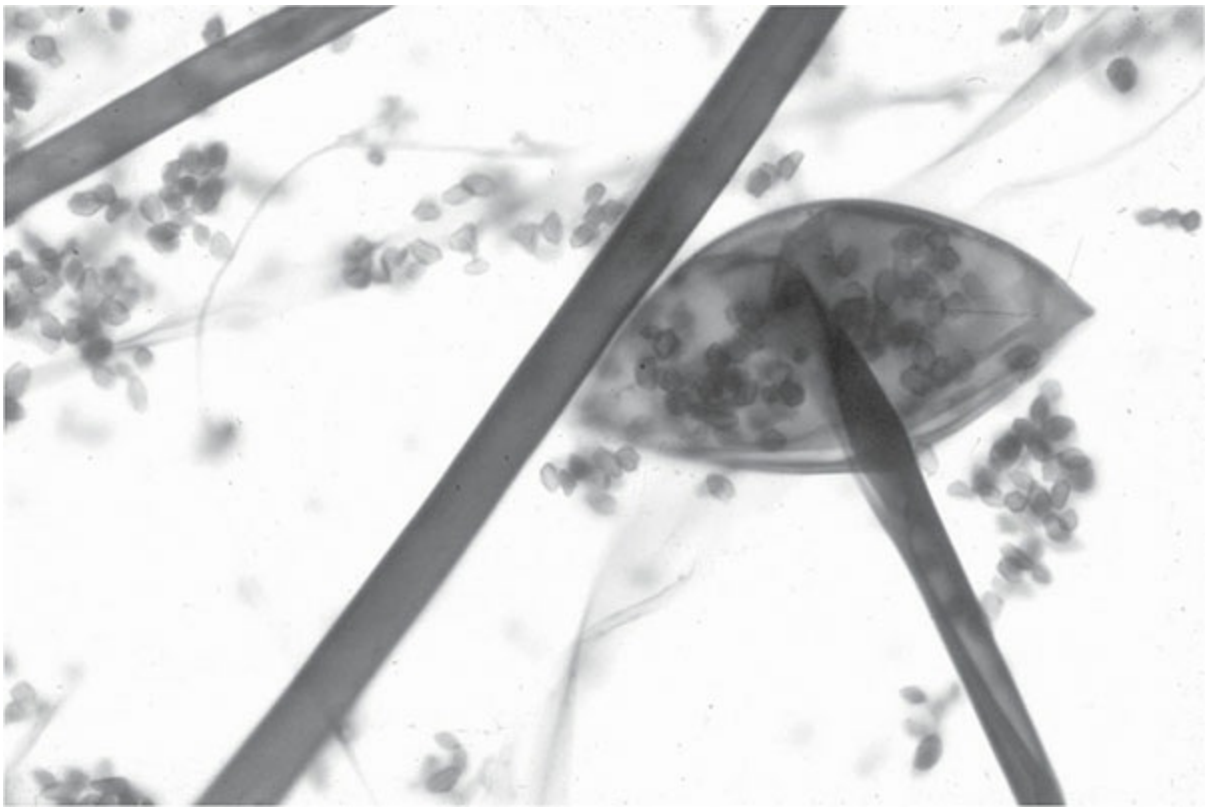


■ **FIGURE 21-7** Photomicrograph of the broad, ribbonlike hyphae of *Zygomycetes* species. Note the clear cytoplasm and the absence of transverse septations in this field.

■ **FIGURE 21-8** Photomicrograph of a sporangium of *Mucor* species, the asexual reproductive structure produced by many of the *Zygomycetes*.

■ **FIGURE 21-9** Photomicrograph of the rootlike rhizoids of *Rhizopus* species, an important differentiating feature within the *Zygomycetes*.

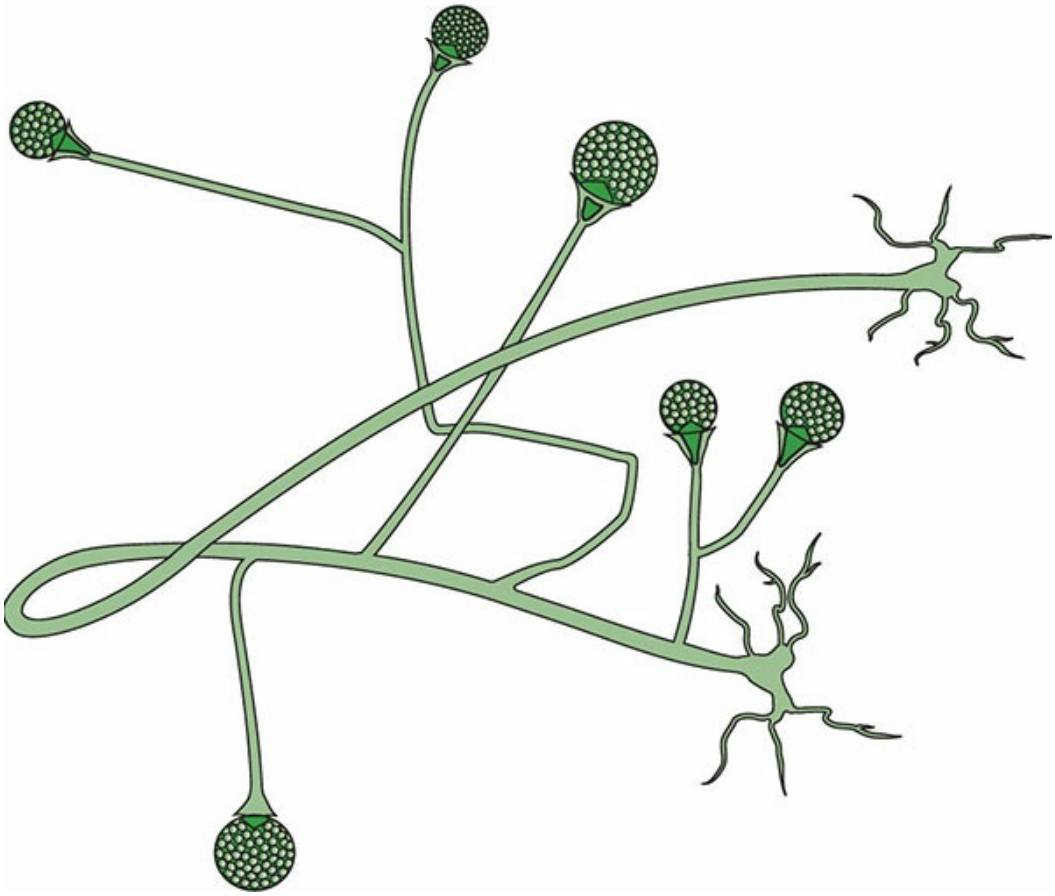
■ **FIGURE 21-10** Photomicrograph of the fruiting structure of *Rhizopus* species. The rhizoids (*arrow*) in *Rhizopus* occur at the base of the sporangiophore in what is termed a "nodal" location. In this preparation, the sporangiophores have burst releasing the sporangiospores.



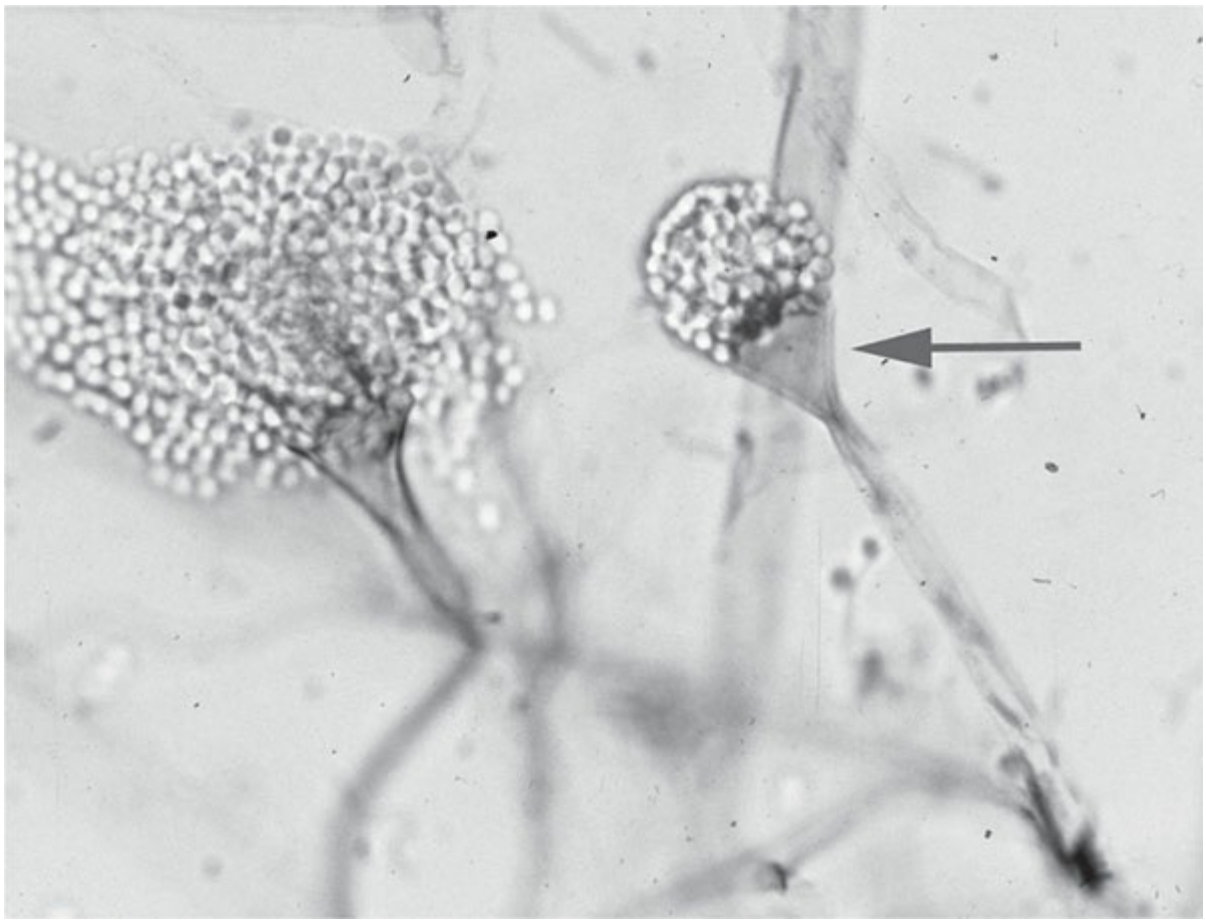
■ **FIGURE 21-11** Close-up view of a post-mature sporangium of *Rhizopus* species illustrating the characteristic umbrella-like collapse.

rootlike structures called rhizoids (Fig. 21-9).

***Lichtheimia* (Formerly) Species.** *Lichtheimia* spp. share with *Rhizopus* spp. the production of rhizoids. The rhizoids of *Lichtheimia* spp., however, differ from those of *Rhizopus* in that they are generally more delicate and have an origin from the hyphae between the conidiophores, a derivation known as "**internodal**" (Fig. 21-12). The sporangiophores may branch. A secondary feature of importance, particularly



■ **FIGURE 21-12** Photomicrograph and sketch of the fruiting structure of *Lichtheimia* species, illustrating the characteristic internodal derivation of the conidiophores. Note the intranodal location of the rhizoids (i.e., not at the base of the sporangiophore).



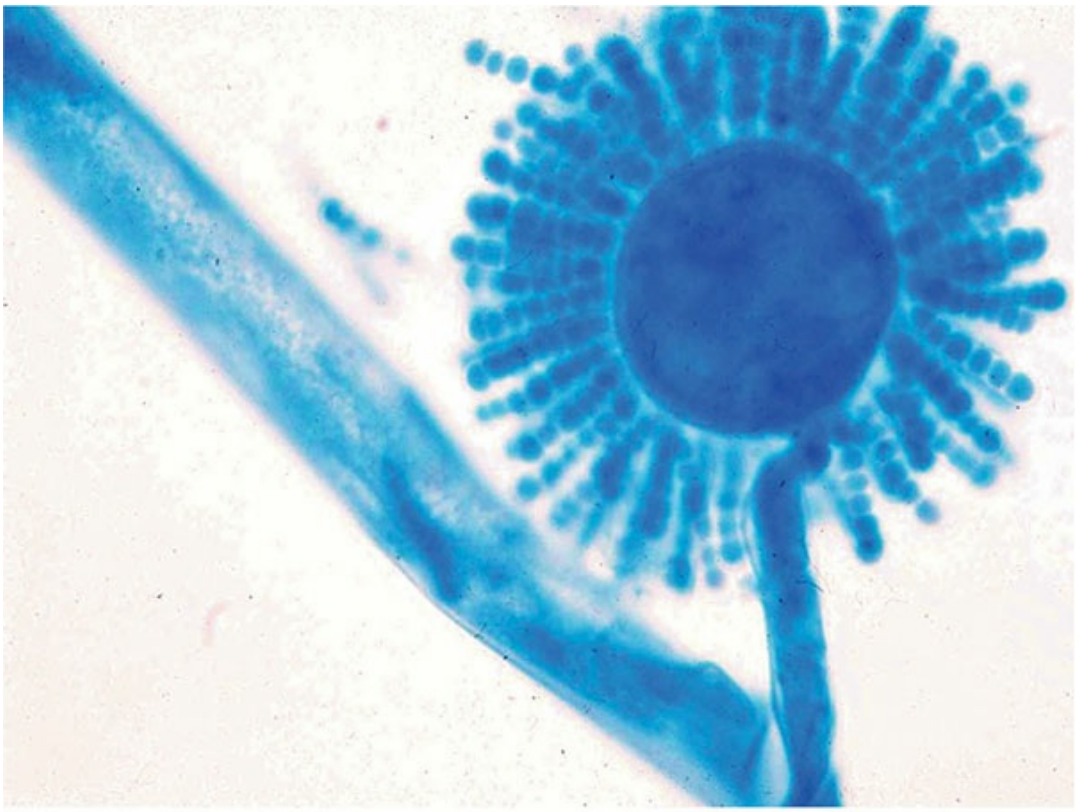
■ **FIGURE 21-13** Higher magnification of the apophysis (*arrow*) of *Lichtheimia* species, the funnel-like expansion of the terminal end of a sporangiophore.

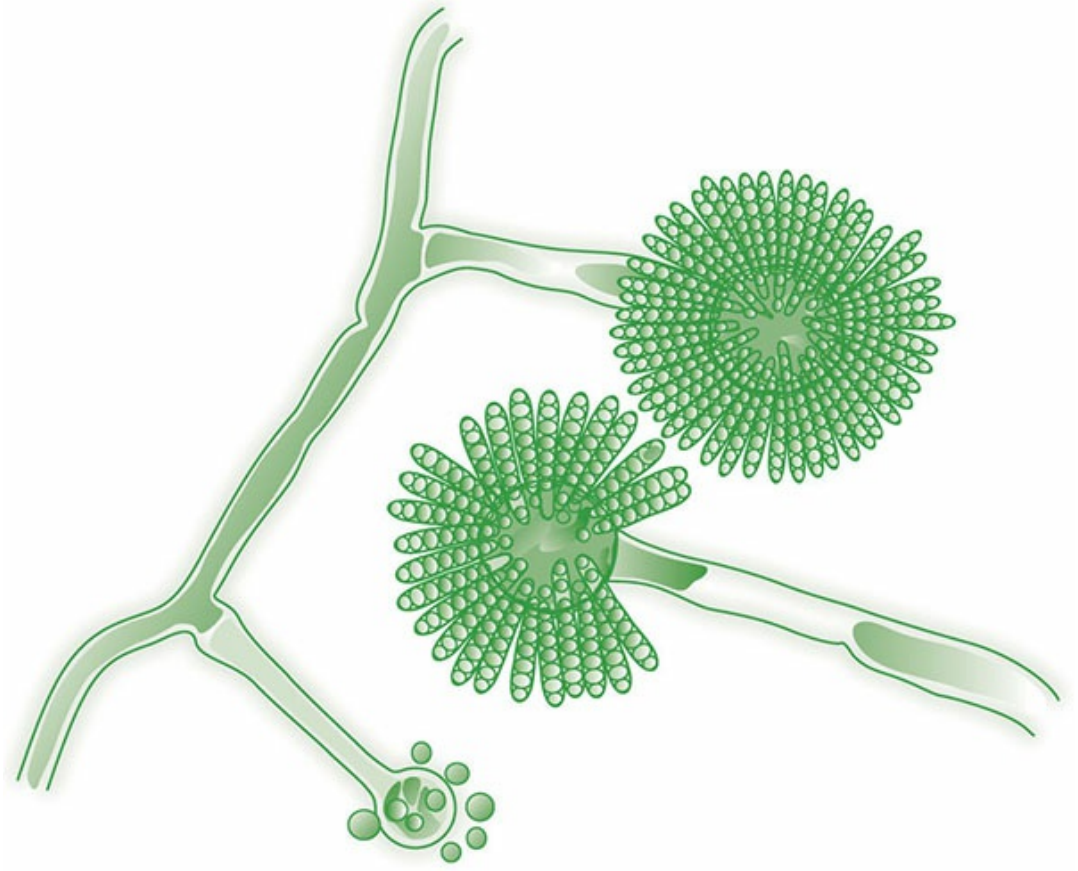
in strains in which the rhizoids are poorly developed, is the funnel-like expansion of the terminal conidiophore into a structure known as the **apophysis** (Fig. 21-13).

***Syncephalastrum* Species.** *Syncephalastrum* spp. differ from the other *Zygomycetes* in the shape and arrangement of the sporangia. Rather than being in the shape of a spherical sporangium, *Syncephalastrum* produces **merosporangia**, which are cylindrical and arranged as "daisy petals" around a relatively small (10 to 50 µm), spherical columella (Fig. 21-14). The sporangiospores are aligned one after another in tandem within each merosporangium. At low magnification, this appearance is reminiscent of *Aspergillus flavus;* however, when the colony morphology, structure of the hyphae and details of the fruiting body are scrutinized, the differentiation is readily

made. Rudimentary rhizoids are occasionally seen with this fungus.

***Circinella* Species.** The key identifying feature of *Circinella* spp. is the distinctive backward curve of the sporangiophores. The sporangiophores are borne laterally from the hyphae, curve directly in reverse and terminate in a globose sporangium that usually is filled with brown-staining





■ **FIGURE 21-14** Photomicrograph and sketch of the fruiting structure of *Syncephalastrum* species, illustrating the radiating sporangia around a spherical columella. At low magnification, these can be mistaken for an *Aspergillus* species by the novice mycologist.

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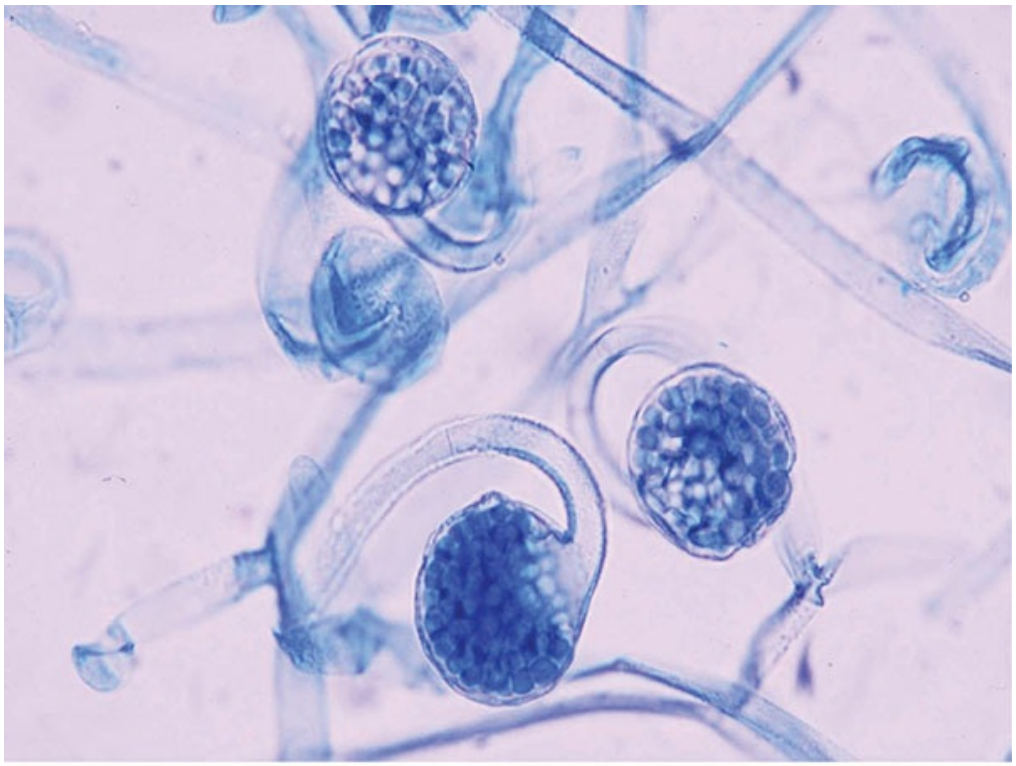
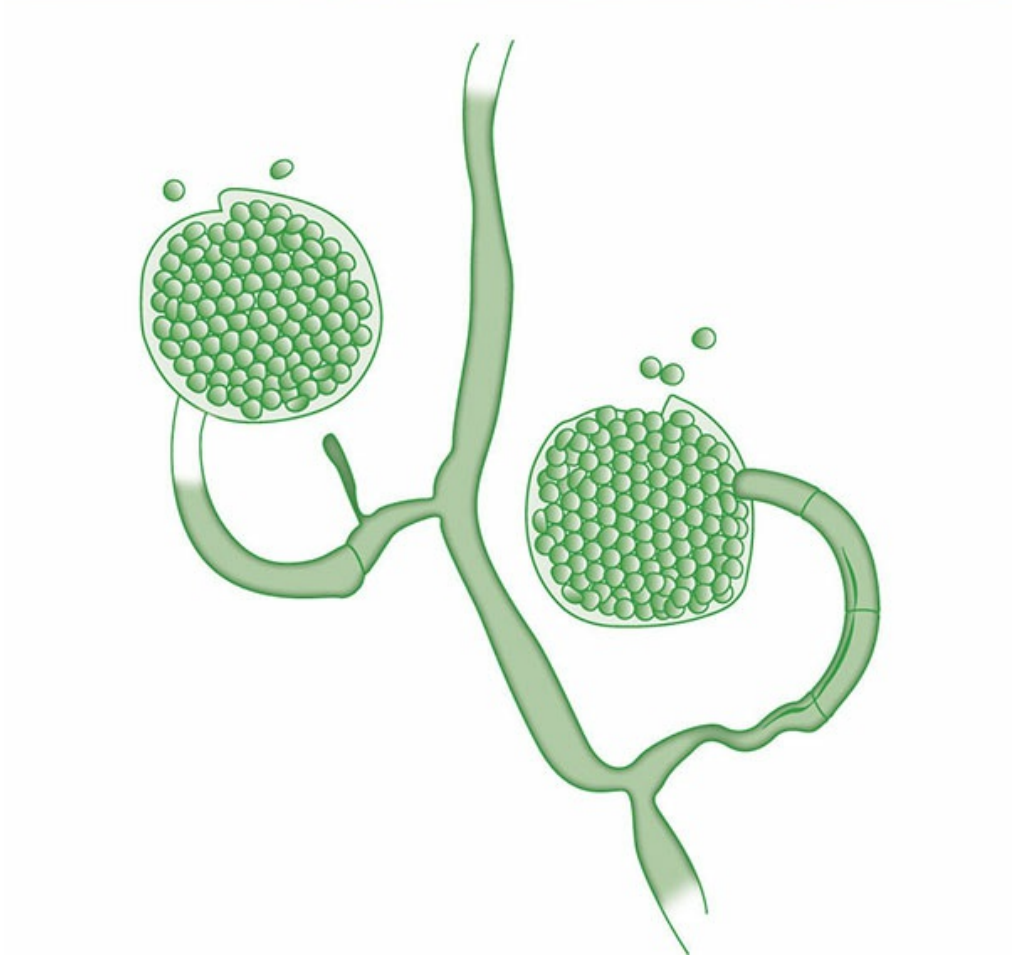
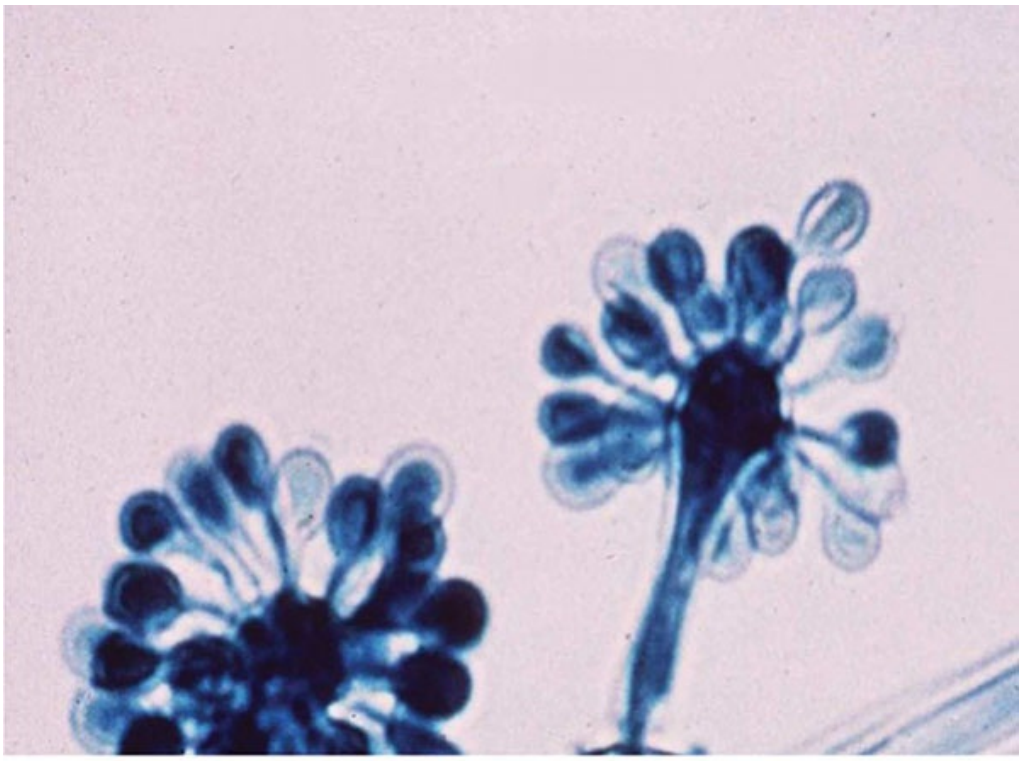
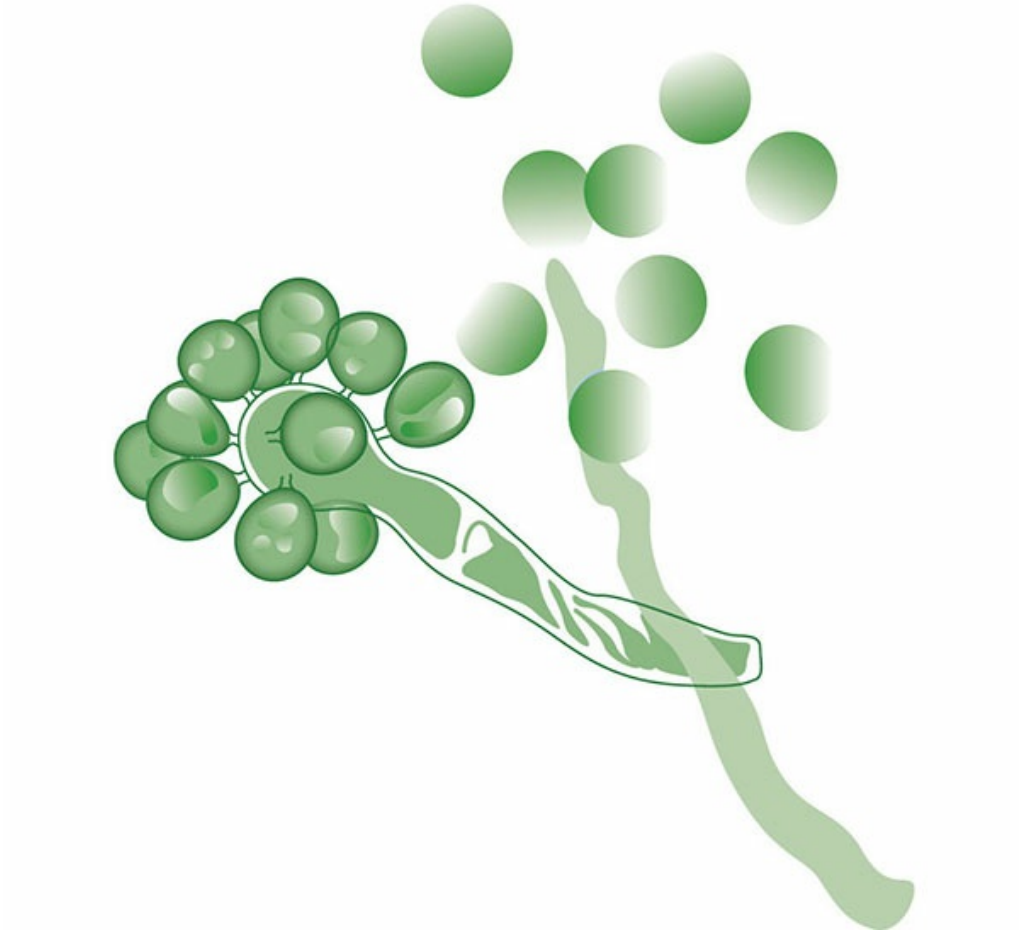




■ **FIGURE 21-15** Photomicrograph and sketch of the fruiting structure of *Circinella* species, illustrating the characteristic backward curve of the sporangiophores.



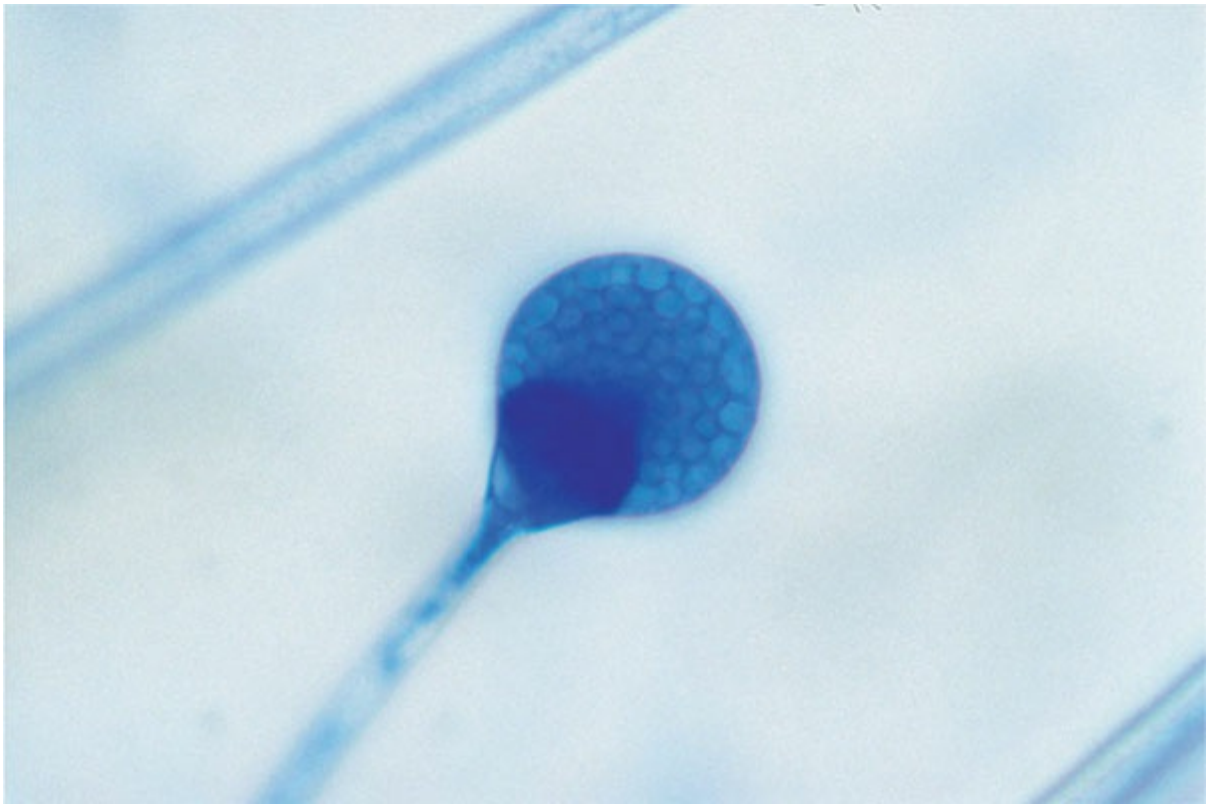


■ **FIGURE 21-16** Photomicrograph and sketch of the fruiting structure of *Cunninghamella* species, illustrating spherical sporangiola arising from the surface of a globe-like columella.

sporangiospores (Fig. 21-15). *Circinella* spp. do not produce rhizoids.

***Cunninghamella* Species.** The sporulation of *Cunninghamella* spp. differs from that of the other *Zygomycetes* by producing specialized spherical spores, called **sporangiola,** from the surface of a large, globose columella; unlike the other *Zygomycetes* described, these are not encased within a sporangium (Fig. 21-16). A tiny hairlike **denticle** may be observed at higher magnification which connects each sporangiole with a spot on the columella.

***Mucor* Species.** The identification of *Mucor* spp. is often made after each of the other *Zygomycetes* described has been excluded. *Mucor* spp. do not produce rhizoids. Sporangiophores are derived singly from the mycelium and may be either branched or not branched. Each sporangiophore ends in a slightly bulbous columella, which extends within a spherical smooth-walled sporangium (Fig. 21-17). The sporangiospores are spherical or ellipsoid and may be hyaline or pigmented yellow-brown.



■ **FIGURE 21-17** Photomicrograph of the fruiting structure of a *Mucor* species. Note the lack of rhizoids. The sporangiophores terminate in a globe-like columella within the sporangia that contains sporangiospores.

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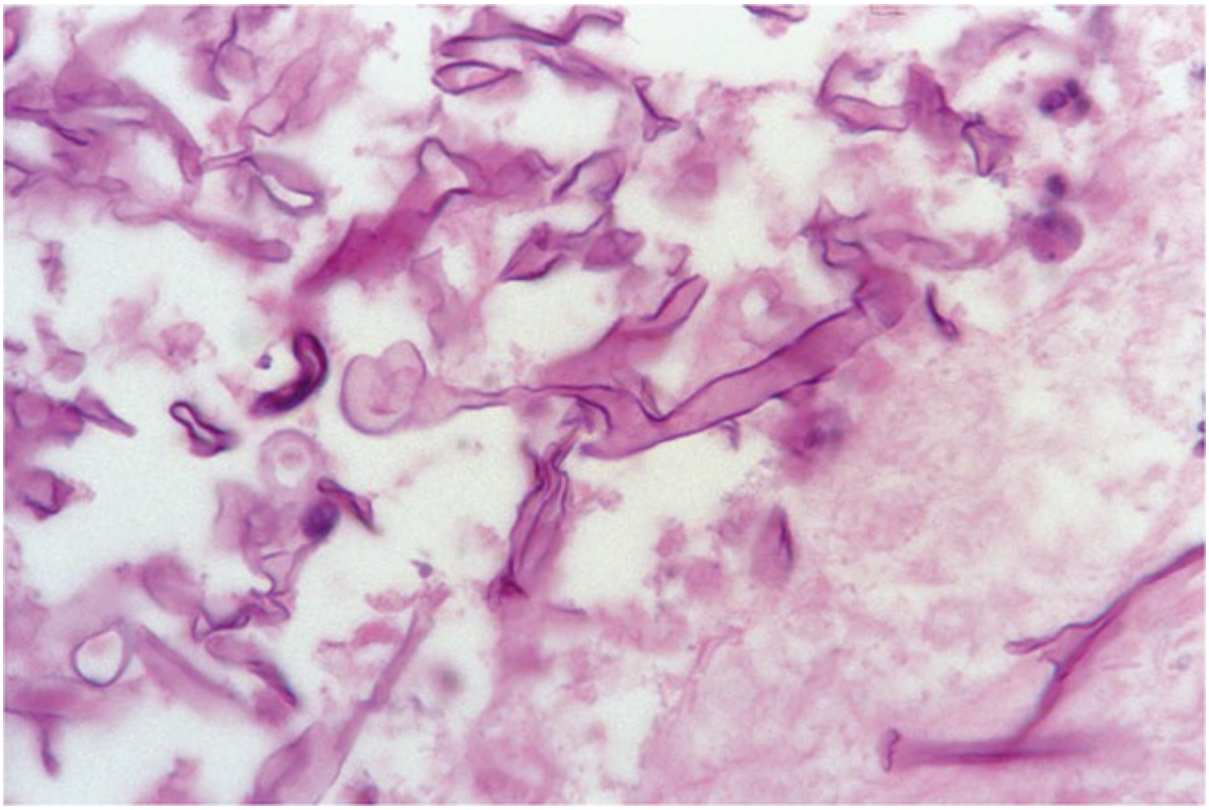

■ **FIGURE 21-18** Photomicrograph of a tissue section illustrating irregular-sized, broad, fragmented aseptate-to-pauciseptate hyphae characteristic of one of the *Zygomycetes* species (H&E, 400×)

Histopathology of Infections Caused by the Zygomycetes.

The ribbonlike, broad, pauciseptate-to-aseptate hyphae of the *Zygomycetes* are generally distinct from the hyphae of other filamentous fungi in tissue sections. The hyphae range from 3 to 25 μm in width, have non-parallel walls, and often tend to break up into small fragments (Fig. 21-18). The hyphae often do not stain well with periodic acid–Schiff (PAS) or Gomori methenamine silver (GMS) fungal stain. The background tissue reaction is typically purulent, and both intact and fragmented hyphae are usually seen amid neutrophils and neutrophilic debria. Occasional septa

may be observed, which is why the designation of pauciseptate is preferred to aseptate. A newer term **mucoraceous hyphae** has been suggested to describe the hyphae from these fungi. The *Zygomycetes* also have a predilection for invading blood vessels, causing hemorrhagic infarction, which is one reason that the infected tissue often appears charred. These fungi often track along the perineurium, as a mechanism of local invasion. In addition to invasive zygomycosis, these fungi can also present as a fungus ball in body cavities; in such locations, the typical fruiting heads complete with sporangia and sporangiospores may occasionally be observed.

The clinical syndromes produced by the *Zygomycetes* are presented in Clinical Correlation Box 21-1.

Hyaline Molds and Hyalohyphomycosis

Ajello[6] originally coined the term "Hyalohyphomycosis" to represent a group of opportunistic fungal infections caused by

CLINICAL CORRELATION BOX 21-1 Zygomycosis

The fungi belonging to the *Zygomycetes* are widely distributed environmental inhabitants of soil, dung, and vegetative matter. Humans usually become infected in the upper respiratory tract through the inhalation of airborne spores, although ingestion of contaminated foodstuffs may result in primary gastrointestinal disease, or direct inoculation of traumatic breaks in the skin and mucous membranes may lead to primary mucocutaneous infection. A proclivity for the hyphae to invade the walls of blood vessels once a primary infection is established results in the dissemination of mycotic thrombi and the formation of metastatic foci in many organs.

Zygomycosis most commonly occurs as opportunistic infections in immunocompromised hosts. Host risk factors include diabetes mellitus, particularly during periods of acidosis, neutropenia, sustained immunosuppressive therapy (as may occur following bone marrow transplantation),[60] the prolonged use of antibiotics, and a breakdown in the integrity of the cutaneous barrier secondary to trauma, surgical wounds, needle sticks, or burns.[233] These authors also indicate that angioinvasive disease is a common complication, resulting in vascular thrombosis, infarction of the involved tissue, and tissue destruction from the action of proteases, lipases, and mycotoxins. Release of mycotic thrombi from the primary sites may lead to disseminated disease. Rhinocerebral, pulmonary, cutaneous, and disseminated forms of disease are most commonly encountered.

A review by Prabhu and Patel[226] reiterates most of the background risk factors

already mentioned. In particular, hematologic malignancy, bone marrow or peripheral blood stem cell transplantation, neutropenia, solid organ transplantation, diabetes mellitus with or without ketoacidosis, overuse of corticosteroids, and deferoxamine therapy for iron overload are cited as causing patients to be predisposed to infection. Emphasis is placed on reducing the high mortality rates, reaching as high as 100%, depending on underlying diseases. Successful outcomes depend on early diagnosis, treatment of the underlying medical condition, surgery when indicated, and amphotericin B therapy. High-dose lipid formulations of amphotericin B and the use of hyperbaric oxygen are the particular strategies that have shown potential value in the treatment of zygomycosis, as outlined by Gonzalez, Rinaldi, and Sugar.[103] Of critical importance is the fact that these fungi are innately resistant to voriconazole. *Rhizopus* is the genus of *Zygomycetes* recovered most frequently in culture from human infections, followed in incidence by *Mucor* species. Other *Zygomycetes* are less frequently encountered. *Cunninghamella* species are infrequently encountered in clinical laboratories. Nevertheless, several case reports indicate that this organism has been recovered as the causative agent of each of the more common forms of zygomycosis—rhinocerebral, pulmonary, cutaneous, and disseminated disease. Kontoyianis et al.[155] describe the common

clinical predispositions of *Cunninghamella bertholetiae* infections; namely, previous corticosteroid therapy, prolonged severe granulocytopenia, and diabetes mellitus. Fever and pneumonia were the most common clinical presentations in this series of patients, usually an indication of disseminated disease. *Cunninghamella* infections are usually rapidly progressive, and the outcome in most cases reported in the literature has been almost uniformly fatal. Aggressive treatment with amphotericin B, resection of infected tissues, and control of the underlying disease may reverse this trend.

Rhinocerebral Disease

This form of infection often begins as sinusitis, progressing to local invasive disease, with edema of the eyelids, proptosis, malar anesthesia, and internal and external ophthalmoplegia. In many instances, infection is rapidly progressive, although slowly progressive, chronic forms have also been reported.[121] A thick, dark, blood-tinged nasal discharge may be observed; material debrided from the sinus may appear blackened and hemorrhagic because of vascular invasion and infarction. Meningitis and cerebritis may develop by direct extension, and are usually fatal. Localized cerebral disease is uncommon, occurring most frequently among IV drug users.[187] Nenoff et al.[197] report an ascending cerebral infection in a patient after transplantation, in whom bilateral total amaurosis (blindness) developed. Cerebral disease has also been reported in patients with AIDS, presumably related to drug

injection. Confusion and mood disturbances are more common presenting signs of cerebral involvement. Space-occupying lesions and brain abscesses are seen in drug addicts. Once cerebral symptoms set in, the process is often rapidly fatal. Noninvasive fungus ball infections of the nasal sinuses may also be seen.

Pulmonary Disease

Ill-defined, finely nodular or diffuse infiltrates may be seen radiologically.[193] **Fungus ball involvement** in preexisting natural or postinfection cavitary lesions may also be seen. Chest pain, hemoptysis, and cough productive of purulent or blood-tinged sputum are common clinical presentations. Subacute pulmonary disease is usually intrabronchial, with the formation of mucin plugs. The pleura may occasionally be involved. Pulmonary infections occur in immunocompromised hosts, including lung transplant recipients.

Cutaneous Disease

Cutaneous disease usually occurs secondary to trauma and soil contamination,[277] in patients with burns,[56] and episodically, from direct implantation with Elastoplast bandages.[214] Occasionally, subcutaneous involvement via hematogenous spread may complicate disseminated disease. Lesions ranging from small violaceous plaques to cellulitis, ulceration, and gangrene may be seen. *Zygomycetes* belonging to the order *Entomophthorales* have recently been cited in cases of subcutaneous disease, particularly in children and young adults in tropical or subtropical locales. Gugnani[105] has reviewed infections caused by *Basidiobolus ranarum*, presenting as fluctuant granulomatous lesions primarily of the legs and trunk, secondary to skin trauma and/or bites of insects. Potassium iodide, amphotericin B, and azole drugs have been successfully used in treatment of these cases.

Disseminated Disease

Virtually any organ may be involved, spreading from primary infections in the lung, sinuses, and gastrointestinal tract.[235] Most commonly affected are individuals who are immunosuppressed because of their age (the very young and the very old), drug therapy, or underlying diseases such as hematologic malignancies (particularly during periods of profound leukopenia), diabetes mellitus, and lupus erythematosus.[133] Although not an AIDS-defining illness, disseminated disease is more likely to occur in HIV-positive patients, as reviewed by Van den Saffele and Boelaert.[280] Metastatic spread occurs from the propensity of hyphae to invade the vasculature, resulting in thrombosis and the release of mycotic emboli into the circulation. Zygomycosis has also been reported in cases of iron overload, specifically in patients undergoing hemodialysis who are receiving deferoxamine therapy.[26] The growth of many microorganisms, including members of the *Zygomycetes*, is enhanced by an increased concentration of iron. The relationship between zygomycosis and deferoxamine therapy hinges on the primary chelating action of the drug, resulting in an increased

local iron concentration that stimulates proliferation of the fungus.

a variety of rapidly growing, saprophytic molds. He thought this designation would be less confusing to physicians than using individual names, such as penicilliosis, paecilomycosis, fusariosis, and the like. Since that time, however, laboratory personnel have become more proficient in making genus identifications of the various fungi included in this group, and physicians have had additional experience in the clinical applications of laboratory reports that list the genus or species names of the more commonly recovered hyaline molds. Culture identification of the causative agent of hyalohyphomycosis should be made. It is important to distinguish these fungi, given more options than ever for systemic antifungal drugs and that some of these fungi are resistant to amphotericin B. Culture-based identification is particularly important, since *Aspergillus* spp. and other hyaline molds that may appear similar in histologic sections. Each mycologist must determine the extent to which genus/species identifications are reported to best suit the needs of the physicians being served.

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Aspergillus *Species and Aspergillosis*

Aspergillus species are commonly discussed as a separate subset of the hyaline molds, given their ubiquity in nature and the frequency with which they are recovered in clinical laboratories as agents of mycotic diseases. *Aspergillus* spp. are widely distributed in nature; they are found in soil, on decaying vegetation, and on a wide variety of organic matter. The inhalation of spore-contaminated dust is the most common mode of infection in humans, resulting in sinusitis or bronchopulmonary disease. Schubert[249] describes three invasive (acute necrotizing, chronic invasive, and granulomatous invasive) and two noninvasive (fungal ball and allergic fungal) forms of fungal rhinosinusitis.

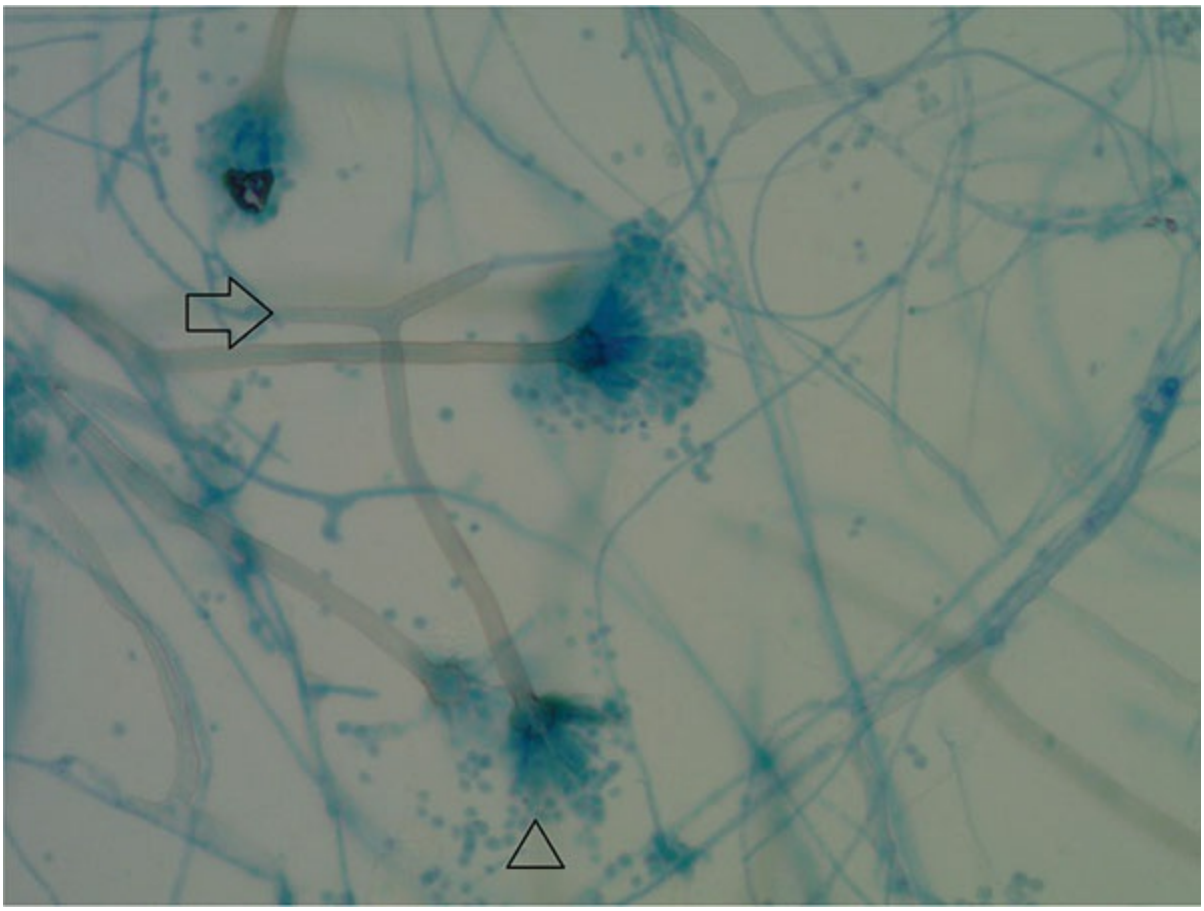
There may be an increase in the rate of infection during periods of building construction, particularly in zones surrounding hospitals, so controlling dust leakage from areas of construction into patient care areas is important. The report many years ago of an outbreak of aspergillosis among cancer patients, related to the fire-proofing operation in the resident hospital is apropos.[4] Surveillance studies should always include sampling of hospital air filters, which commonly reveal high fungal spore counts.[258]Ventilation ducts in particular may become contaminated with dirt and fragments of vegetative matter that become airborne from the disruption of the soil and vegetative undergrowth.[208] The incidence of nosocomial aspergillosis is in direct proportion to the mean ambient airborne spore count, which is highest when mini-

bursts of spores are released into the air via the shaking out of contaminated clothing or from disruption of accumulated contaminated dust on floors or other surfaces during cleanup operations.[232] Patients who are immunocompromised or who are receiving immunosuppressive drugs, notably bone marrow and organ transplant recipients, and those with hematologic malignancies, are particularly susceptible to infection. Therefore, some institutions have opted to hepafilter the air that will enter to rooms of highly immunocompromised patients.

Laboratory Presentation. The *Aspergillus* species most commonly encountered in clinical laboratories include *Aspergillus fumigatus, A. flavus, A. niger,* and *A. terreus.* The majority of serious infections are caused by *A. fumigatus*.[161] *A. nidulans* is also mentioned in this section because most strains readily produce the sexual or telomorphic forms in culture. These forms, termed cleistothecia, enclose meiotically-derived, lemon-shaped ascospores.

Colony Morphology. Colonies of *Aspergillus* spp. can be suspected in culture if an isolate grows within 3 to 5 days and has a distinct outer margin (i.e., a white apron at the advancing area of growth). The appearance as described below may vary depending on the culture medium being used. Early colonies may have a cottony consistency; however, with maturity, the surface becomes more granular as conidia are produced. Descriptions of the colonies when grown on SDA and characteristic of each of the species mentioned above follow.

Microscopic Features. Microscopically, *Aspergillus* species are characterized by the production of uniform, 4 to 6 μm in diameter, **hyaline, septate hyphae** with parallel walls. The **45-degree angle dichotomous branching** so characteristic of the invasive mycelium seen in tissue sections is less commonly seen in microscopic mounts made from culture plates. A specialized hyphal segment, known as a **foot cell,** serves as the base of origin of the **conidiophore** (Fig. 21-19). Conidiophores terminate in a swollen **vesicle,** from the surface of which are borne one (**uniseriate**) or two (**biseriate**) rows of cells. **Phialides** arise directly from the vesicle in a uniseriate conidiophore, whereas with the biseriate conidiophore, there is an inner row of cells are called **metulae** that arise from the vesicle which in turn give rise to the phialides. The phialides are the **conidiogenous cells** that give rise to chains of pigmented conidia (Fig. 21-20). The length, width, and texture (i.e., rough vs. smooth) of the conidiophores; the size and contour of the vesicle; the presence/absence of metulae (i.e., uniseriate vs. biseriate); the arrangement of the phialides; and the color and size of the conidia are features used in making species identifications. The presence of additional "special structures" that may be produced by some species of *Aspergillus,* if present, is useful for the identification of these species. These include the cleistothecia (Fig 21-21) mentioned above, as well as Hülle cells and aleuroconidia.



■ **FIGURE 21-19** Photomicrograph of an aspergillum *(arrowhead)*, the fruiting body of *Aspergillus* species, from *A. nidulans*. The conidiophore (i.e. the aspergillum in this instance) is derived from a foot cell *(arrow)*.

Aspergillus fumigatus. Colonies of *A. fumigatus* are granular to cottony and usually have some shade of blue to blue-green pigmentation (Image Plate 21-1C).

Microscopically, there is a single row of phialides that covers the upper two-third of a club-shaped vesicle (Fig. 21-22). The conidia are spherical, usually smooth, and arranged in a columnar fashion. The conidiophores are relatively long (300 to 500 µm), and, as denoted above, each is derived from a special segment of a septate hypha known as a foot cell (Fig. 21-19).

Aspergillus flavus. Colonies of *A. flavus* are granular to wooly and present with some shade of yellow, yellow-green, or yellow-brown (Image Plate 21-1D).

On microscopic examination, the phialides cover the entire vesicle and the conidia are arranged in what appears to some to resemble a daisy-petal-like arrangement. The conidiophores may be either uniseriate or biseriate (Fig. 21-23). The conidiophores are relatively long (500 to 800 µm). The length of the conidiophores proximal to the vesicle is distinctly roughened, which is a key feature used for the

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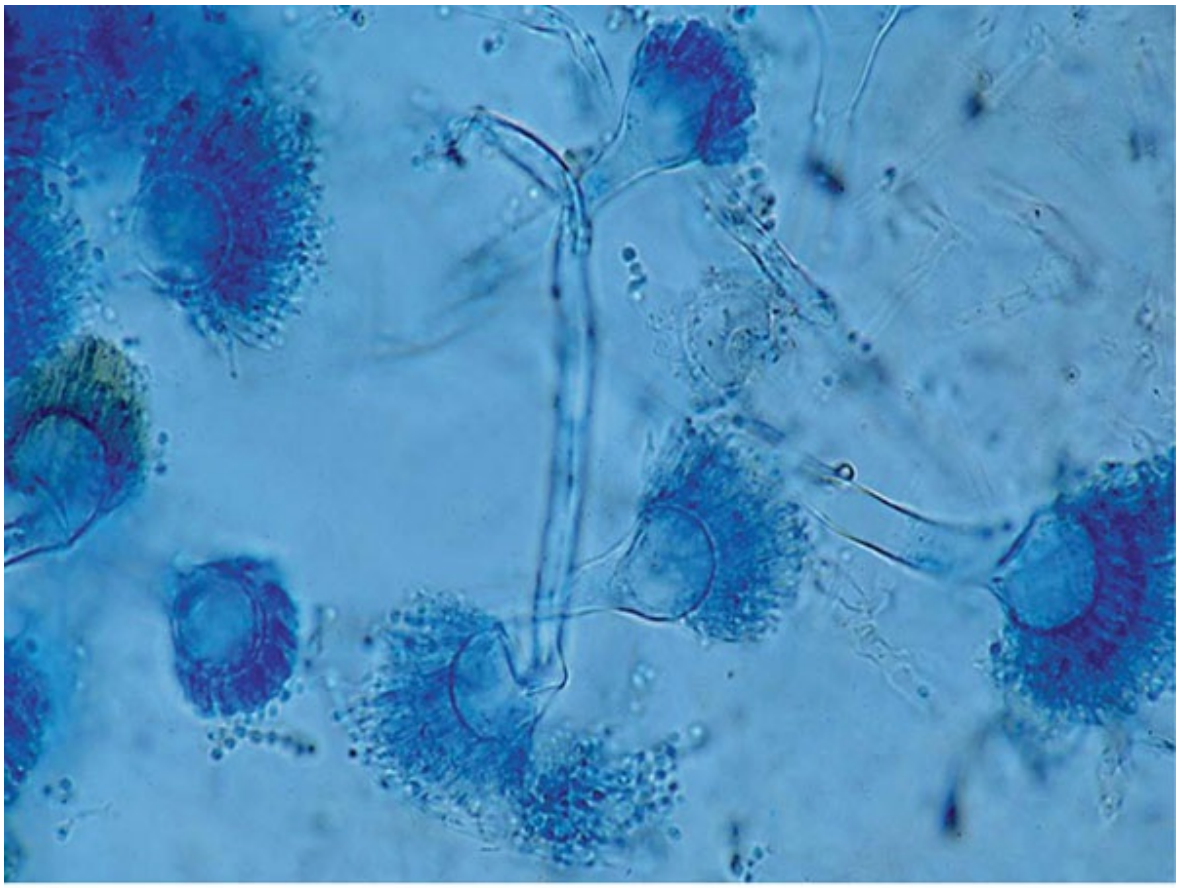
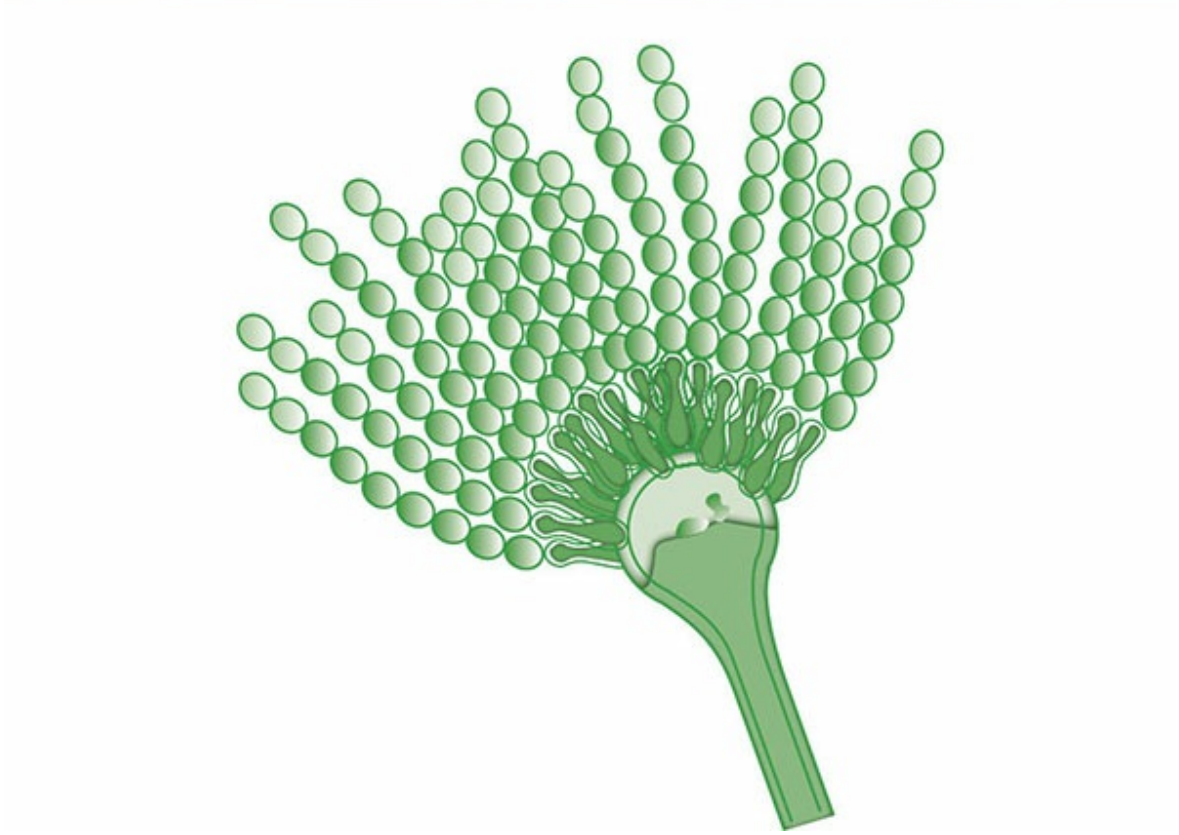
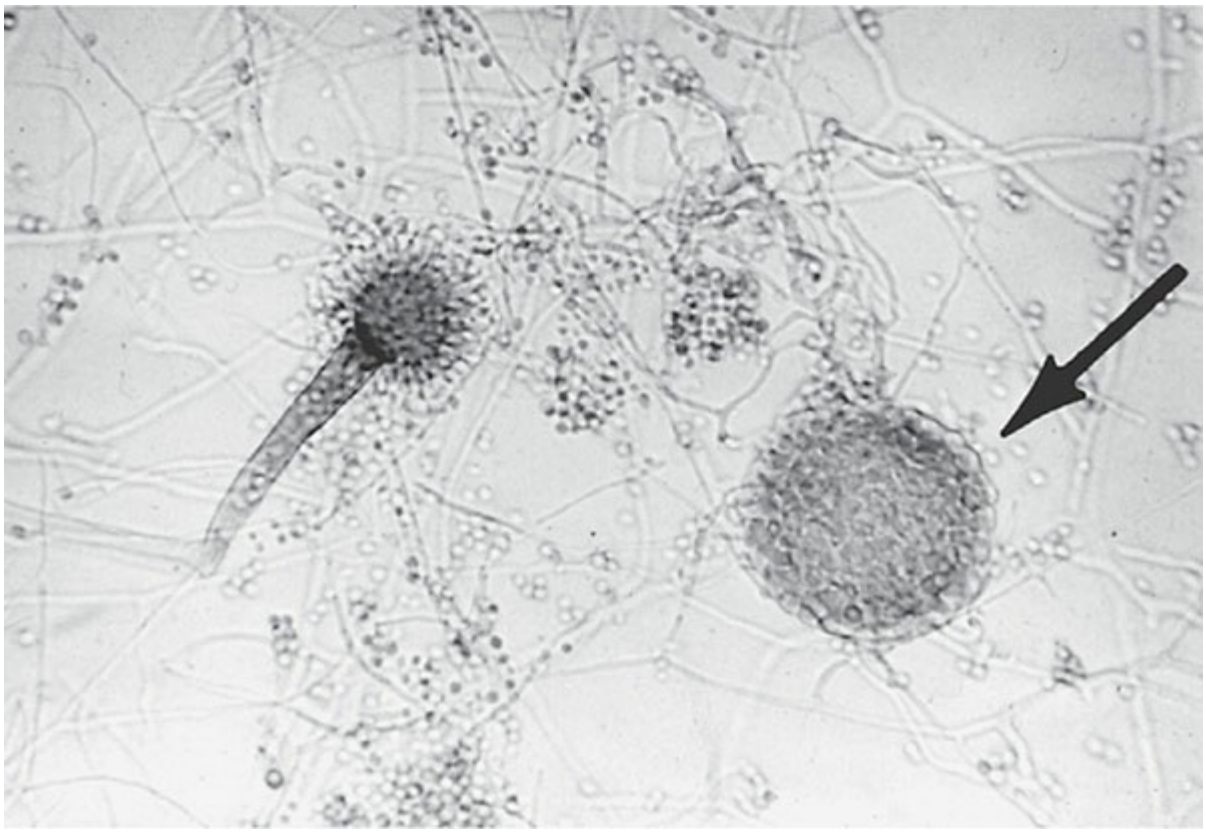
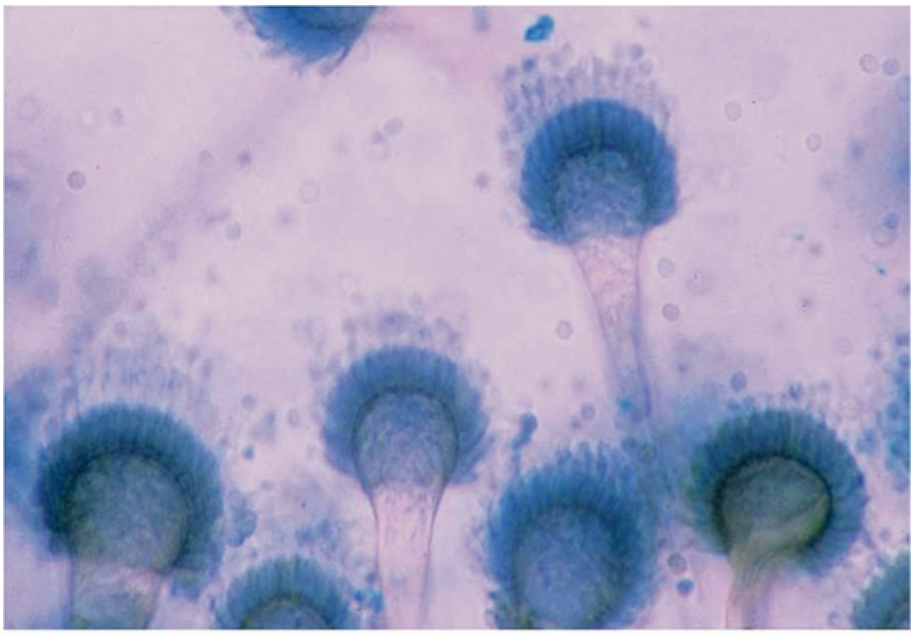




■ **FIGURE 21-20** Photomicrograph and sketch of a generic fruiting head of an *Aspergillus* species, illustrating the swollen vesicle giving rise to phialides from which chains of conidia are produced.



■ **FIGURE 21-21** Photomicrograph of a cleistothecium (*arrow*) of *Aspergillus glaucus,* illustrating the sexual or telomorphic form of reproduction. A typical anamorphic fruiting head (i.e., aspergillum) is seen to the left.



■ **FIGURE 21-22** Photomicrograph and sketch of the fruiting structure of *Aspergillus fumigatus,* illustrating a single row of phialides derived from the top half of a vesicle from which chains of conidia are produced.

identification of this species. The conidia are spherical, smooth, or slightly roughened with maturity, and have a yellow-brown pigmentation.

Aspergillus niger. The surface of a mature colony of *A. niger* is covered by a dense aggregate of jet-black conidia, giving a characteristic peppered effect (Image Plate 21-1E). The reverse of the colony is buff or yellow-gray, in contrast to the deeper brown-black pigmentation of the dematiaceous fungi to be described below.

Microscopically, there is usually profuse conidiation, with dense aggregates of single and short chains of jet-black, rough (echinulate) conidia. The entire surface of the vesicle is covered (Fig. 21-24, A). The conidiophores of *A. niger* are biseriate and the vesicle is globose (Fig. 21-24, B and C).

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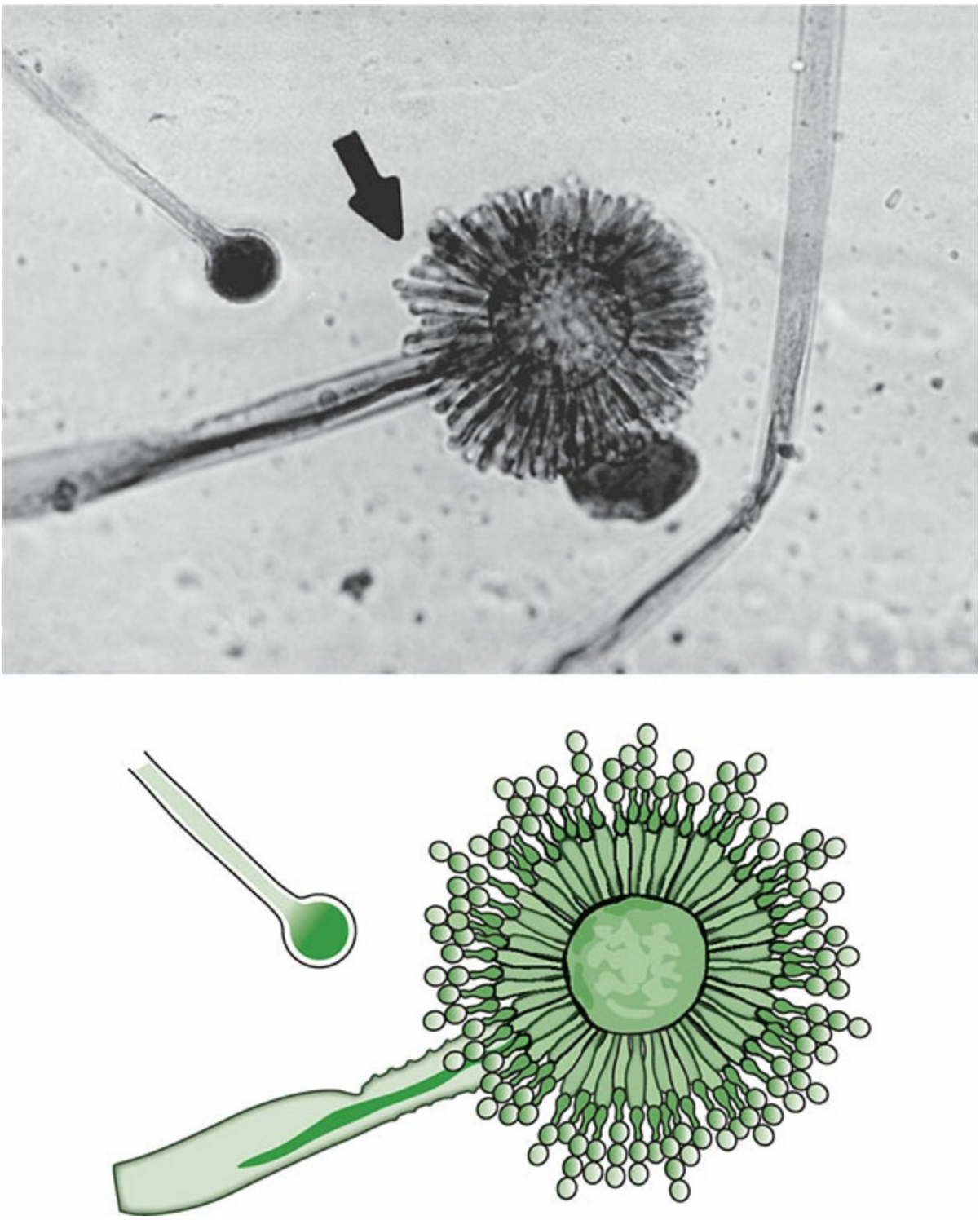


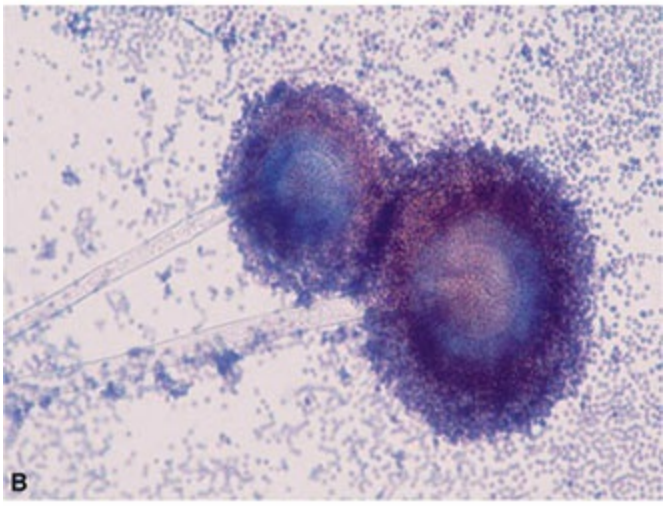
■ **FIGURE 21-23** Photomicrograph and sketch of the fruiting structure of *Aspergillus flavus,* illustrating a central spherical vesicle supporting a double row of phialides derived from the entire surface.

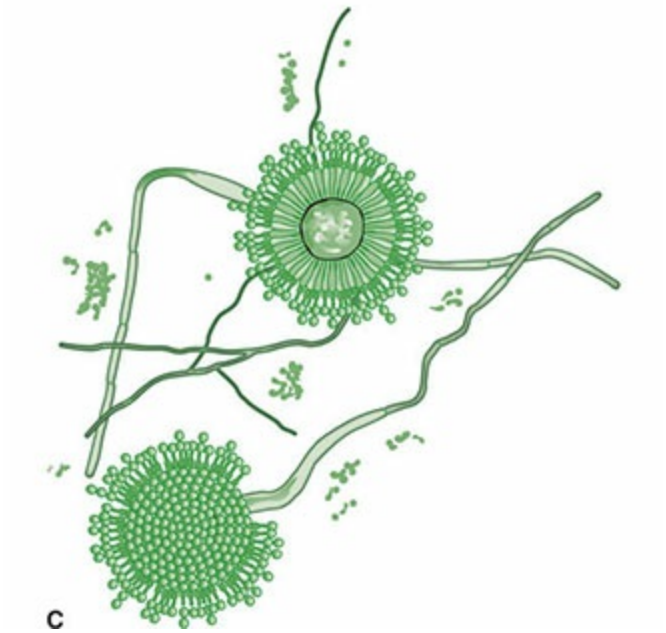
Aspergillus terreus. Colonies of *A. terreus* are granular, radially rugose, and cinnamon buff, brown, or orange-brown (Image Plate 21-1F). This color is not commonly encountered, so when seen it should raise the possibility of *A. terreus*, which is important not to misidentify for therapeutic reasons.

The fruiting bodies (i.e., conidiophores) of *A. terreus* are similar to those of *A. fumigatus* in that phialides cover the upper two-third of the vesicle. Closer examination, however, reveals that the vesicles of *A. terreus* are smaller, the phialides are much longer, and the conidiophore is biseriate; however, the phialides and metulate are somewhat interdigitated, which obscures the line of division between the two cells (Fig. 21-25). The definitive identification of *A. terreus* can be achieved by demonstrating spherical aleuroconidia that arise directly from subsurface hyphae; a dig mount is used to demonstrate these structures (Fig. 21-26).

Aspergillus nidulans. *Aspergillus nidulans* is rarely encountered in clinical laboratories, but a brief discussion is included here because this species commonly produces sexual reproductive structures (cleistothecia and ascospores) in culture, which are discussed below. The colonies are generally white or gray-white, cottony to granular in consistency, and may have radial rugae (Image Plate 21-2G). A dark stippled effect may be noted with mature colonies as cleistothecia are produced. ***Aspergillus glaucus*** is another *Aspergillus* species that should be considered if cleistothecia and ascospores are seen. The colony generally is cottony to

A


B


C

■ **FIGURE 21-24** These photomicrographs (**A, B**) and sketch (**C**) of *A. niger* demonstrate the long conidiophores characteristic of this species (**A**). The conidiation is often dense and arises around the entire vesicle (**B**). The biseriate nature of the the conidiophores is more clearly seen in the drawing (**C**) than in the photomicrographs.

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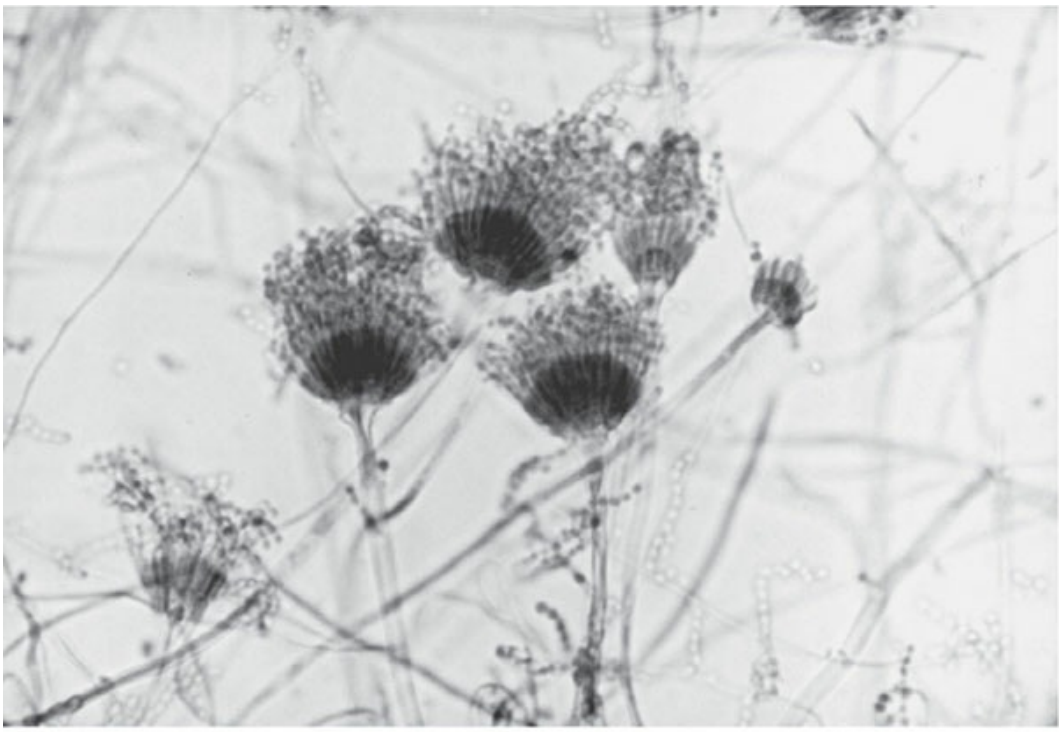
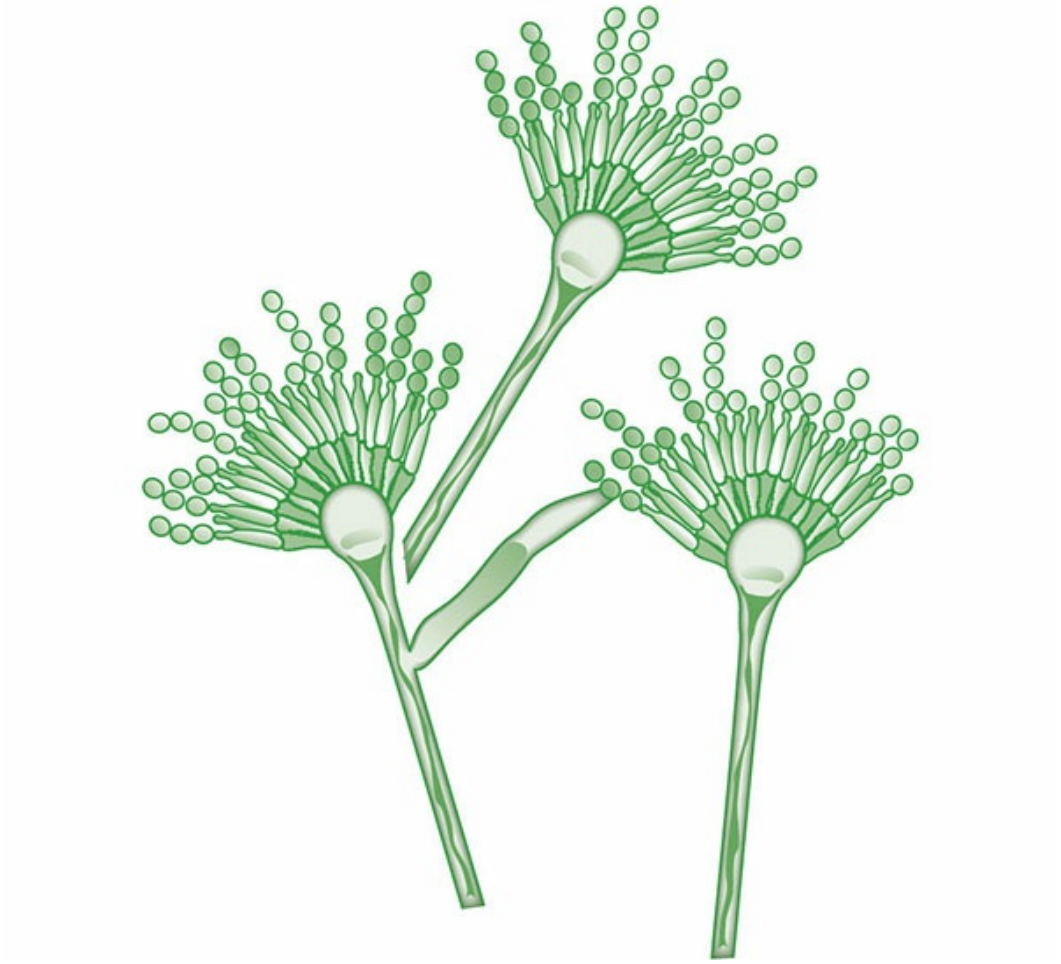
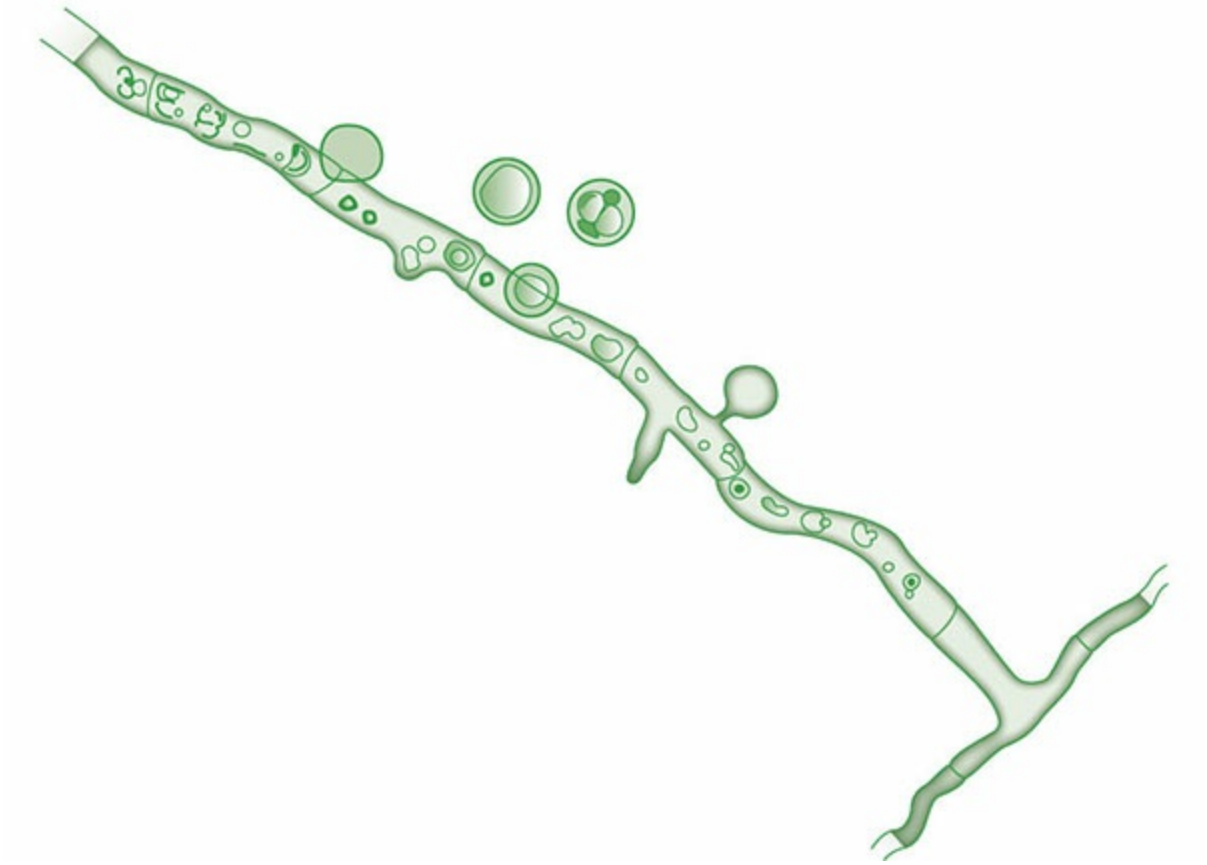
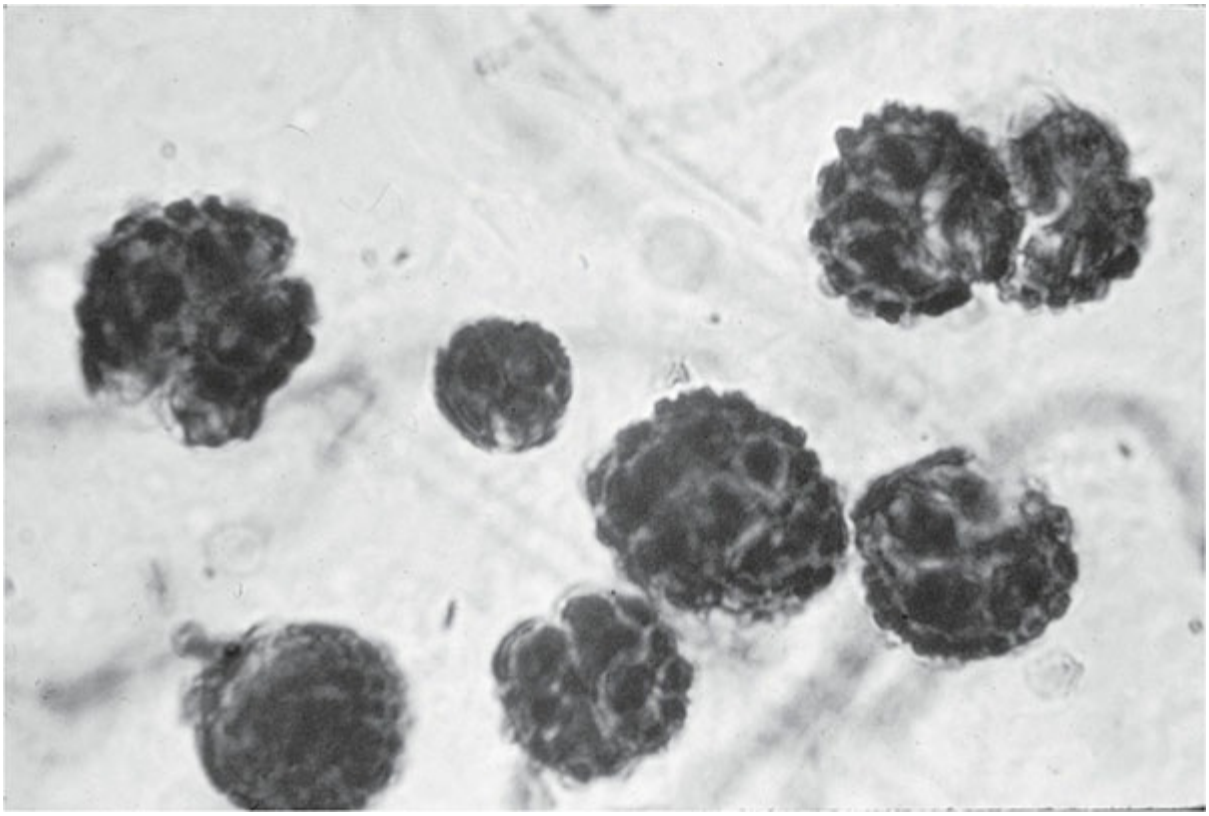




■ **FIGURE 21-25** Photomicrograph and sketch of the fruiting structure of *Aspergillus terreus* revealing conidiophores terminating in a swollen vesicle from the top half of which are derived a double row of sterigmata giving rise to chains of conidia.



■ **FIGURE 21-26** Photomicrograph and sketch of subsurface vegetative mycelia of *Aspergillus terreus,* illustrating spherical aleuroconidia characteristic of the species.



■ **FIGURE 21-27** Photomicrograph of several cleistothecia of *Aspergillus nidulans*; these saclike structures contain ascospores.

granular, and has a variegated green and yellow pigmentation (Image Plate 21-1H).

The aspergilli, belonging to the class *Ascomycetes,* are capable of sexual recombination through the union of two meiotically-derived (i.e., haploid) cells. The sexually reproductive structure that occurs in these and some other medically important fungi are spherical structures without a pore or opening; these are termed **cleistothecia** (Fig. 21-27). Within each cleistothecium are myriad asci, each containing four or eight **ascospores**. In addition to cleistothecia, some strains also produce spherical, hyaline bodies known as Hülle cells; these are the large spherical bodies in Figure 21-28. The precise function of Hülle cells is unknown. However, detection of the presence of Hülle cells is similar to the detection of cleistothecia in that their presence limits the identification of possible *Aspergillus* species to those able to produce these structures. Neither cleistothecia nor Hülle cells are commonly seen in clinical specimens.

Histopathology. The hyphae of *Aspergillus* spp., as observed in stained histologic sections or cytologic preparations, are characteristically hyaline, septate, and regular in outline and have parallel walls. They average from 3 to 6 µm in diameter and are divided by transverse septa (Fig. 21-29). The regular dichotomous branching is at approximately 45-degree angles. Although these features are helpful in differentiating these hyphae from those of *Zygomycetes*, they are not helpful in differentiating *Aspergillus* species from other causes

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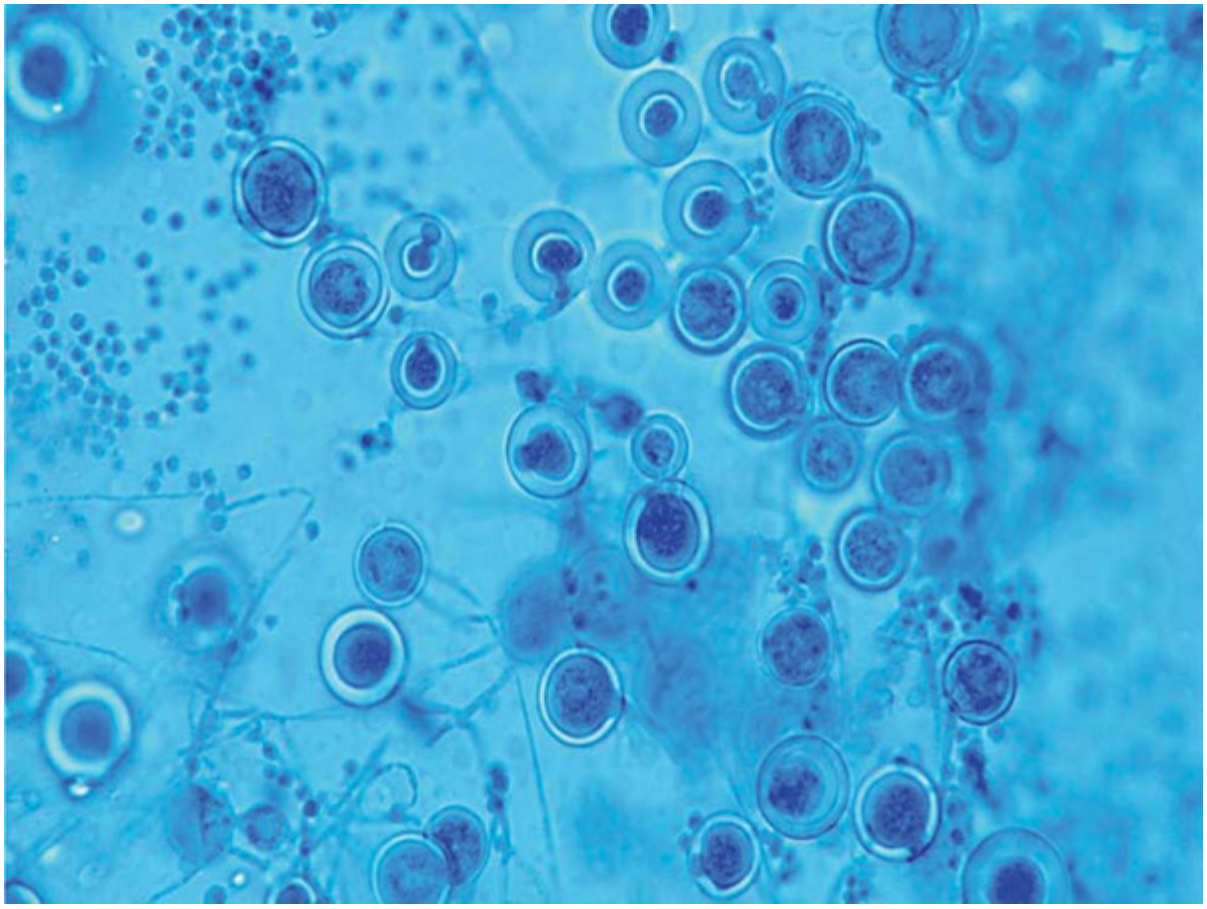
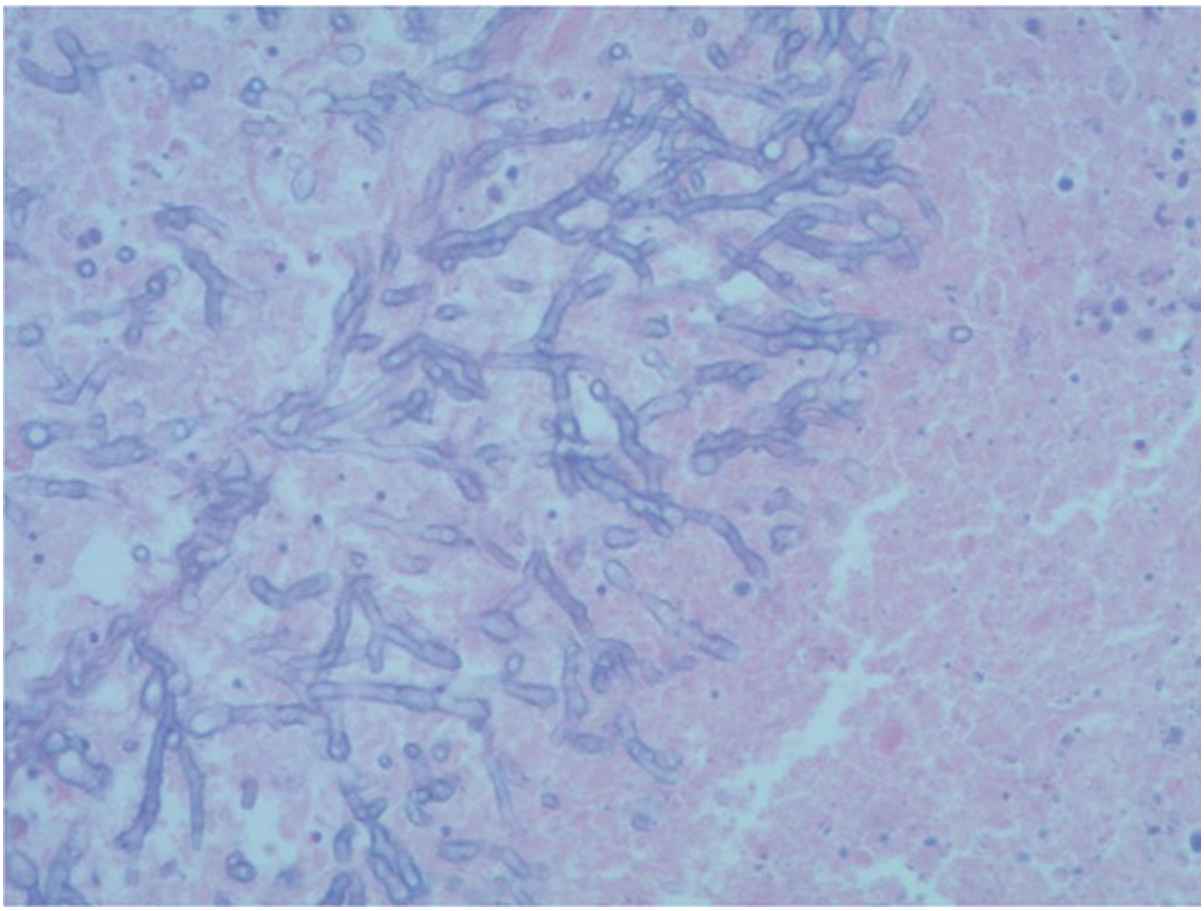


■ **FIGURE 21-28** Photomicrograph of clusters of thin-walled, spherical, hyaline Hülle cells produced by *Aspergillus nidulans*.



■ **FIGURE 21-29** The dichotomously branching hyphae of *Aspergillus fumigatus* are illustrated in the necrotic tissues of this patient with invasive aspergillosis.

of invasive hyalohyphomycosis, such as *Fusarium* spp. or members of the *Pseudallescheria boydii* complex. The hyphae of *Aspergillus* species are usually visible in hematoxylin and eosin (H&E) sections, but are better highlighted using the PAS and GMS stains. The tissue reaction in immunocompetent hosts infected with these fungi may first be purulent, but in some disease states may become granulomatous. In severely immunocompromised hosts, in which infections are most likely to occur, hyphal invasion of the tissues is accompanied by a minimal-to-no cellular response, depending on the degree of neutropenia. In such instances, tissue necrosis may be the only response to injury. *Aspergillus* species, like the *Zygomycetes* and some other fungi, have a particular predilection to invade blood vessels, causing thrombosis and hemorrhagic infarction.

When a fungus colony grows, usually in a noninvasive manner, within a preexisting cavity, such as in a nasal sinus, bronchiectatic airway or within a congenital or inflammatory lung cyst, the lesion is known as a "fungus ball." The hyphae often appear amorphous and stain poorly. Fruiting heads with well-formed vesicles and

chains of conidia may be seen within cavities that are connected to open bronchi and exposed to air (Fig. 21-30). The lining of the cavity is often intact, with no evidence of extension into the surrounding tissue. Although any filamentous fungus can cause a fungus ball, *Aspergillus niger* is recognized as a common cause of fungus ball formation. The presence of black, rough (echinulate) conidia is a clue to the presence of this mold, if sporulation occurs. In addition, this fungus produces oxalic acid as a metabolic by-product and birefringent oxalate crystals, which have a "sheaf of wheat" appearance, may be seen. It is important that the exact crystal morphology is seen, since other types of crystals may be produced by other fungi.

■ **FIGURE 21-30** This photomicrograph from an *Aspergillus* fungus ball illustrates a typical aspergillum with the conidiophore terminating in a club-shaped vesicle upon which the phialides arise. (H&E stain, 400×)

Bronchopulmonary aspergillosis is a disease that is really misnamed, since fungi other than *Aspergillus*, as well as aerobic actinomycetes, cause the disease. This is caused by the inhalation of large quantities of these organisms. The aerosolization of hyphae fragments and filamentous bacteria when the material (e.g., harvested corn or grain) on which these organisms are growing is disturbed. The bronchi and bronchioles of the patient often are dilated and filled with viscid, mucinous material in which are

trapped cellular debris, many eosinophils, scattered neutrophils, lymphocytes, plasma cells, Charcot-Leyden crystals and hyphal or bacterial fragments. Invasion into tissue is not seen.

Diagnosis Using Nonculture Techniques. The galactomannan assay is a sandwich enzyme-linked immunosorbent assay (ELISA) kit that is currently available in many reference laboratories. This assay provides a means for the detection and monitoring of circulating galactomannan.[185] Although variation in sensitivity and specificity of this procedure has been found in different clinical trials and research studies, if used appropriately, this assay represents a major advance in the early detection of invasive aspergillosis in patients at risk for disseminated disease. Under study are the possible factors that affect the release of the *Aspergillus* antigen, the nature of the epitope that reacts with the monoclonal antibody used in the ELISA, its diffusion from the site of infection, and the manner in which it binds to substances present in the blood. This assay is best used to *monitor* the galactomannan

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levels in the blood of a patient at risk, rather than attempts to use it as a single, diagnostic test. Monitoring levels allows the clinician to follow trends and diminishes the chance of acting on a single spuriously high false-positive reaction.

Musher et al.[195] evaluated the performance of the galactomannan assay (GM EIA, Bio-Rad, Benicia, CA) in the detection of *Aspergillus* antigen or DNA in bronchoalveolar lavage fluid obtained from patients with invasive pulmonary aspergillosis (IPA). In the study of 47 patients with IPA and 46 control patients, the GM EIA assay performed with a sensitivity of 76% and a specificity of 94%. These authors cite the relatively low cost, ease of processing, and rapid results yielded by this assay using bronchoscopy as a viable alternative to an invasive biopsy to establish a diagnosis of invasive aspergillosis, particularly in high-risk patients. Similarly, Hayden et al.[122] found that galactomannan antigenemia preceded clinical, microbiologic, and radiologic evidence of invasive aspergillosis in pediatric oncology patients.

The several clinical presentations of aspergillosis are presented in Clinical Correlation Box 21-2.

CLINICAL CORRELATION BOX 21-2 Aspergillosis

Aspergillosis may present as well-defined clinical syndromes involving a variety of sites and organ systems: pulmonary, disseminated, central nervous system, cutaneous,

endocardial, and naso-orbital. Pulmonary aspergillosis is the most common, since the infective conidia usually gain access to the body through inhalation. Three broad categories that have been used to describe the involvement of the lungs by *Aspergillus* species are (1) the fungus ball, (2) allergic bronchopulmonary aspergillosis, and (3) invasive aspergillosis, which may be further subcategorized.[254]

These clinical forms of pulmonary disease are distinct entities, but invasive aspergillosis could develop from either of the former, depending largely on the immunologic status of the patient. Disseminated aspergillosis, which most commonly occurs in severely immunosuppressed patients, often begins as IPA. Dissemination may involve any organ in the body, including the brain with the formation of a fungal brain abscess.

Fungus Ball

A fungus ball is a mass of hyphae (i.e., a mycelium) from one or more molds that are growing in a saprophytic nature in a preexisting cavity. Old cavitary lung lesions caused by tuberculosis are a favored site of growth, in areas with a high prevalence of tuberculosis. The sinus cavities, pathologically dilated airways (i.e., bronchiectatic) and the external auditory canal are also sites wherein fungus balls may occur. A wide variety of fungi may be the cause of a fungus ball, but *Aspergillus* species, particularly *A. niger*, are well-recognized causes. A fungus ball that is caused by an *Aspergillus* species may be termed an aspergilloma. Interestingly, in many instances the fungus may outgrow its saprophytic food supply and die; thus, the fungus ball identified by the surgical pathologist or seen in the direct examination may not grow in culture. When the fungus is present in a cavity that connects with the air supply, then conidiation may occur; therefore, an aspergilloma may be definitively identified on a morphologic basis, if an aspergillum fruiting head is detected.

In most instances, the fungus ball remains confined to the cavity without invasion of the surrounding parenchyma. Regardless, the histopathologist should examine all associated tissue to assure the absence of invasion. Importantly, Nolan et al.[200]warn that aspergillomas should not be considered an indolent condition, as instances of intermittent or exsanguinating hemoptysis have been reported; in addition, erosion into adjacent structures may result in morbidity or death.

Allergic Bronchopulmonary Aspergillosis

Allergic bronchopulmonary aspergillosis is somewhat of a misnomer in that it is a usually caused by the inhalation of a variety of aerobic actinomycetes and fungi, which may or may not include *Aspergillus* species. Hallmarks of disease include peribronchiolar inflammation, infiltrating eosinophils, and Charcot-Leyden crystals trapped within mucin plugs expectorated in sputum specimens. Lee et al.[162] suggest that the following criteria pertain to make a diagnosis of allergic

bronchopulmonary aspergillosis: episodic bronchial obstruction, peripheral blood eosinophilia, cutaneous reactivity to *A. fumigatus* antigen, precipitating serum antibodies to *A. fumigatus*, elevated total serum IgE, history of pulmonary infiltrates, elevated serum IgE and serum IgG to *A. fumigatus*, and proximal bronchiectasis.

In cases of bronchopulmonary aspergillosis, serum IgE and IgG against *Aspergillus* fumigatus may be elevated in both serum and bronchoalveolar lavage specimens. The most common *Aspergillus* species causing allergic bronchopulmonary disease are *A. flavus* and *A. fumigatus*.

Invasive Pulmonary Aspergillosis

This form occurs almost exclusively in patients who are immunosuppressed or have neutropenia, particularly in those with leukemias and lymphomas.[283] The disease often presents as pneumonia (cough, fever, signs of respiratory distress). Pleural invasion may result in chest pain and a pleural friction rub. Because of the propensity for advancing hyphae to invade blood vessels, disseminated disease with metastatic spread to the central nervous system and other organs is possible.

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Although *Aspergillus* fumigatus and less commonly *Aspergillus* flavus are the two species most commonly involved,[161] *Aspergillus terreus* has also recently been incriminated. Woods and Goldsmith[303] reported four cases of disseminated disease caused by *A. terreus*, and reviewed five additional cases reported in the medical literature. In a more recent review of invasive *A. terreus* infections, Iwen et al.[134] culled 13 patients from the literature, 10 of whom died following distant dissemination of the disease. Most of the involved strains were resistant to amphotericin B.

Disseminated Aspergillosis

Because *Aspergillus* species has a propensity to invade into blood vessels, widespread dissemination into virtually any tissue or organ remains a threat in any cases of localized disease. Denning and Stephens,[67] in a review of over 2,000 cases of aspergillosis culled from the literature, found that aspergillosis in bone marrow transplant recipients is particularly devastating, with a mortality rate of 94% in reported cases, despite therapy. The overall rate of response to amphotericin B therapy of all cases reviewed in this series was 55%.

Ho and Yuen[127] postulate that the high mortality rate caused by invasive aspergillosis among bone marrow transplant recipients is the delay in diagnosis because an inflammatory response is blunted by immunosuppression.

Although AIDS per se is not considered a risk factor for IPA, the underlying risk factors, including leukopenia, use of corticosteroids, and IV drug abuse, put these patients at particular risk. In a series of patients with AIDS with aspergillosis reviewed by Singh et al.,[259] 79% were found to have one or more of these known predisposing risk factors. Invasive aspergillosis most commonly occurs in the later stages of AIDS, when the $CD4^+$ count is low, and when diagnosed it has a dismal prognosis. Only early diagnosis can improve the prognosis.[144]

Miscellaneous conditions associated with *Aspergillus* infections include otitis externa ("swimmer's ear," a chronic local inflammation of the auditory canal characterized by itching, pain, and scaling), which is most commonly caused by *A. niger*. Most cases can be cured by topical antifungal therapy and avoiding water exposure. *Aspergillus* sinusitis, often indistinguishable clinically from viral, bacterial, or allergic sinusitis and occurring in immunocompetent hosts, has occurred in at least 29 reported cases since 1987.[47] Several isolated cases of aspergillosis involving virtually every organ system have been reported, with predisposing conditions in these studies most commonly related to prolonged neutropenia, hematologic malignancies, spinal trauma or surgery, and prolonged used of corticosteroids.

Other Hyaline Septate Molds

Colony Characteristics. Display 21-2 outlines some of the other important hyaline septate molds or members of the hyalohyphomycetes. These can be suspected if rapidly growing, cottony, wooly, or granular colonies are recovered in culture within 3 to 5 days, usually presenting with a variety of surface colors, often of the pastel variety. The colonies are typically entire and have an outer margin, with the exception of *Gliocladium* spp. and *Trichoderma* spp., the colonies of which grow border to border over the surface, forming a green or yellow "lawn." Colonies appear more or less granular on the surface, depending on the degree of sporulation. The reverse of hyaline molds is light gray or buff in color.

Although in the past many of these were considered to be nonharmful saprophytes, several of the hyaline molds have emerged during the past two decades as agents of opportunistic fungal infections with significant morbidity and mortality. The patients at risk for infections by these fungi are the same as those at risk for invasive aspergillosis, namely those with severe underlying illnesses and compromised host defenses. These emerging infections are directly related to the success of medical community in controlling or curing certain diseases, but with therapeutics that produce either a transient or permanent immunosuppression. Examples include chemotherapeutics producing bone marrow suppression, immunosuppressive regimens used to control rejection in patients who have undergone transplantation, and new immunomodulating

drugs, like tumor necrosis factor-α inhibitors used to control or suppress inflammation in diseases such as rheumatoid arthritis, among others. The opportunistic fungi that cause disease in this setting include *Fusarium* species, *Acremonium* species, *Paecilomyces* species, *Pseudallescheria boydii* complex, and *Scedosporium prolificans*, among others.

The individual hyaline molds of medical significance, and most commonly recovered in clinical laboratories, are divided into three subgroups, depending on whether the conidia are: (1) produced in chains from phialides, (2) produced in clusters from conidiophores, or (3) borne singly and directly from the hyphae (Display 21-2). Each of these subgroups is presented in the following paragraphs.

Genera of Hyaline Filamentous Molds Producing Conidia in Chains

The hyaline molds featured here are characterized by the production of conidia in chains (Display 21-2).

***Penicillium* Species.** The colonies of *Penicillium* spp. are granular and entire, and generally those most commonly encounter produce surface colors in various shades of green, although yellow and yellow-brown variants are sometimes encountered (Image Plate 21-2A). Color is an important feature for the differentiation of these organisms from *Paecilomyces* species, which may produce a superficially similar conidiophore; *Paecilomyces* are not green. The vast majority of *Penicillium* species that are encountered are contaminants. It is important to accurately identify these both to finalize the culture and to differentiate these fungi

from *Paecilomyces,* which may be a pathogen. One species of *Penicillium*, *Penicillium marneffei*, has been shown to be a pathogen in patients with AIDS. This species is a dimorphic pathogen, producing a yeast-like form in the human body, and an infection similar to that of disseminated histoplasmosis in this population. This species, which is geographically limited to Southeast Asia, produces a wine-red pigment that diffuses into the agar (Image Plate 21-2B). The identity of isolates suspected to possibly represent *P. marneffei* can be confirmed by demonstrating thermal dimorphism or through molecular methods.

Microscopically, all *Penicillium* species produce **"penicillus"** (brush) structures. These are a conidiophore with the terminal production of primary metulae and secondary phialides, the latter of which produces chains of conidia (Fig. 21-31). It is important to observe that the tips of the phialides are blunt, in contrast to the long and tapered phialides produced by *Paecilomyces* and *Purpureocillium*. The conidia are spherical, regular in size, and evenly staining, representing an **acropetal** type of

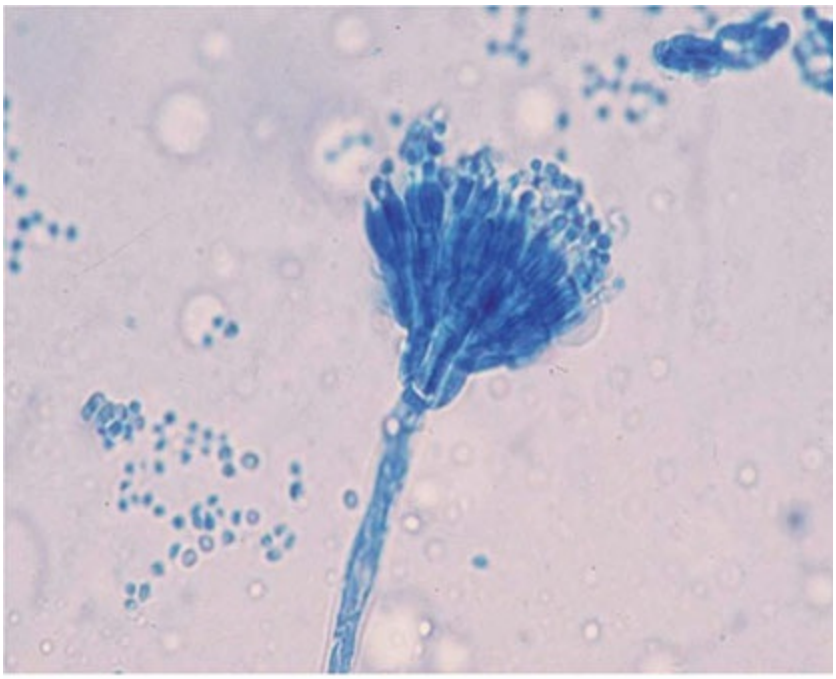
conidiation in which identical conidia are produced in sequence from the tips of the conidia previously formed (Box 21-1).

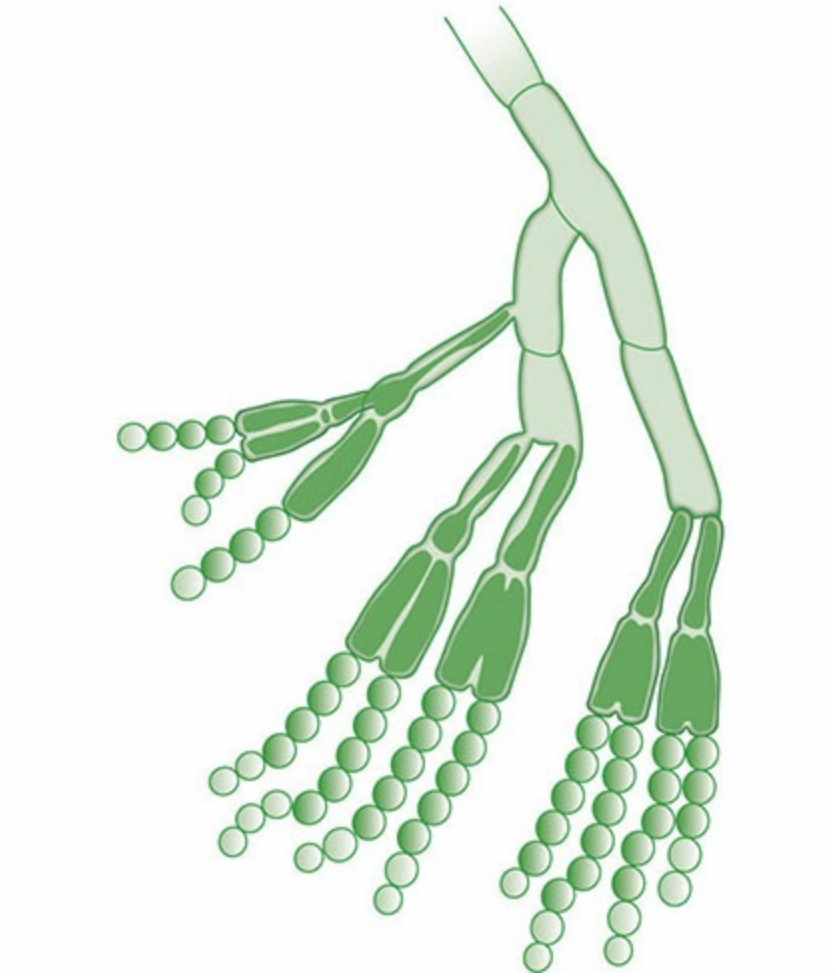
***Paecilomyces* and *Purpureocillium* Species.** In the past, both these fungi were included in the genus *Paecilomyces*, but subsequently the species *lilacinus* has been reassigned to the newly formed genus *Purpureocillium.* The colonies of *Paecilomyces variotii* are usually buff, tan or light brown, whereas those of *Purpureocillium lilacinus*, not surprisingly, are light purple. The surface of the colonies of these fungi tends to be quite granular because of the production of myriad conidia (Image Plate 21-2C).

Two important microscopic observations are useful for the identification of *Paecilomyces* or *Purpureocillium* spp. The tips of the phialides of these organisms are **long and tapered**, terminating in a point, in contrast to the blunt ends of phialides seen with *Penicillium* spp. (Fig. 21-32). Secondly, the conidia are oval to elliptical, irregular in size, and uneven in staining, representing **basipedal** conidiation in which each new conidium is formed directly from the tip of the phialide, pushing the previous conidium ahead of it. Therefore, the "older" conidia at the tips are larger and more deeply staining than those produced previously.

***Scopulariopsis* Species.** There are two *Scopulariopsis* species of medical importance, a dematiaceous species, *S. brumptii,* and the hyaline species, *S. brevicaulis* covered here. The colonies are characteristically yellow-brown to buff and granular and develop radial rugae emanating from the center to the periphery (Image Plate 21-2D).

The identification of *Scopulariopsis* spp. can be made microscopically from observation of the size and morphology of the conidia. The conidia are two to three times larger than those of *Penicillium* spp. and *Paecilomyces* spp., are entirely spherical, except for the presence of a distinct flat or truncated base. They are arranged in chains, with the truncated base of each successive conidium attached to the round terminus of the previous conidium (Fig. 21-33). Initially, the conidia are smooth; however, as the colony matures, the outer wall becomes distinctly roughened (**echinulate**). Sharp focus of the microscope objective may reveal a short, rodlike connection between adjacent conidia. The production of conidia for *Scopulariopsis* spp. is **annellogenic;** that is, when each new conidium is produced there remains behind a portion of the thickened truncated base or scar known as an **annellide**. When a new conidium is formed, it pushes the previous conidium forward and the chain of conidia is formed.





■ **FIGURE 21-31** Photomicrograph and sketch of the fruiting structure of *Penicillium* species, illustrating the branching phialides from which are produced chains of spherical conidia.

Identification of Hyaline Molds Producing Conidia in Clusters

This group of hyaline fungi is characterized by the formation of conidia in clusters. The conidia may be elongated or

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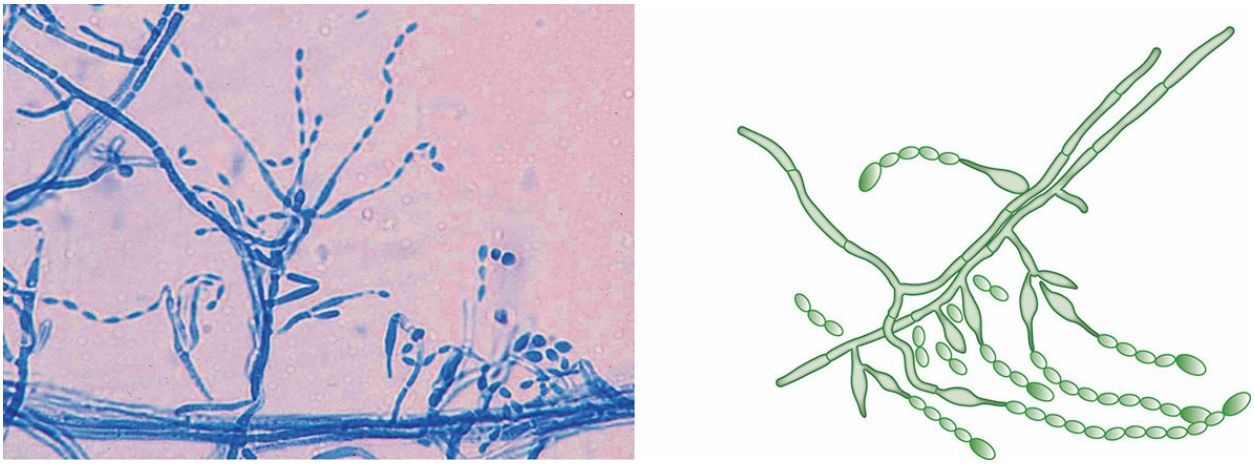
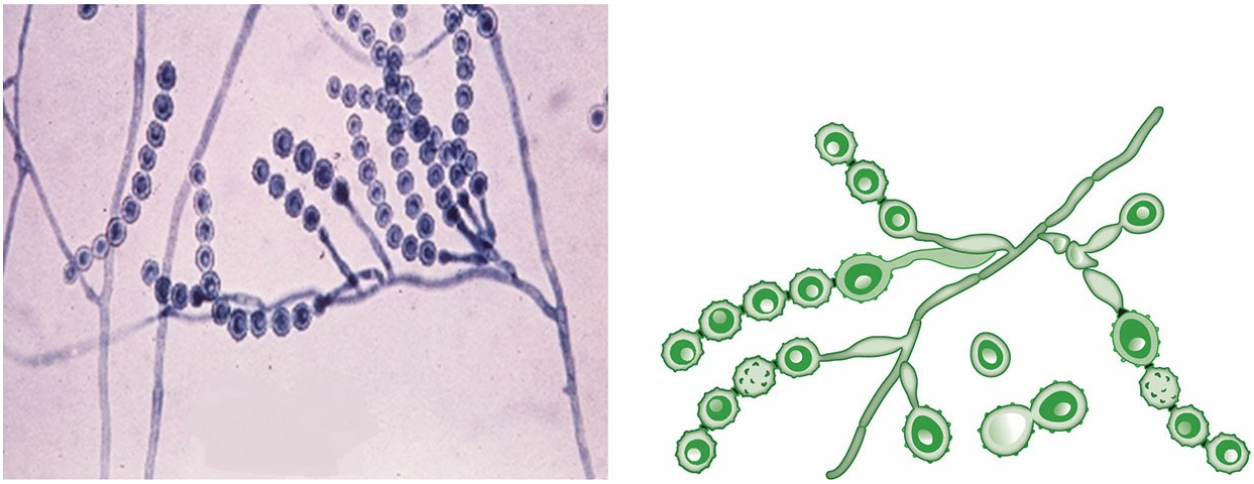


■ **FIGURE 21-32** Photomicrograph and sketch of the fruiting structure of *Paecilomyces* species, illustrating the long tapered phialides from the tips of which are produced chains of elliptical conidia. Note: the terminal conidium is often slightly larger than the rest.



■ **FIGURE 21-33** Photomicrograph and sketch of the fruiting structure of

Scopulariopsis species, illustrating the chains of characteristic large, circular annelloconidia.

elliptical, as with *Acremonium* and *Fusarium* spp., or spherical, as with *Gliocladium* and *Trichoderma* spp. Among these four, *Fusarium* spp. are unique in that they produce both microconidia and macroconidia. Guidelines for the separation of these three species are outlined in Display 21-2.

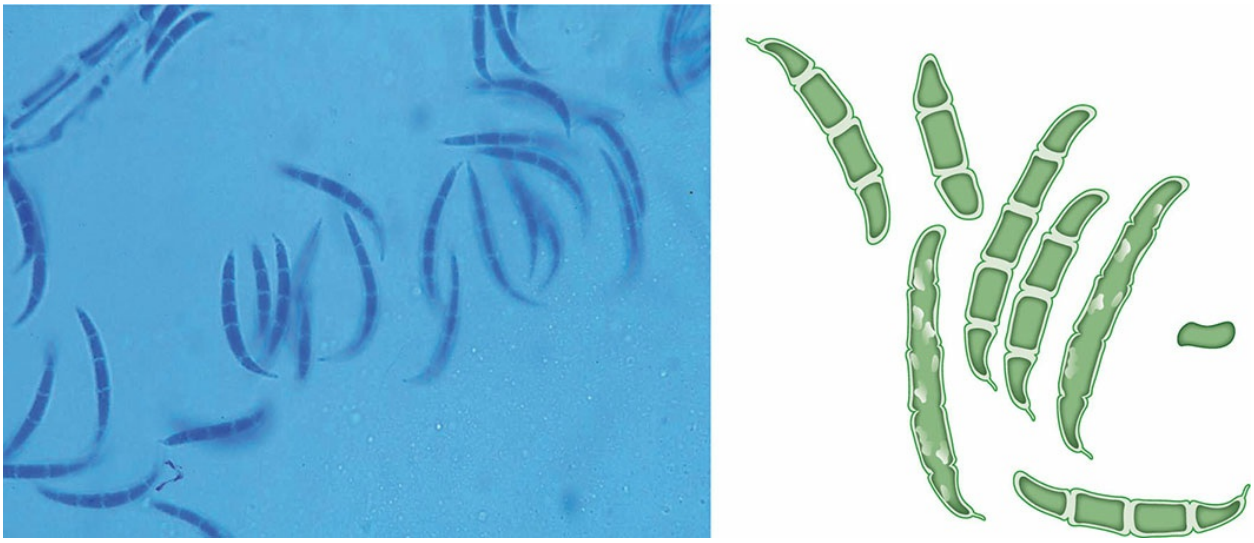
***Acremonium* Species.** The colonies of *Acremonium* spp. generally appear less wooly or granular than other hyaline molds, but rather are smooth, glabrous, or cottony in appearance because of the production of a very delicate mycelium. Colonies may be white or various shades of light pastel green or yellow (Image Plate 21-2E).

As observed microscopically, the conidia of *Acremonium* spp. are elongated to spherical, and arranged in loose clusters (Fig. 21-34). Each cluster is borne at the tip of a long, slender, delicate conidiophore that ends in a blunt tip. Important for the identification of *Acremonium* is the presence of a septum at the base of the tapered conidiophore (i.e., a basal septum). Macroconidia are not observed.

***Fusarium* Species.** There are a wide variety of *Fusarium* species, many of which are plant pathogens. In most instances, attempts are not made at species-level identification. These are important to promptly identify, as they are an important cause of hyalohyphomycosis (i.e., fusariosis) in immunocompromised patients. The colonies are generally cottony or wooly, and have distinctive pastel coloration; the surface of these may be lavender, pink, deeper red, or magenta. The reverse side also typically displays some pigmentation (Image Plate 21-2F).

Of the hyaline filamentous molds reviewed here, *Fusarium* spp. are unique in that they produce both **microconidia** and **macroconidia.** The microconidia are similar to those produced by *Acremonium* spp. The key feature used for the identification of *Fusarium* spp. is the observation of long, sickle-shaped, multicelled macroconidia; transverse septa separate the cells from one another within the macroconidia (Fig. 21-35). Exact focusing on the hilar cell reveals a hairlike extension, which designates this as the **"foot cell."** The

■ **FIGURE 21-34** Photomicrograph and sketch of the fruiting structure of *Acremonium* species, illustrating the delicate conidiophore supporting a loose cluster of elliptical conidia arranged in a "diphtheroidal" pattern.



■ **FIGURE 21-35** Photomicrograph and sketch of the long, multicelled, canoe-shaped macroconidia characteristic of *Fusarium* species.

presence of a foot cell is helpful in separating *Fusarium* spp. (foot cell present) from *Cylindrocarpon* spp. (foot cell absent), the latter being a soil organism rarely encountered in clinical laboratories.

***Gliocladium* Species.** The colonies of *Gliocladium* spp. are green or green-yellow and extend from edge to edge of the agar plate as a "lawn" of growth, without a margin. The surface is generally powderlike (Image Plate 21-2H).

The regular-sized, spherical conidia of *Gliocladium* spp. as observed

microscopically are also tightly clustered, and appear similar to those produced by *Trichoderma* spp. (below). However, in contrast to *Trichoderma* spp., in which each conidium is derived at the tip of a single conidiophore, the cluster of conidia produced by *Gliocladium* spp. is supported at the tips of three or four tapered conidia on what appears to be a rudimentary penicillus structure (Fig. 21-36).

***Trichoderma* Species.** The colonies are similar to those of *Gliocladium* spp., also producing a granular "lawn" of growth that extends from one edge of the agar plate to the other. Colors tend to be yellow and green.

The conidia of *Trichoderma* spp. as observed microscopically are regular in size, spherical, and tightly clustered (Fig. 21-37). The conidia form adherent to one another at the tip of the conidiophore. Although a similar spore mass is produced by *Gliocladium* spp., those produced by *Trichoderma* spp. are located at the tips of individual tapered phialides. Although usually smaller to some these appear similar to those of *Paecilomyces* spp. in that the conidiophore is derived laterally from the hyphae.

Identification of the Genera of Hyalohyphomycetes Producing Conidia Singly

For many years, *Pseudallescheria boydii* was thought to be the teleomorphic or sexual form of *Scedosporium apiospermum*. However, as with several other groups of fungi, DNA sequence analysis of taxonomically-important genes

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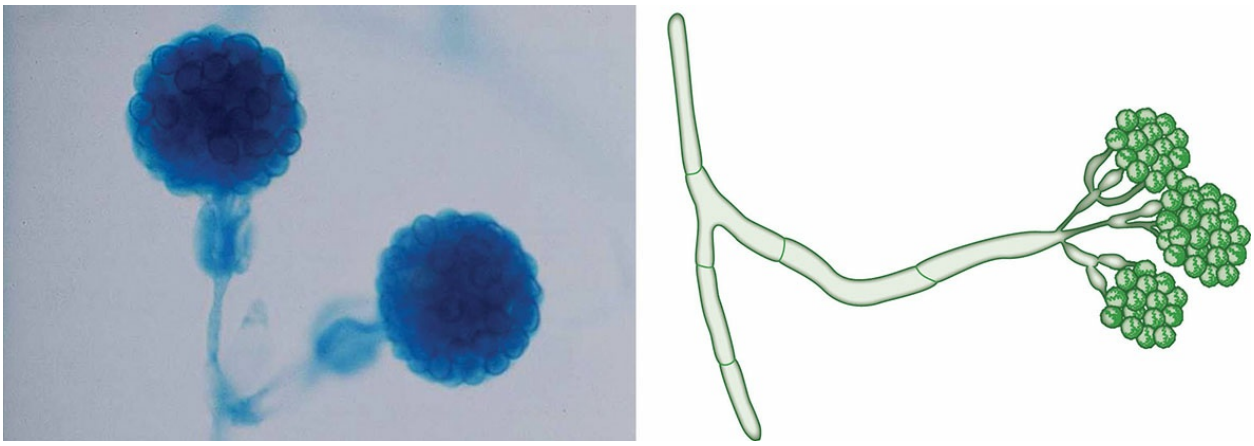
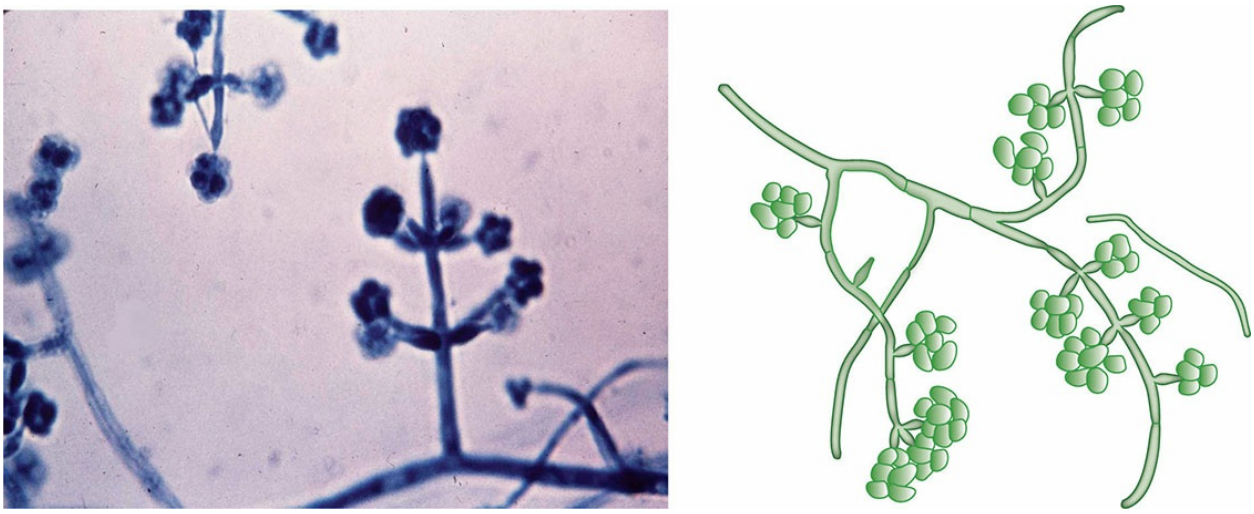


■ **FIGURE 21-36** Photomicrograph and sketch of the fruiting structure of *Gliocladium* species, illustrating the fingerlike phialides supporting compact clusters of spherical conidia.



■ **FIGURE 21-37** Photomicrograph and sketch of the fruiting structure of *Trichoderma* species, illustrating the laterally placed tapered phialides supporting compact clusters of spherical conidia.

has disclosed the short comings of purely morphologically-based taxonomy. It is now known that there are several different organisms within the group we will refer to as the *Pseudallesheria boydii* complex. This group produces colonies with the so-called "house-mouse gray" coloration. The reverse may be buff to light brown and darkens with time. The colony morphology of the other fungi covered here (i.e., *Chyrsosporium* etc.) is not distinctive. These colonies are usually entire, white to gray, smooth to slightly granular, and often rugose. Microscopic examination is required to make a genus separation, as discussed below. This group of hyaline fungi is characterized by the formation of conidia singly from the tips of individual, straight, or branched conidiophores. The unique microscopic features of each of these species are described below, as outlined in Display 21-2.

***Pseudallescheria boydii* Complex.** The members of the *Pseudallescheria boydii* complex are an important cause of infection in immunocompromised patients. Interestingly, there is also an association with near-drowning events. Microscopically, the hyaline septate hyphae are present from which arise non-descript, straight conidiophores. Members of the *P. boydii* complex produce characteristic thin, smooth-walled, pyriform-to-ovoid, conidia 3 to 5 μm in diameter from the tips of unbranched conidiophores (Fig. 21-38). It is important to note that the conidiophores are not swollen at the base or "inflated," which is the characteristic of a dematiaceous *Scedosporium* variant, *S. prolificans* (Fig. 21-39). Mature conidia take on a dark pigmentation with age, thus resulting in the house-mouse gray colony previously described. The tips of the conidiophores actually extend when a spore is produced, with the formation of annellides, which are difficult to see with light microscopy, but

are readily evident in scanning photomicrographs. The annelloconidiation also imparts a flattened end at the smaller end of the pyriform conidia. In some strains, there may be the parallel bundling of conidiophores to form the graphium anamorph (Fig. 21-40). Detached conidia may occur singly or may aggregate in loose clusters.

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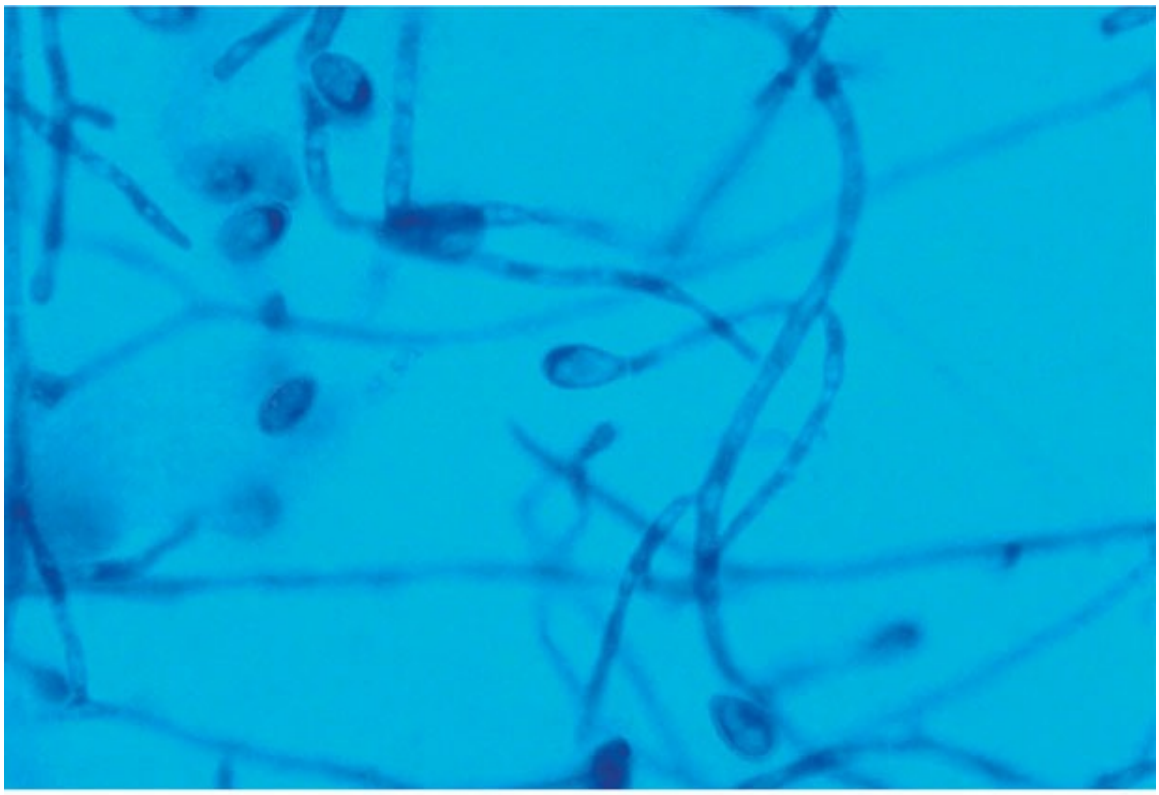
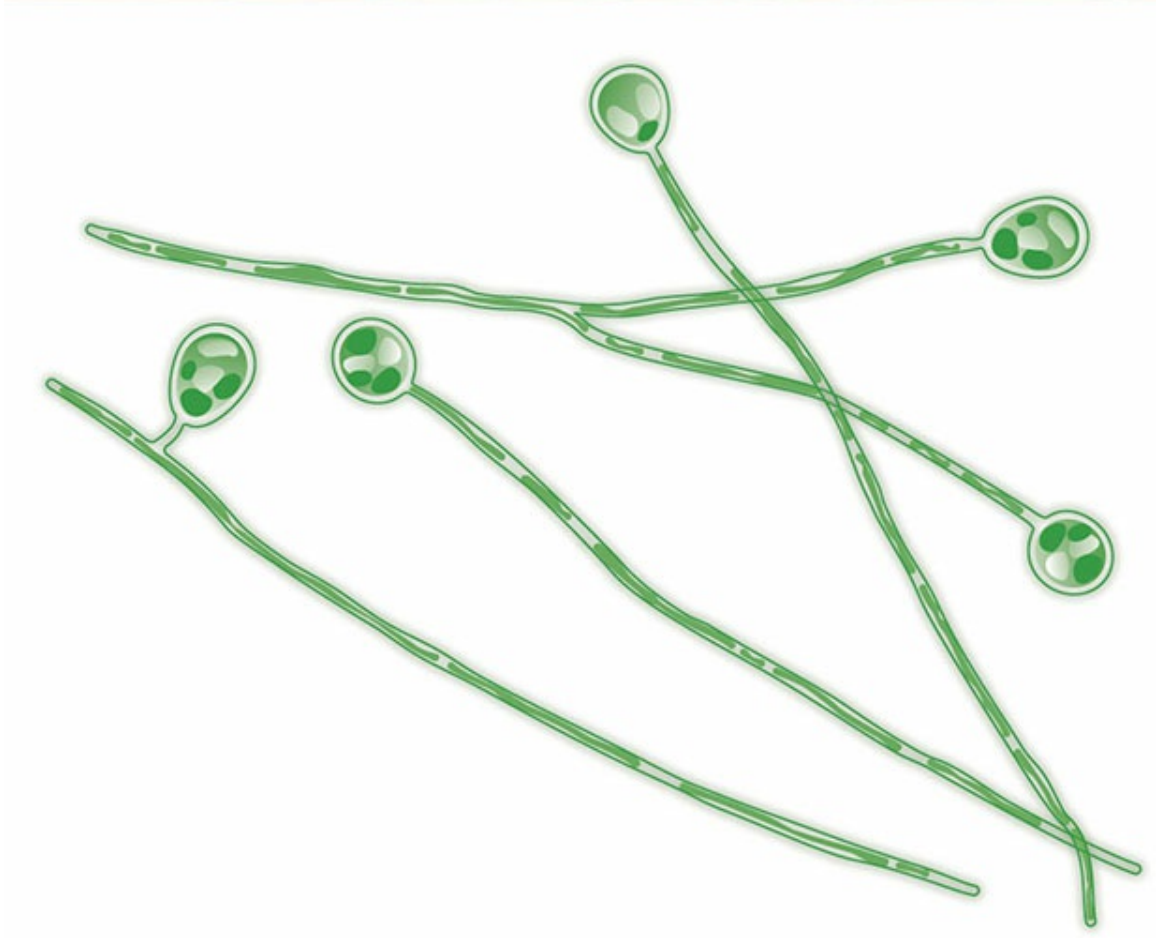
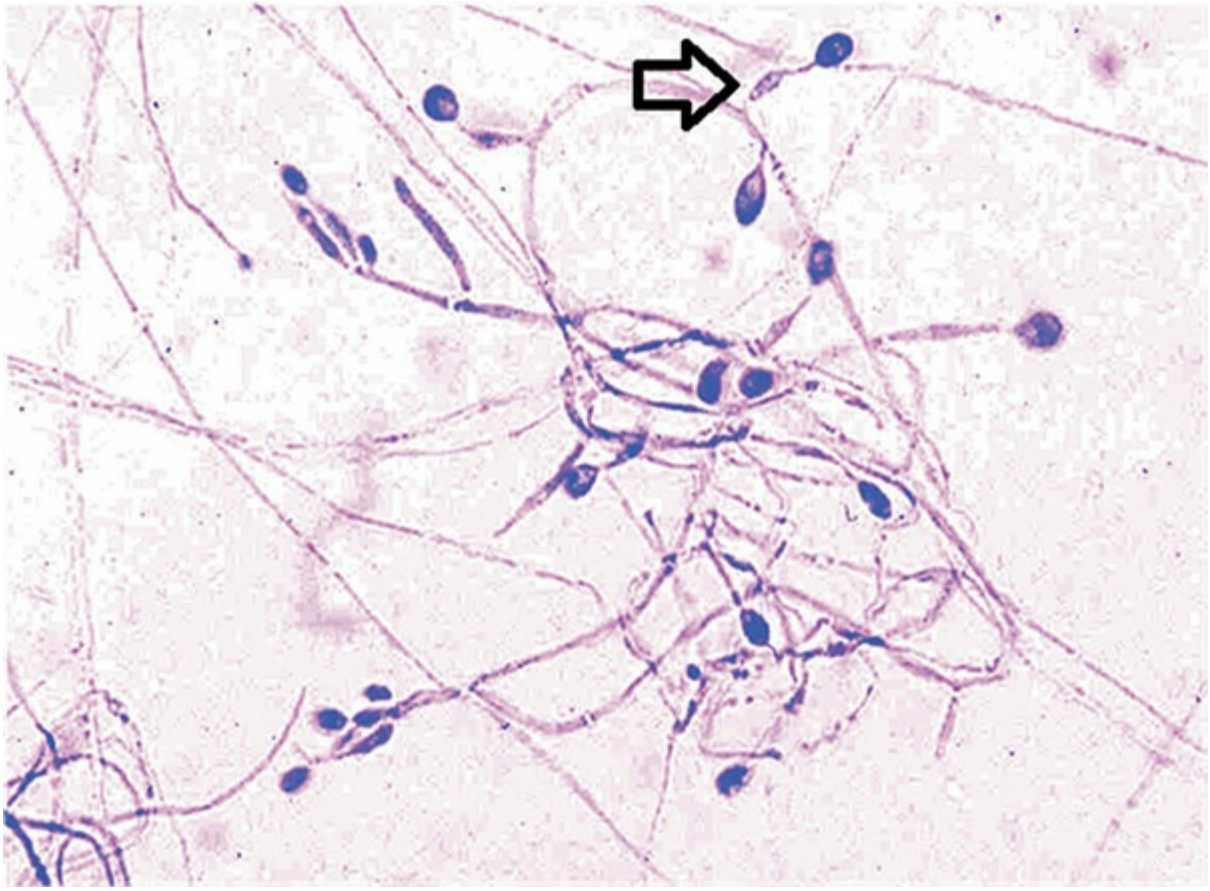
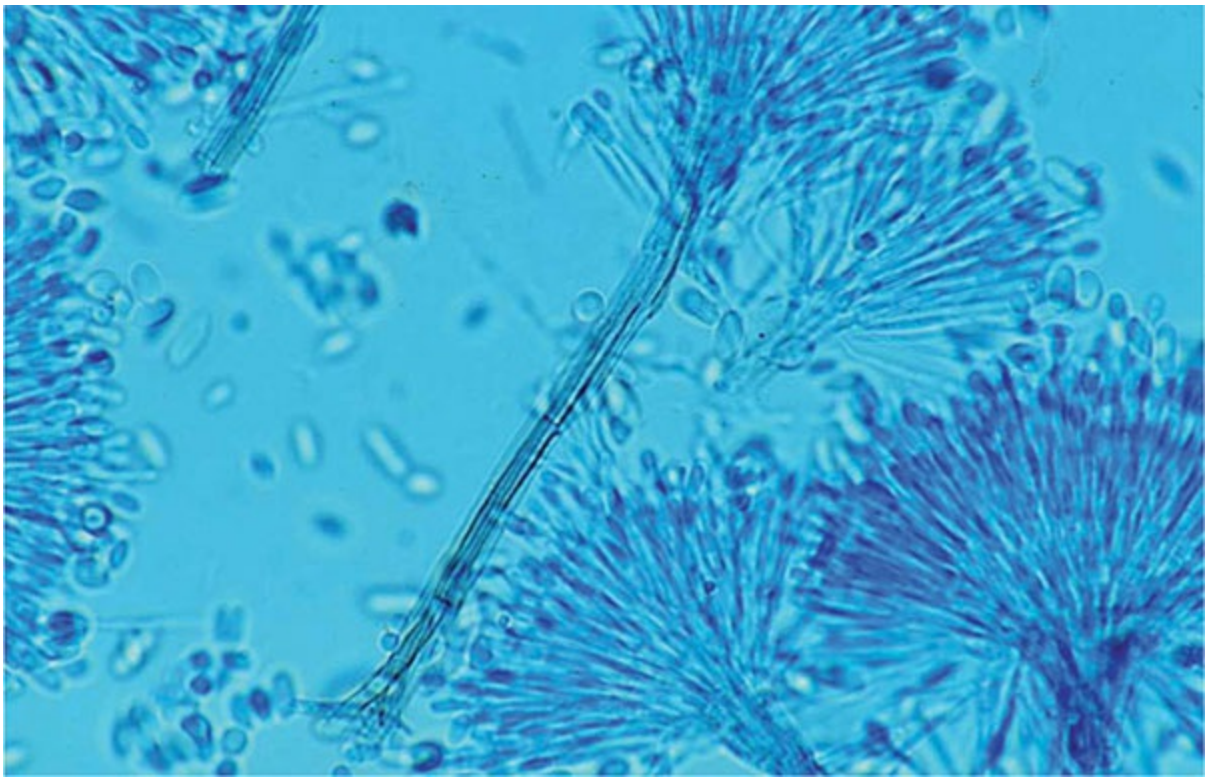




■ **FIGURE 21-38** Photomicrograph and sketch of the long, straight, delicate conidiophores, each supporting a single oval, dark-staining conidium characteristic of a member of the *P. boydii* complex.



■ **FIGURE 21-39** Photomicrograph of the conidia of *Scedosporium prolificans,* illustrating the characteristic urn-shaped swelling of the base of the conidiophore (*arrow*).



■ **FIGURE 21-40** Photomicrograph of the graphium anamorph of a member of the *P. boydii* complex, which is arranged in fan-like bundles simulating sheaves of wheat. (Compliments of Dr. Glenn Roberts, PhD.)

***Chrysosporium* Species.** The colonies of *Chrysosporium* spp. are not distinctive. These are white to gray, cottony, or wooly, and develop after 2 to 4 days of incubation. Microscopically spherical, subglobose to pyriform conidia are borne singly at the tips of long lateral conidiophores; these closely resembling the conidia of *Blastomyces dermatitidis* (Fig. 21-41). The bases of the conidia may appear flattened and scarred from annelloconidiation. The colonies of *Chrysosporium* spp. generally grow more rapidly than those of *B. dermatitidis*; in contrast to *B. dermatitidis* do not grow on cycloheximide-containing selective culture media; and, unlike *B. dermatitidis*, cannot be converted to a yeast form upon incubation at 35°C to 37°C. In practice, nucleic acid probe or other molecular studies (e.g., DNA sequencing) may be performed on colony extracts to exclude the possibility of *B. dermatitidis* and support the identification of *Chrysosporium*.

■ **FIGURE 21-41** Photomicrograph of *Chrysosporium* spp., illustrating spherical to oval-shaped conidia each supported by a short, straight conidiophore ("lollipops"). This organism is a morphologic mimic for members of the *P. boydii* complex and *Blastomyces dermatitidis*.

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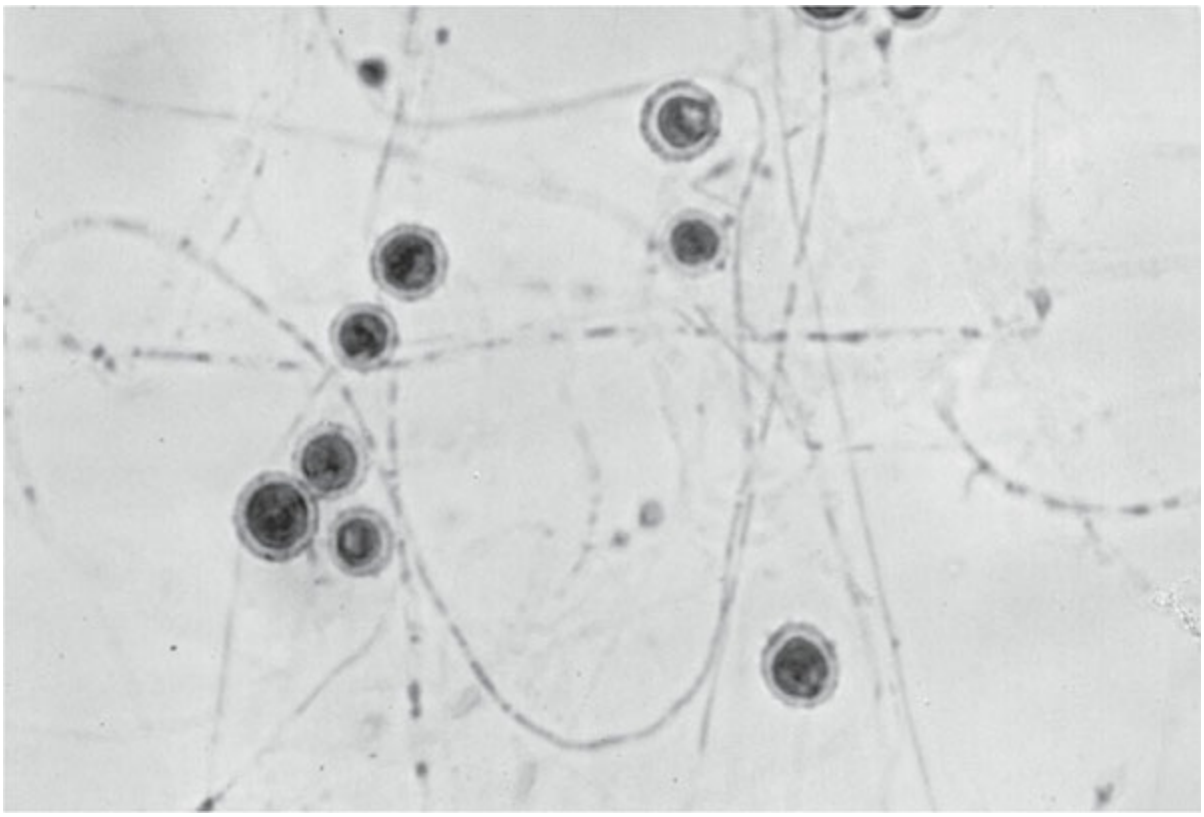
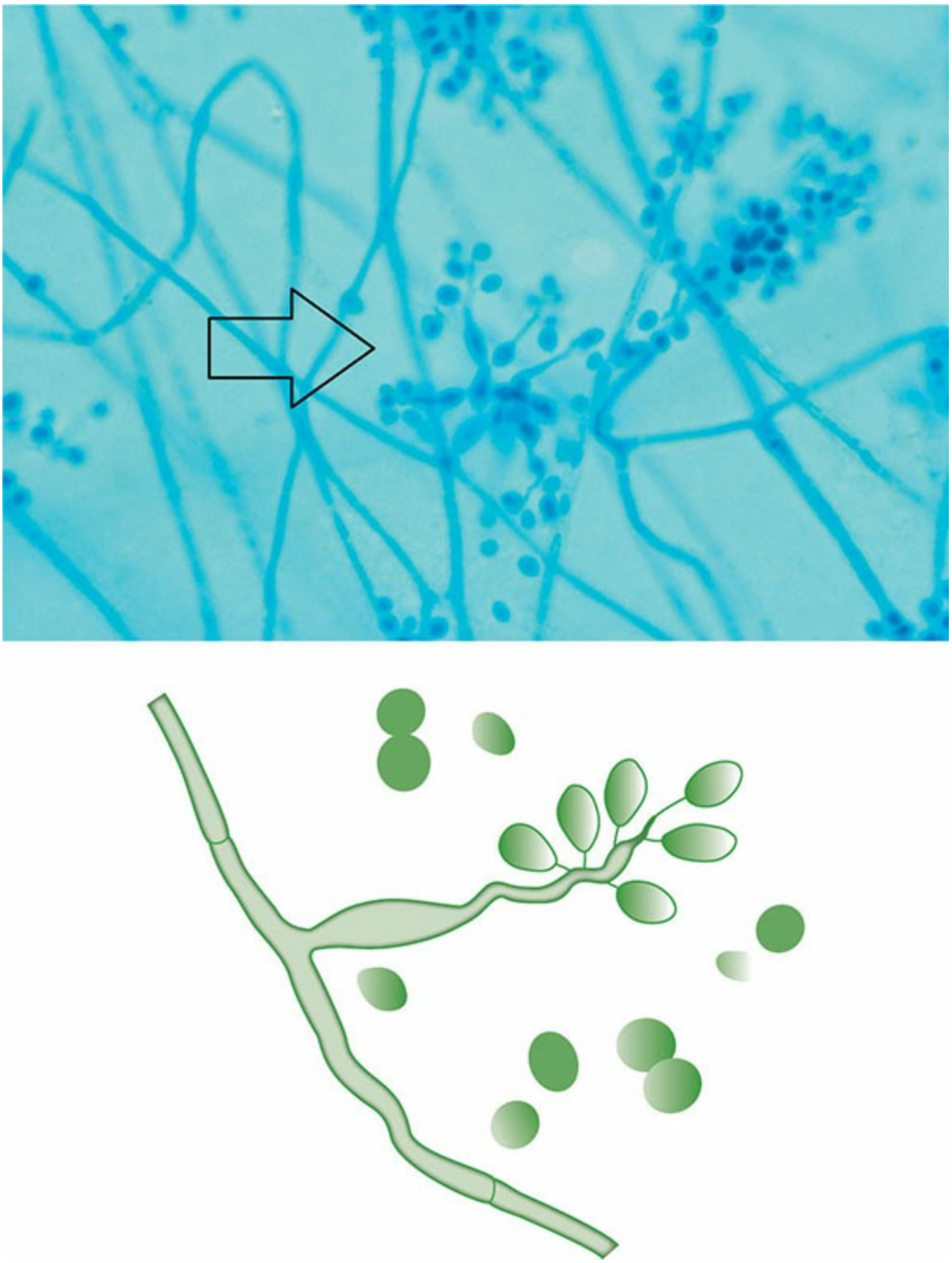


■ **FIGURE 21-42** Photomicrograph illustrating large, spherical, finely echinulate conidia characteristic of *Sepedonium* species.

***Sepedonium* Species.** The colonies of *Sepedonium* spp. are similar to those of *Chrysosporium* spp. described above, and cannot be distinguished visually. Microscopically, the observation of large, spherical, bluntly spiked macroconidia, simulating those of *H. capsulatum,* is characteristic (Fig. 21-42). The colonies of *Sepedonium* species grow more rapidly than those of *H. capsulatum*; unlike *H. capsulatum*, this fungus is inhibited on selective media containing cycloheximide; and, *Sepedonium* species cannot be converted to a yeast form on incubation at 35°C to 37°C. Smaller, ovate conidia, borne singly from short conidiophores, may also be observed in some strains, but are not common. These elongated microconidia are useful in differentiating *Sepedonium* species from *H. capsulatum*, which produce spherical microconidia. In practice, a DNA probe or other molecular technology (e.g., DNA sequencing) is used to exclude the possibility of *H. capsulatum* and support the identification of the fungus as *Sepedonium.*

***Beauveria* Species.** *Beauveria* is a very uncommon human pathogen. This is, however, an important insect pathogen and was responsible for the death of countless silkworms and the decline of the silkworm industry that Louis Pasteur was called on to investigate. The colonies have a white, cottony surface. The reverse of the colony is a

light buff. Microscopically, tiny, globose microconidia are densely aggregated around delicate, short, swollen conidiophore that near the terminus bends in a zigzag manner. This "bent knee" configuration is termed **geniculate** (Fig. 21-43). Each conidium is borne in one plane, after which the conidiophore turns before bearing another conidium in another plane, and so forth. Therefore, the type of conidiation produces conidia from all sides of the conidiophore, a configuration that is termed **sympodial.**



■ **FIGURE 21-43** Photomicrograph and sketch illustrating the delicate, zigzag conidiophores of *Beauveria* spp., with denticles each supporting a single, oval-shaped

conidium.

The various clinical manifestations of opportunistic infections with hyaline molds are presented in Clinical Correlation Box 21-3.

CLINICAL CORRELATION BOX 21-3 Hyalohyphomycosis

Mycetoma, onychomycosis,[106] and mycotic keratitis[160] are the infections most commonly caused by this group of filamentous molds. Reports of other infections, including sinusitis with or without fungus ball formation, meningitis, osteomyelitis, endocarditis, and otomycosis may also be caused by hyaline septate molds. Many of the earlier reports have been summarized by Rippon.[235]

Aspergillus species are the most common cause of hyalohyphomycosis and have been presented in Clinical Correlation Box 21-2. *Fusarium* species and members of the *P. boydii* complex are the next most frequent causes of invasive disease in the immunocompromised host, but it should be remembered that any fungus may cause disease in the profoundly immunosuppressed patient.

Vajpayee et al.[278] found *Fusarium* species to be the most common cause of mycotic keratitis among 156 patients with mycotic corneal ulcer disease, most commonly complicating allergic conjunctivitis and a combination

of antibiotic and corticosterone therapy. A comprehensive review of the taxonomy, mycology, laboratory features, and clinical syndromes related to *Fusarium* species has been published by Nielsen et al.[198] These authors also cite *Fusarium* species as the most common cause of mycotic keratitis in the United States, usually following corneal trauma from ocular implantation of vegetable fragments or soil matter during outdoor activities. Fungal keratitis has been reported in 4% to 27% of contact lens wearers, with *Fusarium* species being recovered most frequently.[296] Low et al.[173] report a case of *Beauveria*-related keratitis that was cured with deep dissection. Predisposing conditions include improper contact lens care, the presence of an underlying corneal infection such as herpes simplex infection, and the prolonged use of local corticosteroid and antibiotic medication.

In a 10-year (1986–1995) retrospective study of patients with hematologic malignancy, Boutati and Anaissie[29] found cases of disseminated *Fusarium* species infection in 43 patients, 40 with disseminated disease and 3 with invasive pulmonary infection. Although drug therapy was efficacious in 13 of these patients, in the main, recovery from the infection occurred only on reversal of myelosuppression and

neutropenia. The recovery of *Fusarium* species from the bloodstream in patients with a variety of malignancies has been reported by Krcmery et al.[157]

Cutaneous infections may occur from direct penetration of the skin by contaminated vegetative material. The author served as a consultant in an unreported case of posttraumatic *Fusarium* mycetoma in a 35-year-old woman. Initially, a species of *Alternaria* species, susceptible to amphotericin B by in vitro studies, was recovered from a superficial biopsy of the lesion. When the patient did not improve on amphotericin B therapy, a deeper biopsy was performed, from which an amphotericin B-resistant strain of *Fusarium* species was recovered. The condition regressed when itraconazole was administered.

Members of the *Pseudallescheria boydii* complex, which includes *Scedosporium apiospermum,* are one of the more common causes of subcutaneous mycetomas in the United States. The presence of soft-to-firm, white-to-yellow, spherical grains points to this organism, but this finding is not specific. Pulmonary infections with the *P. boydii* complex often resemble infections with *Aspergillus* spp., and the hyphae have a similar appearance in stained tissue sections. In addition to causing disease in the immunocompromised, *P. boydii* infections are also associated with episodes of near-drowning. Sinusitis, including fungus ball formation, meningitis, osteomyelitis, endocarditis, mycotic keratitis, endophthalmitis, and otomycosis are other *P. boydii* infections extensively reviewed by Rippon.[235] Perez et al.[217] report a patient with a brain abscess caused by *Pseudallescheria boydii* in association with an infected central venous catheter. Tamm et al.[271] report infections with *P. boydii* in seven lung transplant recipients in whom pulmonary infection developed. This organism was documented in BAL specimens of all seven patients and all showed airway problems, including early ischemic airway stenosis in one and bronchiolitis obliterans syndrome in the other six. Combined treatment with itraconazole and fluconazole was not able to eradicate the infection. Four of the seven patients died with advanced bronchiolitis obliterans 3–35 months after the diagnosis.

Scedosporium prolificans (formerly *S. inflatum*) is a dematiaceous fungus, but covered here as it is morphologically similar to *P. boydii* and other members of this complex. Salkin et al.,[241] who reported 15 human infections. Wilson et al.[300] added another 11 cases of *Scedosporium prolificans* infection to the literature, almost always in patients who had penetrating trauma or surgery. *Scedosporium* species, in particular *S. prolificans* have been found to be resistant to amphotericin B, miconazole, and ketoconazole and other antimycotic agents, one important reason for differentiating these from *Aspergillus* species. Pickles et al.,[225] however, report success in effecting cures through the use of fluconazole, and new triazoles are even more promising.

Alvarez et al.[10] report on a nosocomial outbreak of four fatal infections with *Scedosporium prolificans* in patients with severe neutropenia resulting from leukemia chemotherapy. The infections occurred sequentially within a 1-month period in two rooms during a phase of hospital reconstruction when the patients were

housed in a provisional hematology unit. The authors conclude that, despite the inability to recover *S. proliferans* from the patients' rooms or adjacent corridors, circumstantial evidence indicated a nosocomial outbreak. Two similar related infections have been reported by Simarro et al.[257] Both patients were admitted to the hematologic ward in nearby rooms during building work in the hospital. After a previous episode of bacterial sepsis in the neutropenic phase, which improved with antibiotic treatment, the respiratory status in both patients deteriorated, presenting acute dyspnea, with a lung infiltrate in one of them. A few hours later both patients died. Blood cultures were positive for *S. prolificans*. De Battle et al.[64] described a patient with leukemia who developed a disseminated *S. prolificans* infection during a period of chemotherapy-induced neutropenia. *S. prolificans* was recovered from four blood cultures. At autopsy, disseminated fungal infection was found, with multiple intravascular mycotic thromboses in the lungs, liver, spleen, and other organs. The authors make the plea that every effort be made to protect patients with leukemia from the invasion of saprophytic fungi during periods of therapy-induced neutropenia.

A variety of other hyaline molds may cause disease in the susceptible host. Chan et al.[40] report peritoneal dialysis-related peritonitis caused by *Paecilomyces variotii* in two patients. Each of these patients had received multiple antibiotics as a treatment for bacterial peritonitis, possibly contributing to the emergence of the opportunistic *Paecilomyces* infection.

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Paecilomyces species–related infections have also been reported by Chan-Tack et al.[41] (fungemia in an adult bone marrow transplant recipient), Gucalp et al.[104] (amphotericin B-refractory sinusitis in an immunocompromised adult), Okhravi et al.[204](endophthalmitis following penetrating keratoplasty), and Westenfeld et al.[288] (a patient with subcutaneous infection with prepatellar bursitis). Castro et al.[37] reported a case of deep subcutaneous infection of the left forearm with *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) in a kidney-transplant recipient, which responded to oral griseofulvin therapy. These authors reviewed 42 instances of human mycoses due to *Paecilomyces* species, usually in conjunction with prosthesis implants or immunosuppression. The differentiation of these microscopically-similar fungi is important as most strains of *P. lilacinus* are resistant to amphotericin B, whereas most strains of *P. variotii* are susceptible.

Scopulariopsis spp. have been reported to cause infections of nails, subcutaneous tissue, and the lungs.[274] Phillips et al.[224] report several patients with *S. brevicaulis* mycosis involving the toenail in an allogeneic bone marrow transplant recipient. Other infections cited were hypersensitivity pneumonitis, fungus ball formation in the lung, and deep subcutaneous infections in immunocompromised hosts. Miscellaneous *Scopulariopsis* infections include invasive fungal sinusitis reported by Ellison et

al.[79]and by Jabor et al.,[135] the latter involving invasive nasal destruction in a nonimmunocompromised host; a fatal case of *S. brevicaulis* prosthetic-valve endocarditis reported by Migrino et al.[186]; and a patient with a recurrent subcutaneous infection, occurring in a patient 6 years after liver transplantation, reported by Sellier et al.[251]

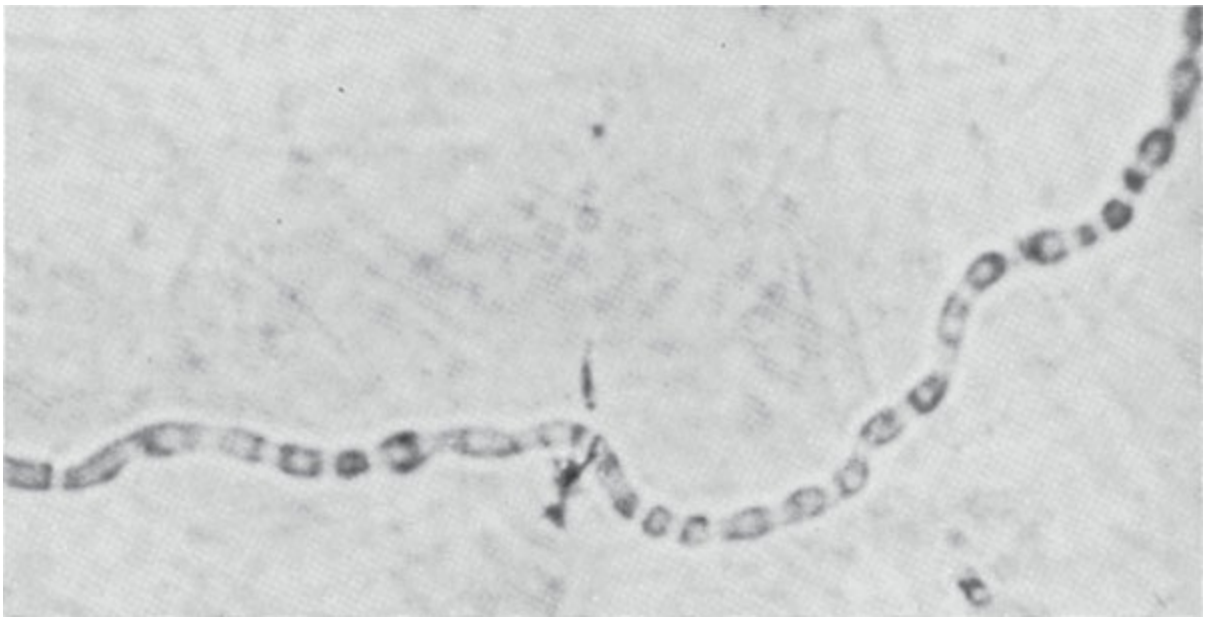
Schell and Perfect[247] report a patient with neutropenia who developed a disseminated infection caused by *Acremonium strictum*. In this patient, the organism was recovered from blood cultures after previous recovery from fecal specimens suggests the gastrointestinal tract as the primary source of infection.

Identification of the Dermatophytes

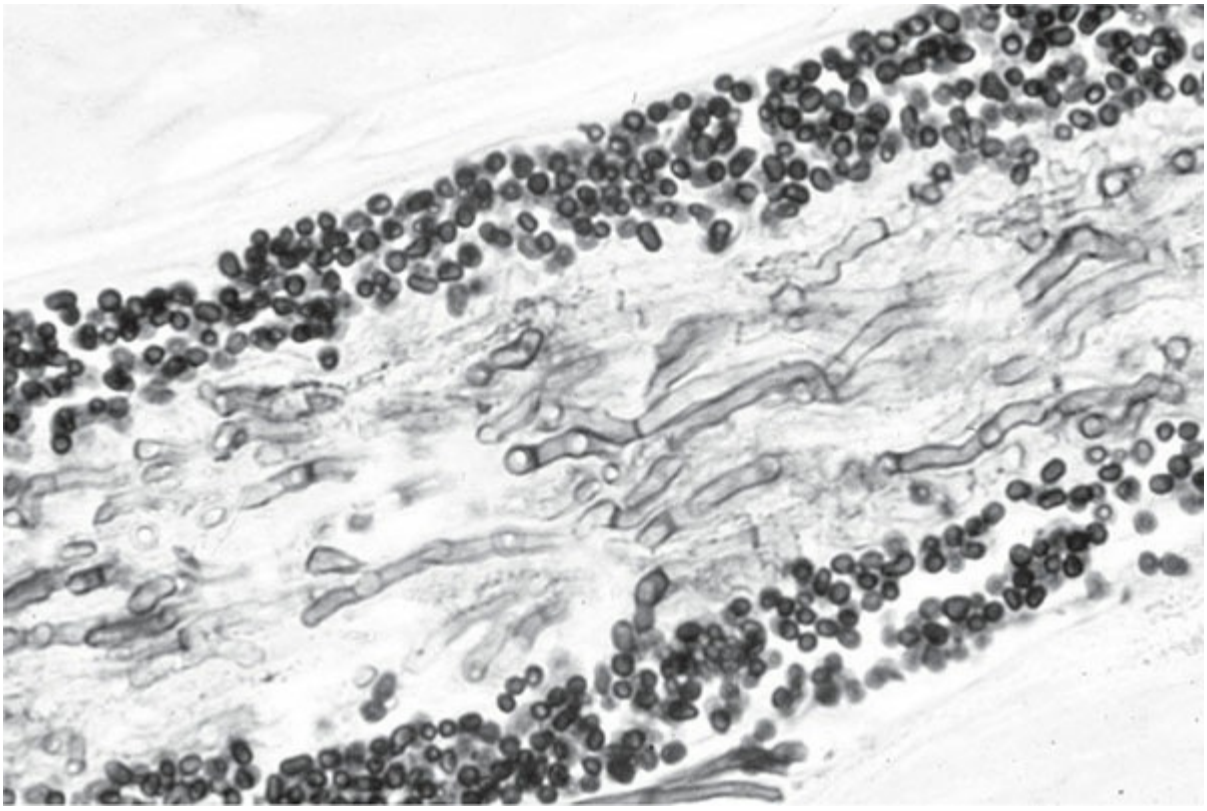
The dermatophytes are a distinct group of fungi that infect the skin, hair, and nails of humans and animals, producing a variety of cutaneous infections, colloquially known as "ringworm." Any mold recovered in culture from specimens labeled skin, nail, or hair should be assessed to determine whether it is a dermatophyte. One must be alert, however, that other pathogenic fungi, including the dimorphic molds, can also involve the skin and may have features, such as delicate, narrow diameter hyphae and resistance to cycloheximide, which are similar to the dermatophytes. In addition, many saprophytic environmental molds may be recovered, especially from nails. Determining the pathogenic nature of these fungi is challenging as they could represent either a contaminant or the cause of infection. Repeated isolation of such fungi, the exclusion of the presence of a dermatophyte, and the demonstration of invasive fungal form by histopathology would provide supportive evidence that the onychomycosis was caused by the fungus.

The direct examination using the KOH/calcofluor white preparation is useful for the direct detection of dermatophytes. Observing typical hyphal segments in direct skin scales (Fig. 21-44) or either ectothrix or endothrix invasion of infected hairs are helpful preliminary findings (Fig. 21-45).

The colonies of the various strains of dermatophytes vary considerably in rates of growth, morphology, and pigment production, sometimes even within the same species. Therefore, the genus and species designation depends on observing microscopic features. In some instances, the species-level identification, particularly for the species of *Trichophyton*, is not possible without supplemental biochemical/biophysical testing. Species-level identification, although of epidemiologic interest, is not necessary for treatment. There are, however, a few colony characteristics, which may be helpful



■ **FIGURE 21-44** Photomicrograph of a KOH mount of skin scraping, illustrating a hyphal segment of one of the dermatophytic fungi. Note that the hyphal fragment is breaking up into tiny arthroconidia (1,000×, oil immersion).



■ **FIGURE 21-45** Photomicrograph taken through a longitudinal section of a hair shaft

from a patient with dermatophytosis. Note the endothrix invasion with septate hyphae centrally and conidia at the periphery (GMS stain, 400×).

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in the identification of a given species; these are discussed below, where appropriate. Representative dermatophyte colonies are illustrated in Image Plate 21-4. The species of medical importance more commonly encountered in clinical laboratories are *Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton tonsurans*, *Trichophyton verrucosum*, and *Epidermophyton floccosum.*

In a survey of dermatophyte infections in the United States conducted by Weitzman,[286] *Trichophyton tonsurans* was the dermatophyte species most frequently recovered (44.5% of cases), and *Trichophyton rubrum* (41.3%) was next in frequency, followed less commonly by *Trichophyton mentagrophytes* (8.5%), *Microsporum canis* (3.5%), and *Epidermophyton floccosum* (1.1%).

The dermatophytes are separated into three genera, *Microsporum*, *Trichophyton,* and *Epidermophyton*, based primarily on differences in the microscopic morphology and modes of sporulation. An approach to the identification of the genera and species of the dermatophytes is presented in Display 21-3.

The genus ***Microsporum*** is characterized by the production of many macroconidia, and few or no microconidia. The macroconidia are multicelled and thick-walled, and have a thick, echinulate, or verrucose cell wall. The species identifications are based on differences in morphology of the macroconidia. The microconidia, if present, are small, hyaline, and tear-drop or elliptical in shape and attach directly to the sides of the hyphae.

The genus ***Trichophyton*** is characterized by the production of many microconidia, and few or no macroconidia. The macroconidia, when formed, in contrast to those of *Microsporum* spp. are thin-walled and smooth. The size and arrangement of the microconidia are important in making a species-level identification. The production of pigment, urease activity, hair penetration capabilities, and the differential growth patterns on culture media with and without thiamine and niacin (*Trichophyton* differential agars) are also used to achieve a species-level identification.

The genus ***Epidermophyton*** is characterized by the production of clavate, smooth-walled, two- to four-celled macroconidia that are borne either singly from the hyphae or, more characteristically, in clusters of two or three. The absence of microconidia is of prime importance in making the identification of *Epidermophyton.*

Identification of Microsporum Species

Microsporum canis. *Microsporum canis* is a zoophilic dermatophyte. Infection is often contracted from the family pet. Growth is rapid, within 3 to 5 days. The colonies are initially white and silky, later developing a lemon-yellow pigment at the colony peripheral apron (Image Plate 21-4A). The reverse of the colony becomes yellow-brown as the colony matures.

Microscopically, both macroconidia and microconidia may be observed, with the former predominating. The production of spindle-shaped (fusiform), multicelled macroconidia that are pointed, and may be slightly turned to one side at the tip, is characteristic of *Microsporum canis* (Fig. 21-46). Scattered microconidia may be seen borne laterally directly from the hyphae. In hair infections, mosaic clusters of microconidia develop on the outside of the shaft (**ectothrix**).

■ **FIGURE 21-46** Photomicrograph and sketch of a multicelled macroconidium of *Microsporum canis,* illustrating the spindle shape, thick echinulate wall, cross septations and tapered tip.

Microsporum gypseum. *Microsporum gypseum* is a geophilic dermatophyte, and infection is usually contracted from contact with soil. Growth is rapid, within 3 to 5 days. The colonies are flat, initially white but turning fawn-brown to reddish-brown on maturity (Image Plate 21-4B). The surface becomes sugary or granular as conidia are produced.

The macroconidia of *M. gypseum* generally are more numerous than found with *M. canis*, are less fusiform and have rounded tips (Fig. 21-47). A history of exposure to soil and possibly infected animals, and the colony morphology may be helpful in making the differential identification.

Microsporum nanum. *Microsporum nanum* is a zoophilic dermatophyte that is associated with pigs and is a relatively rare cause of dermatophytosis.[236] The colonies of *M. nanum* grow rapidly, within 3 to 5 days. The surface is cottony, initially white, and later buff-colored, turning to brownish-red on the reverse side. Tufts may appear on the surface with maturity.

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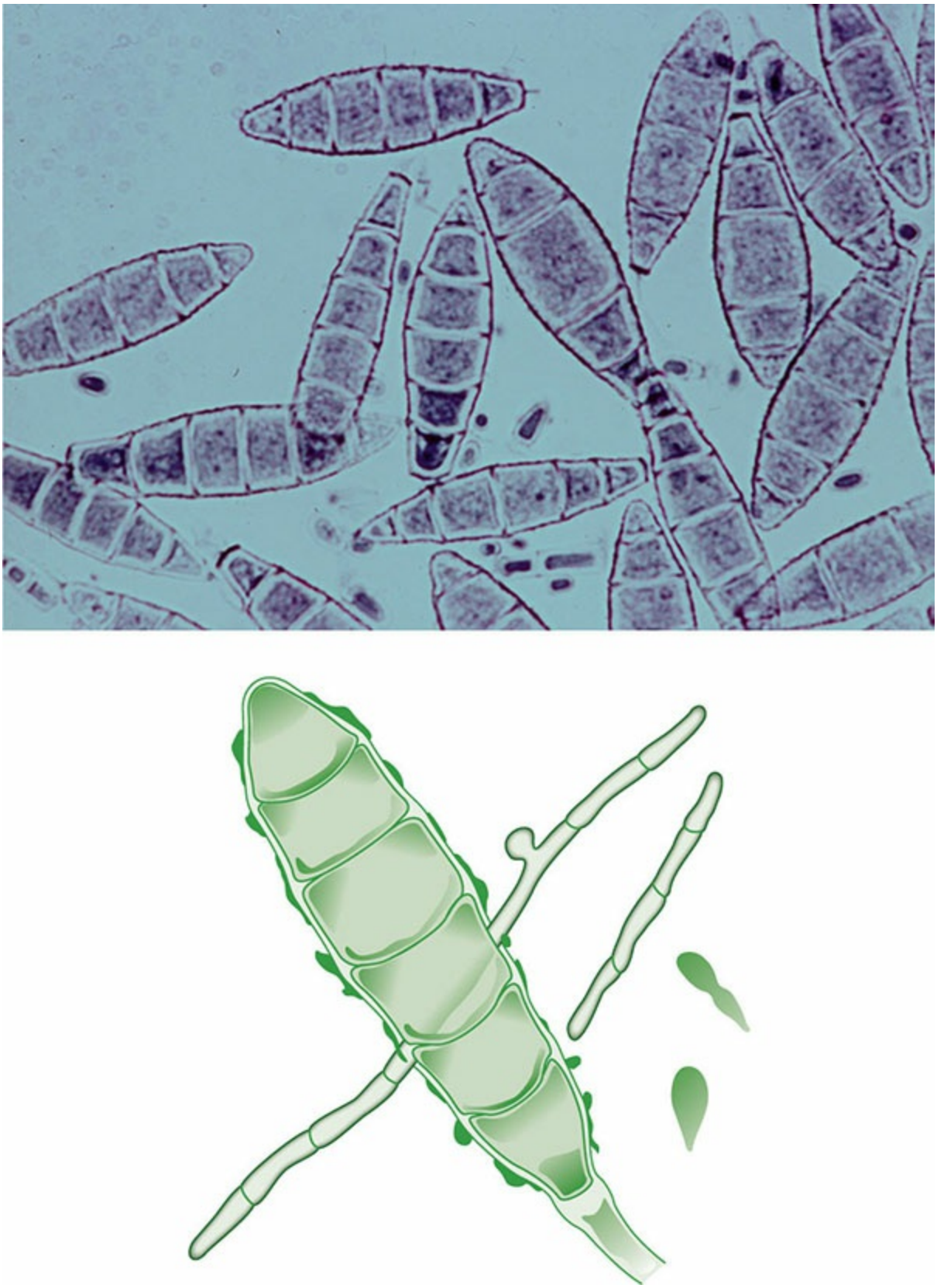
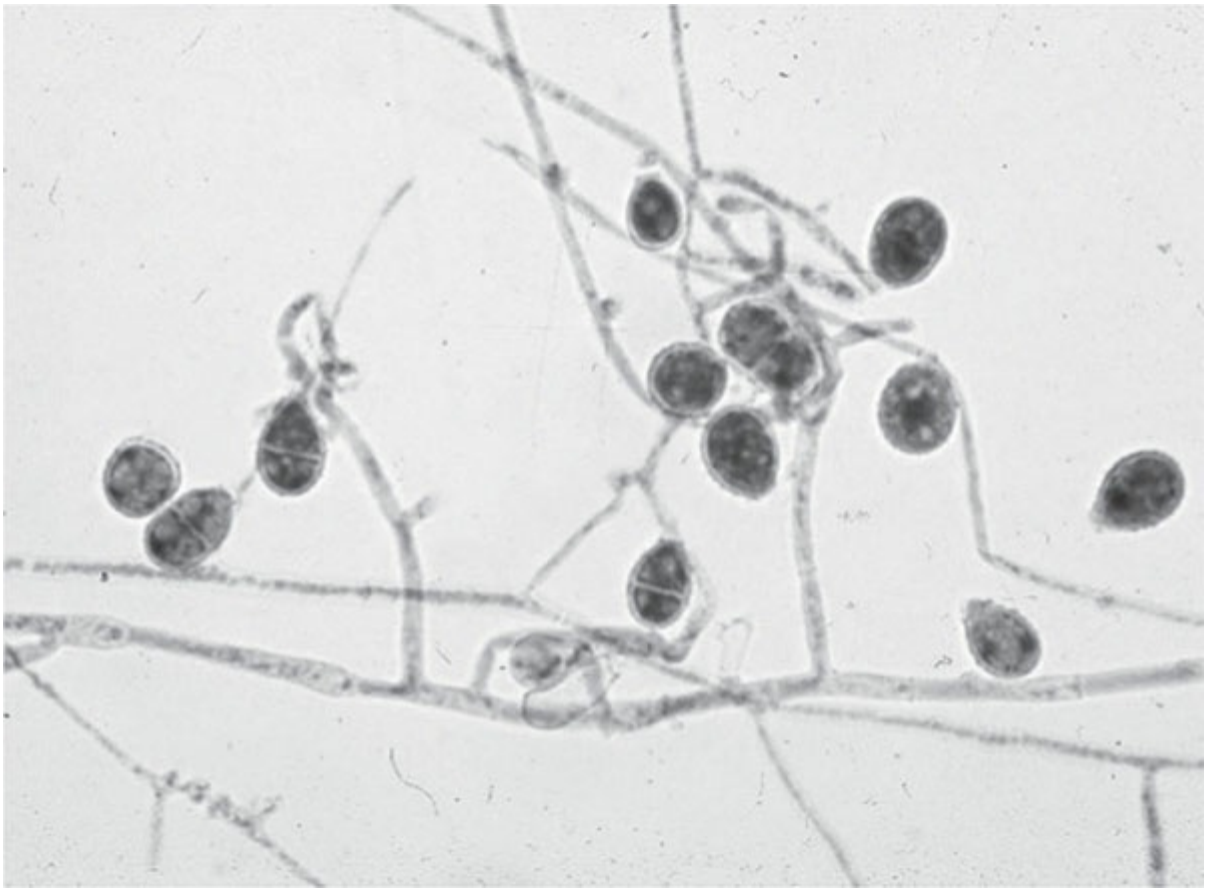


FIGURE 21-47 Photomicrograph and sketch of a multicelled macroconidium of

Microsporum gypseum, revealing a thick, echinulate wall, cross septations and a rounded terminal cell.



■ **FIGURE 21-48** Photomicrograph of the macroconidia of *Microsporum nanum*. They differ from those of *E. floccosum* by being two-celled and having a thick, echinulate wall.

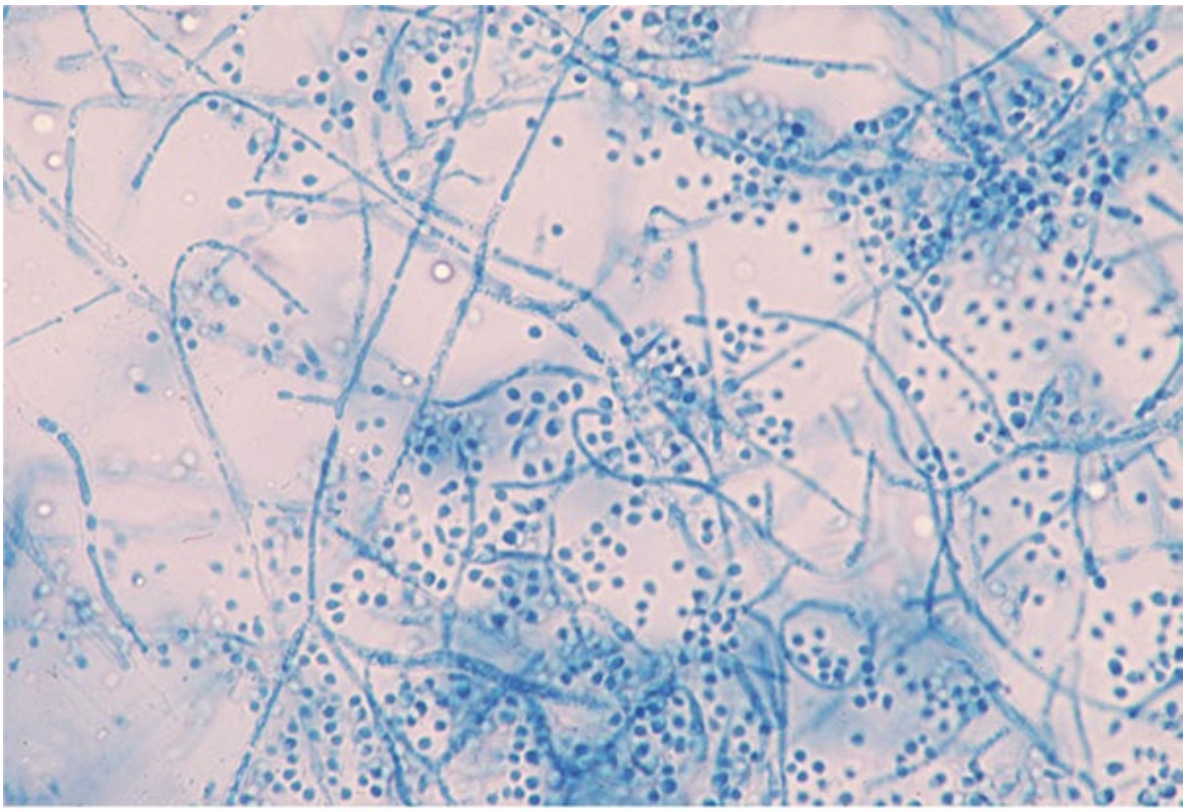
Microscopically, two- to three-celled, oval to clavate macroconidia are characteristically seen. At cursory evaluation, the macroconidia produced may resemble those of *Epidermophyton floccosum*. The cell walls are finely verrucose (Fig. 21-48) in contrast to the conidia of *E. floccosum,* which have smooth walls. Small 3 to 5 mm clavate to cylindrical microconidia are derived from the sides of the hyphae, an additional important observation in differentiating *M. nanum* from *E. floccosum*.

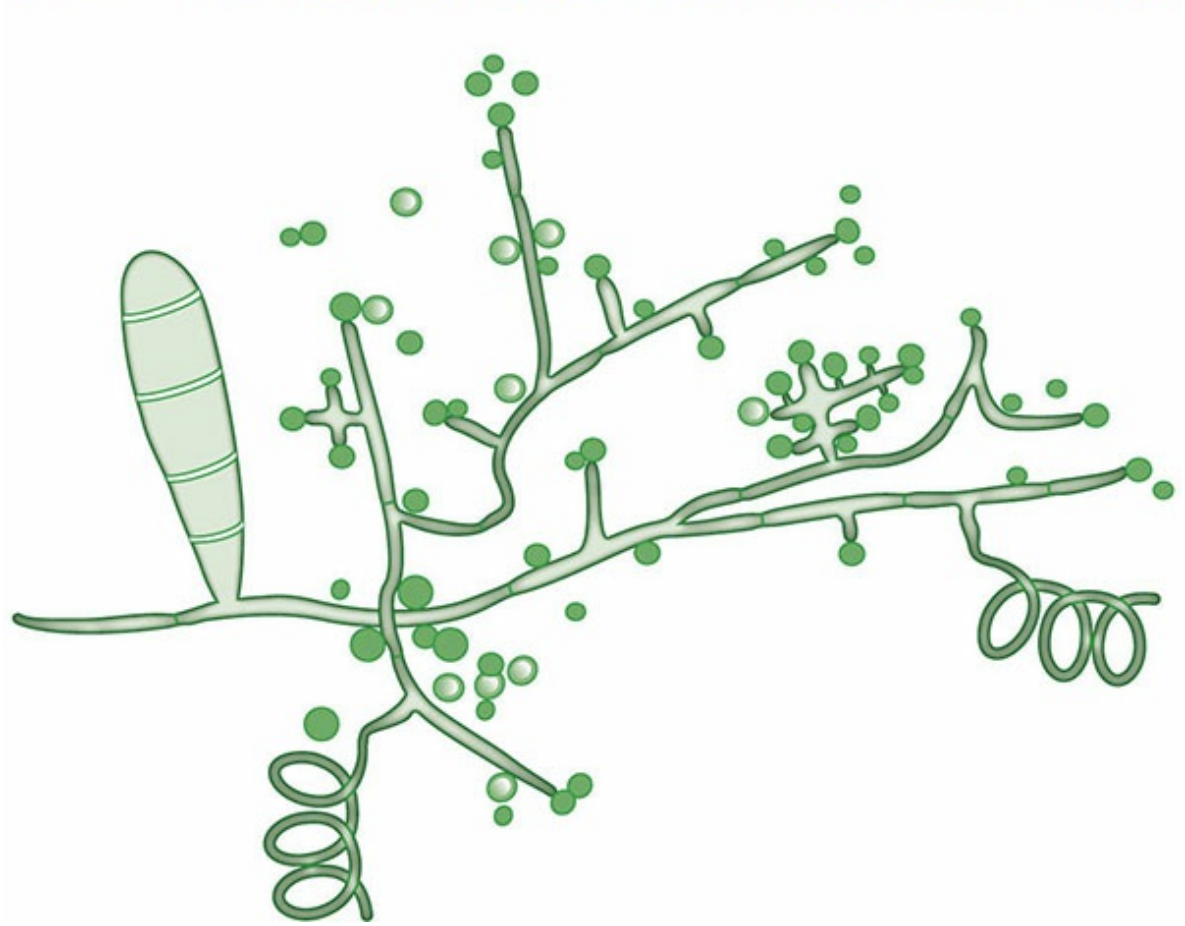
Identification of Trichophyton Species

Trichophyton mentagrophytes. *Trichophyton mentagrophytes* is a zoophilic dermatophyte, and infection may be contracted from a wide variety of animals.

Growth is relatively rapid, with maturation in 3 to 5 days. One of two colony types may be observed: cottony or granular. The cottony variants are initially white, but may become cream to tan with maturity. A central raised central area of cobweblike sterile hyphae often develops. The periphery and reverse of the colony may be rose to tan (Image Plate 21-4D). The granular variants produce flat and spreading colonies with a fine to coarse granular surface. The colonies are initially white or off-yellow, but later become tan to brown. A red pigment similar to that seen with *T. rubrum* may be observed; however, the pigmentation is never as intense as with *T. mentagrophytes*, particularly when colonies in parallel are grown on cornmeal agar or PDA (Image Plate 21-4F).

The microconidia tend to form in loose grapelike clusters (*en grappe*) (Fig. 21-49). Macroconidia are typically absent



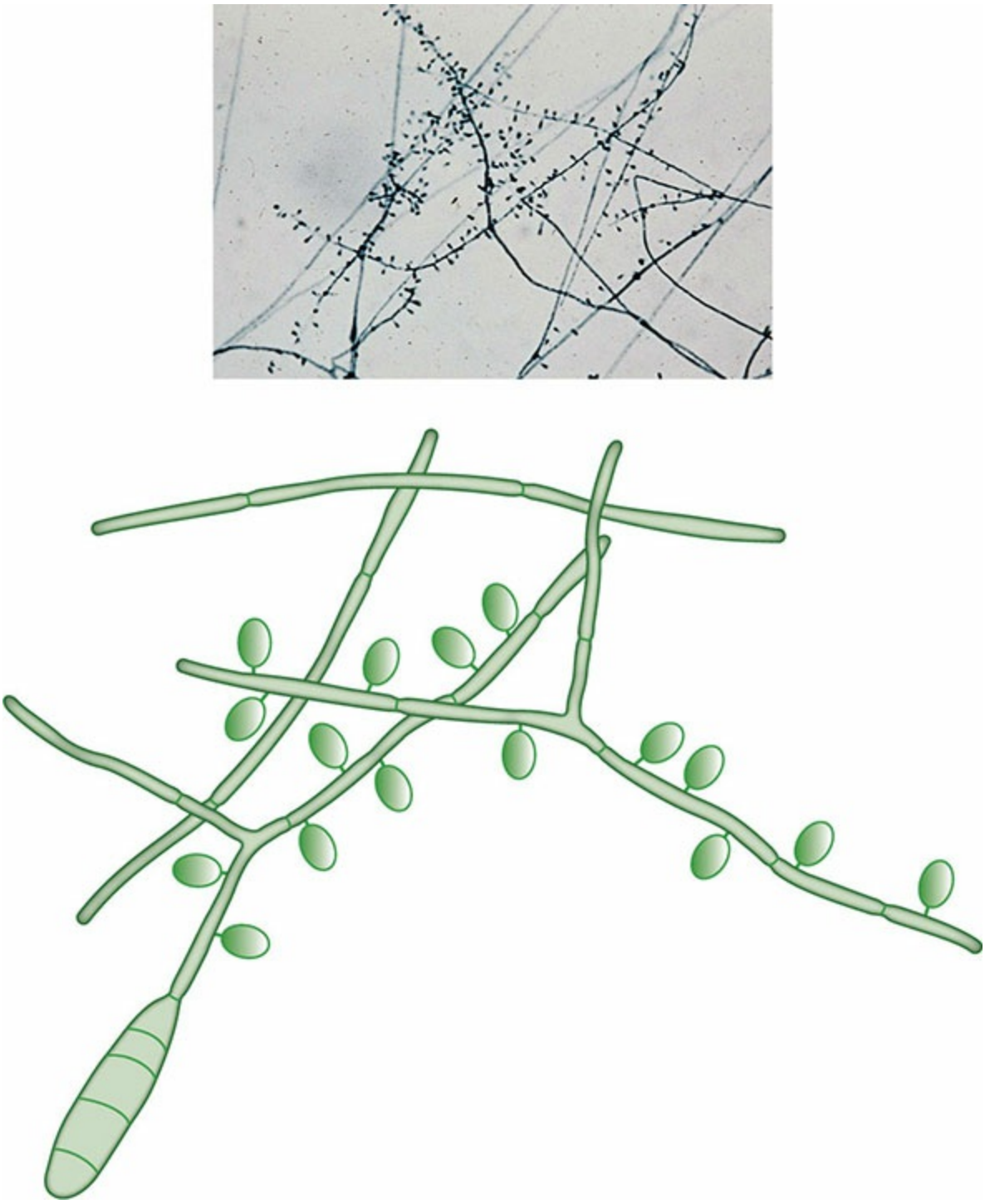


■ **FIGURE 21-49** Photomicrograph and sketch of the microscopic forms of *Trichophyton mentagrophytes,* illustrating tiny, spherical conidia arranged in loose clusters.

or present in small numbers; they are more common in granular cultures. If present, they are long, multicelled, cigar-shaped and have thin, smooth walls. Spiral hyphae and chlamydospores are often seen in the vegetative hyphae. Most strains are urease-positive, and in hair infections the hyphae invade the shafts (**endothrix**). There is equal growth on the *Trichophyton* differential agars.

Trichophyton rubrum. *Trichophyton rubrum* is an anthropophilic dermatophyte that commonly infects the skin and nails. Growth on SDA is relatively slow, requiring 4 to 7 days until maturity. The colony surface is initially white, and the consistency may be cottony, velvety, or granular, depending on the strain, the culture medium being used, and the magnitude of sporulation. As the species name indicates, a key observation is the production of a water-soluble, wine-red pigment on the reverse of the colony that diffuses into the agar (Image Plate 21-4E and F). Pigment production is more intense with colonies grown on cornmeal agar or PDA than on SDA.

Microscopically, the microconidia of *T. rubrum* tend to be teardrop-shaped (pyriform) and are usually distributed on either side of the hyphal strands, producing a "birds on a fence" appearance, rather than the loose clustering seen with *T. mentagrophytes* (Fig. 21-50). Multicelled macroconidia may rarely be observed; if present, they are elongated and cigar-shaped, with smooth, thin walls, similar to those produced by *T. mentagrophytes*. Urease is not produced by this species, and the hair shaft is not invaded in the hair-baiting or hair penetration test. There is equal growth on the *Trichophyton* differential agars.

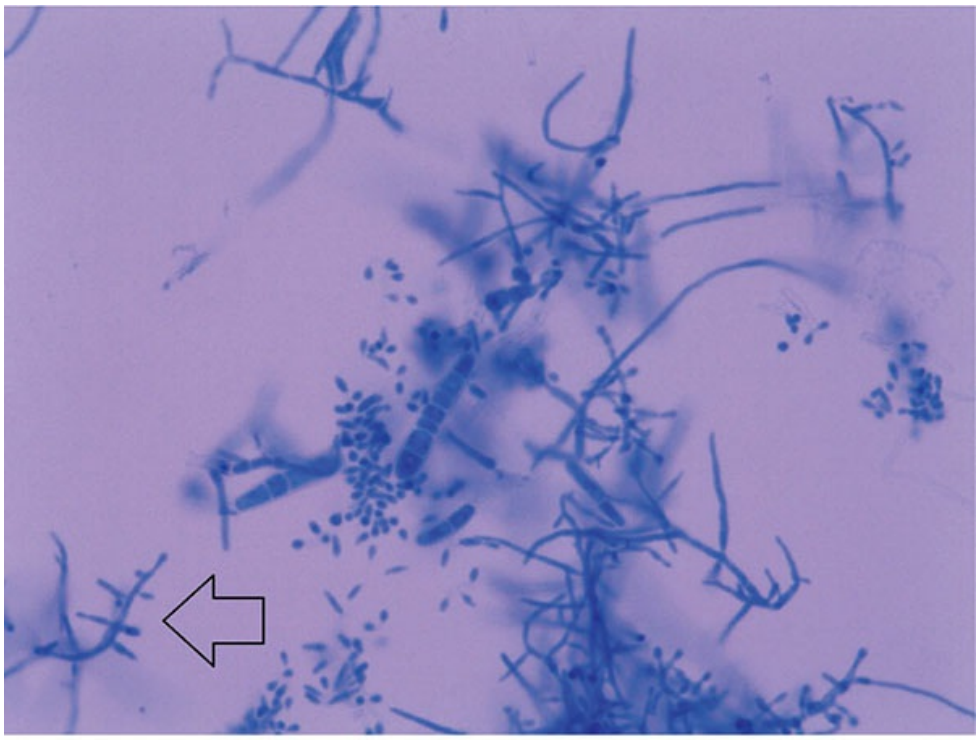


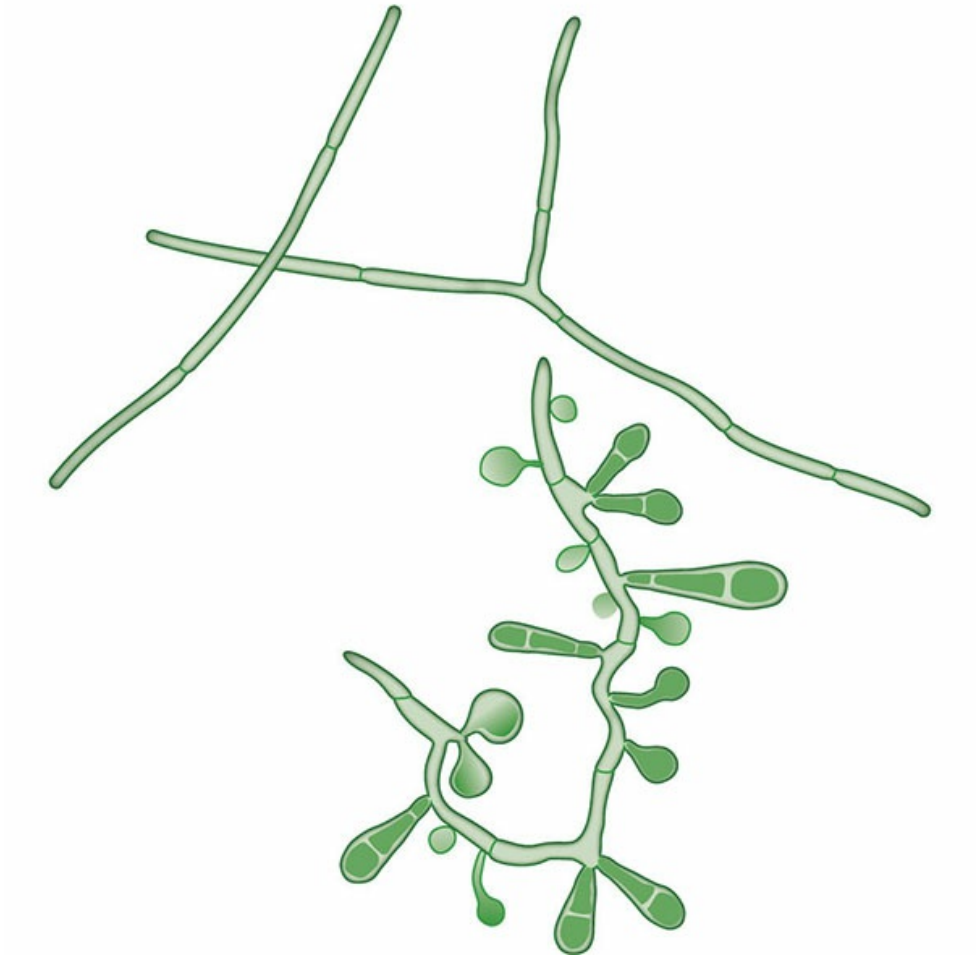
■ **FIGURE 21-50** Photomicrograph and sketch of the microscopic forms of *Trichophyton rubrum,* illustrating the production of tiny conidia from the sides of the hyphae in a "birds on a fence" arrangement.

Trichophyton tonsurans. *Trichophyton tonsurans* is an anthropomorphic

dermatophyte that causes infections of the nails, skin, and scalp. Growth is slow, requiring 7 to 10 days to maturity. The colonies have a characteristic granular, buff surface, with the development of deep radial rugae on maturation (Image Plate 21-4G). The identification can be confirmed by demonstrating poor growth on *Trichophyton* agar 1, which is deficient in thiamine; thiamine is an absolute growth requirement for *T. tonsurans*. There is good growth on *Trichophyton* agar 4, which contains thiamine.

Microscopically, the microconidia are usually distinctive. They vary considerably in size, and range from elongated, club-shaped, or large and balloon-shaped forms may be admixed with smaller oval or teardrop-shaped microconidia (Fig. 21-51). Macroconidia are rarely observed in laboratory isolates.





■ **FIGURE 21-51** Photograph and sketch of the club-shaped and spherical microconidia of *T. tonsurans* free and borne laterally from the delicate hyphae *(arrow)*. Additionally, several smooth and thin-walled macroconidia are present in this preparation.

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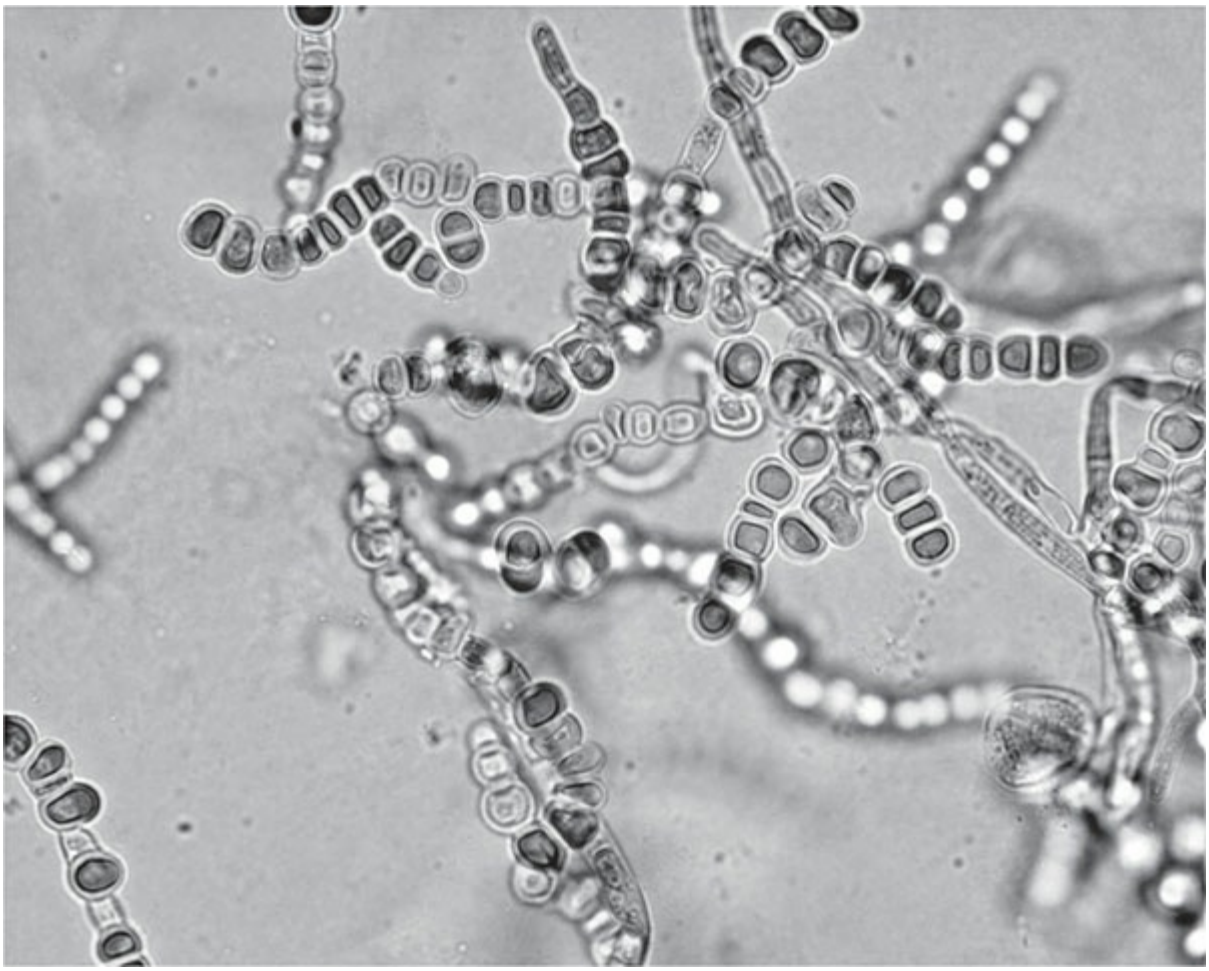


■ **FIGURE 21-52** Photomicrograph of the microscopic appearance of chains of chlamydospores, characteristic of *Trichophyton verrucosum.*

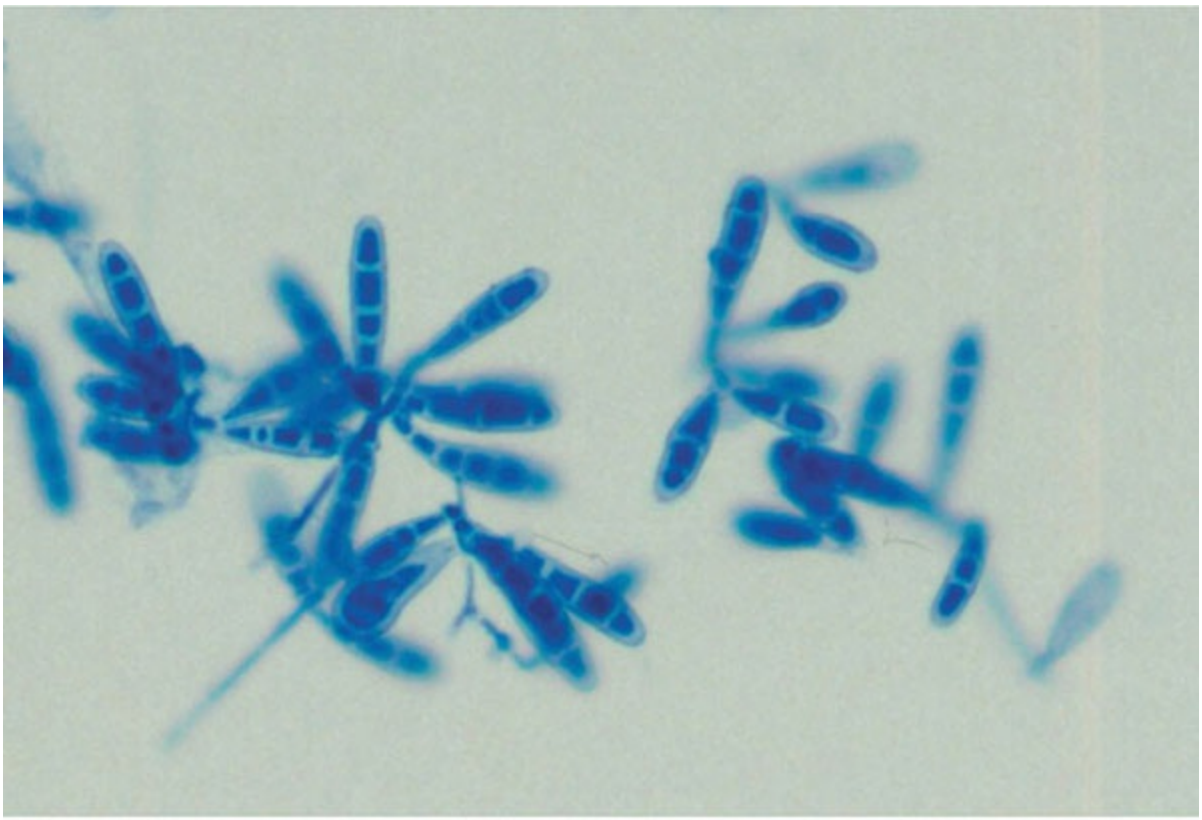
Trichophyton verrucosum. *Trichophyton verrucosum* is a zoophilic fungus that causes infections in cattle. Although it is transmitted by direct contact, it also may be transmitted by fomites. It may infect the skin, hair, and skin. Growth is slow, requiring 7 to 14 days to maturity. Growth is poor in *Trichophyton* agar 1, which is deficient in thiamine and inositol. Growth, however, will be observed on *Trichophyton* agars 2, 3, and 4, all of which contain either thiamine, inositol, or both. Colonies are small,

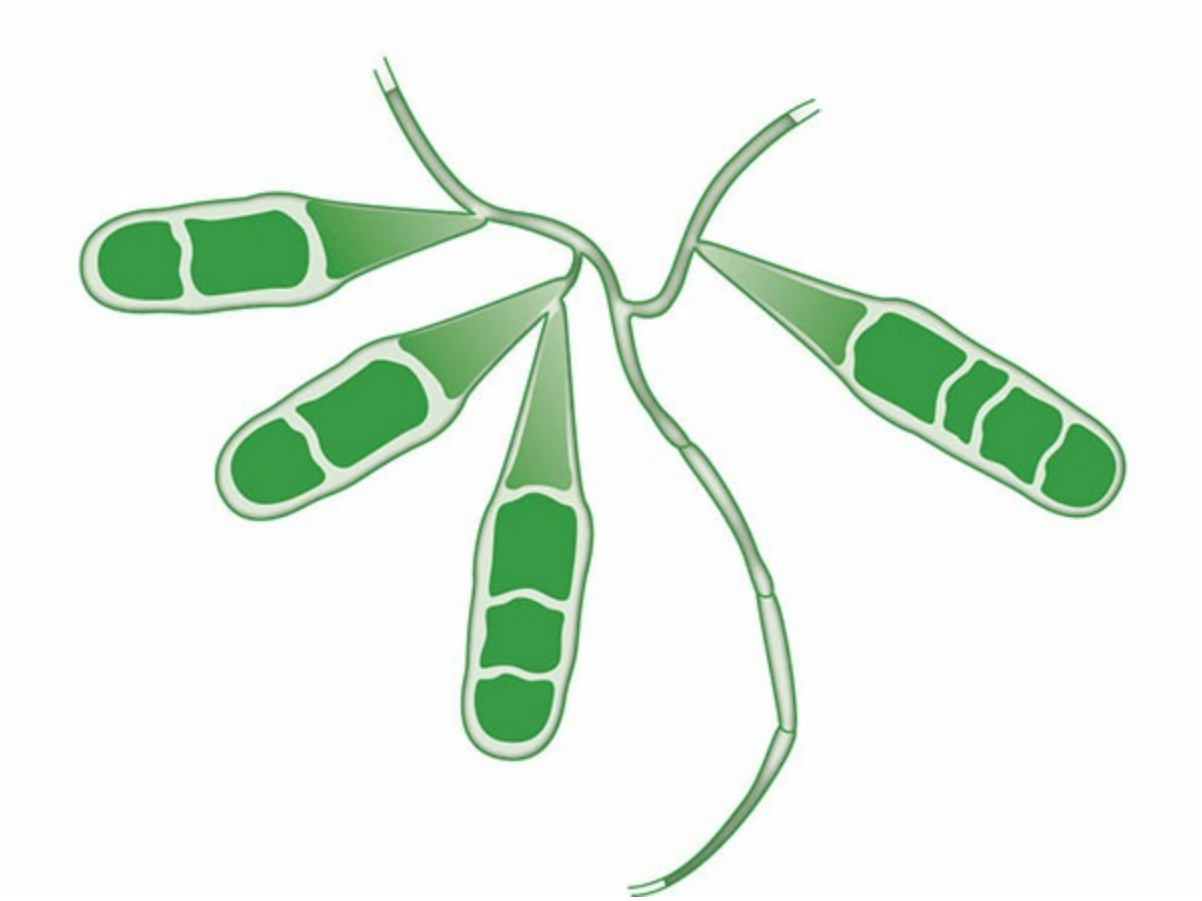
circular, flat, glabrous initially or downy later, and white to off-yellow. The reverse of the colony remains nonpigmented.

Microscopically, sporulation is usually poor. Antler-type hyphae may be observed and also typical are the production of numerous chlamydospores typically arranged in chains (Fig. 21-52). Macroconidia are rare, but are distinctive when present. They are multicelled, have thin, smooth walls, and are thin with a paddle, "string bean," or "rat tail" configuration. Microconidia, when present, are small, pyriform, and borne directly along the sides of the hyphae.

Epidermophyton floccosum. *Epidermophyton floccosum* is an anthropophilic dermatophyte that is an important cause for tinea cruris (jock itch) and tinea pedis (athlete's foot). The colonies grow rapidly, within 3 to 5 days; initially they are gray-white and then develop a distinct, characteristic khaki-green pigment when mature. Yellow-white streamers of hyphae may be seen radiating from the center of the colony to the periphery (Image Plate 21-4H). The surface becomes granular on further maturity as conidia are produced.

Microconidia are never produced—a key observation. Thus, if microconidia are observed in an unknown culture of a dermatophyte, *E. floccosum* can be eliminated from consideration. **Macroconidia** are usually abundantly produced, are typically club-shaped, and have three to five cells and thin, smooth walls (Fig. 21-53). The macroconidia often cluster in groups of three or four. Chlamydoconidia are typically present, particularly in older cultures.





■ **FIGURE 21-53** Photomicrograph and sketch of a cluster of elongated, cylindrical-shaped macroconidia of *Epidermophyton floccosum* with smooth walls and transverse septa. Microconidia are not produced by this fungus.

Diagnosis Using Nonculture Techniques

Molecular diagnostic assays have been described that detect dermatophytes, but these have not been widely employed and are not commonly used. Therefore, this section is limited in scope. Liu et al.[167] have used arbitrarily primed polymerase chain reaction and were able to identify up to 20 of the 25 dermatophyte species or subspecies under investigation. Although the use of such technology may be rarely required, reference is provided here for those instances when more exacting definition is needed.

The clinical manifestations of the various dermatophyte infections are in Clinical Correlation Box 21-4.

The Dimorphic Fungi

A subset of the hyaline molds, the dimorphic fungi, is usually considered separately because they have unique characteristics. First, they are dimorphic; that is, they exist in the mold form in the environment ("room temperature incubation"), whereas yeasts or yeast-like forms are produced during infection (i.e., in situ in the human body). All of these, except,

CLINICAL CORRELATION BOX 21-4 The Dermatophytes

Tinea capitis, Tinea corpora, Tinea pedis, and *Tinea cruris* are the names given to the various forms of dermatophyte infections. Onychomycosis refers to infections of the nails. With the advent of more effective oral antifungal therapy, the laboratory identification of the dermatophytes is now less frequently required. In most clinical practices, a KOH or KOH/calcofluor white mounts of skin scales, nail scrapings, or hairs are observed for the detection of typical hyphae that tend to break up into arthroconidia. Therapy is commonly instituted without obtaining a culture, if the direct examination demonstrates fungal elements and the examination is performed in the physician's office (i.e., provider performed microscopy). Although the vast majority of infections are superficial, there have been reports of locally invasive

disease, which is more likely in an individual with a compromised immune system.[250]

The term "tinea," dating back to the Middle Ages, refers to the circular holes in garments produced by the clothes moth, an appearance similar to the ringlike lesions of the skin produced by dermatophytic fungi. The term is now used to describe the various clinical syndromes caused by the dermatophytes. Tinea infections are also common in dogs, cats, horses, cattle, and other animals, providing a source for human zoophilic infections. The following is a brief summary of the several clinical types of tinea infections.

Tinea capitis—Ringworm of the Scalp
Several types of infection may be observed:

1. Gray-patch ringworm, a communicable ectothrix infection of children caused by *Microsporum audouinii* or *M. canis*;
2. Inflammatory ectothrix infection with *T. mentagrophytes*, of animal origin;
3. Black-dot ringworm, an endothrix infection in which infected, degenerate hairs break off at the skin surface producing what appears to be a black dot, caused by *T. tonsurans*; and
4. Fungating exophytic masses (kerions) produced by *T. tonsurans*, or favus infections caused by *T. schoenleinii* (in Scandinavia and northern Europe) and by *T. violaceum* (in southern Mediterranean Europe).

During the past 50 years, *T. tonsurans* has replaced *T. audouinii* as the predominant etiologic agent of *tinea capitis* in the United States and Western Europe.[76,107,299] *T. tonsurans* infections are contagious, as evidenced by an outbreak of *tinea corporis* infections among four healthcare workers in an inpatient rehabilitation ward who contracted the disease from an infected patient.[166] The contagious spread of *T. tonsurans* has become a nuisance among young competitive wrestlers. As the lesions appear somewhat different in a subset of youth wrestlers compared with the pediatric population as a whole, the term "*tinea gladiatorum*" has been applied to this form of infection.[152] *Tinea capitis* is most frequently obtained either from direct contact with an infected person or with a variety of fomites; an asymptomatic adult carrier state may contribute to persistence of infection in a given setting.[19] In a study of dermatophyte infection in 202 children residing in Kuwait, al-Fouzan et al.[7] found that *tinea capitis* was the most common infection and *M. canis* was the most prevalent species (96% of patients in this series). Although in the great majority of infected patients the infection remains confined to the superficial skin, reports have indicated that the fungus can invade deeper tissues in immunocompromised hosts. King et al.[148] report a subcutaneous papular infection in a kidney-transplant recipient wherein invasive fungal elements were seen in a deep biopsy of the skin. Similarly, deep subcutaneous penetration of erythematous nodules of the scalp and face has been seen in a patient with AIDS.

Tinea corporis—Ringworm of the Body
Typical annular lesions on the skin of the smooth parts of the body that have a spreading, hemorrhagic border are caused most commonly by *T. rubrum*, *T. mentagrophytes*, and *T. tonsurans*. *T. tonsurans* is being recovered with increasing frequency as the cause of *tinea corporis* in the United States. *T. rubrum* in particular is well suited to survive on the surface of the skin, leading to chronic infection, often enduring for the lifetime of the patient.[58] The mannans of *T. rubrum* appear to be better able to suppress cell-mediated immune reactions than are mannans from other fungi, thus modulating host response and permitting survival. *T. rubrum* also can survive off the human body as spores in desquamated skin scales, promoting person-to-person transmission in various human habitats. Zoophilic *M. canis* and geophilic *T. gypseum* infections are also occasionally encountered in clinical practice.

Tinea barbae—Ringworm of the Bearded Area
This zoophilic infection has most commonly been found in farm and dairy workers. *T. mentagrophytes* is the most common agent recovered from human infections, and the lesions tend to be inflammatory. *Trichophyton verrucosum* is also commonly associated with *tinea barbae*, acquired from the hide of dairy cattle. Sabota et al.[239] have reported *T. verrucosum* infection in five patients, three of whom had severe pustular *tinea barbae* and two of whom had eruptions of the forearms. All five patients were dairy farmers. A KOH preparation showed hyphae, and cultures yielded *T. verrucosum* in all three instances. *T. verrucosum* can cause pustular *tinea barbae*

in farmers that may be mistaken for a *Staphylococcus aureus* infection by clinicians, including infectious disease experts. The answer to the simple question "Are you a dairy farmer?" may suggest the possibility of *T. verrucosum* in the proper clinical setting.

Tinea cruris—Ringworm of the Groin
The lesions tend to be circinate and serpiginous, with inflammatory, vesicular, enlarging margins, most commonly caused by *Epidermophyton floccosum*. This infection may reach epidemic proportions in athletes, soldiers, and ship's crews, among whom towels, linen, and clothing may be shared.

Tinea pedis—Ringworm of the Feet (Athlete's Foot)
This is the most common fungal infection in humans, typically manifesting as itching, scaling, or seeping skin lesions on the soles of the feet and/or between the toes. Infections are most common during the warm, humid months. *T. mentagrophytes, T. rubrum*, and *E. floccosum* are the dermatophyte species most commonly recovered. The increased amount of keratin on the soles of the feet and the palms of the hands

makes these two sites selectively vulnerable to infection with *T. mentagrophytes* and other dermatophytes. The capability of *T. rubrum* to survive as spores in desquamated skin scales makes these hyperkeratotic areas particularly vulnerable to contracting infections from contaminated bath towels, locker room floors, and other human habitats.

Tinea unguium—Ringworm of the Nails
Tinea unguium is the term used to describe involvement of the nails by dermatophyte fungi, to be differentiated from onychomycosis, which refers to nail infections caused by a wide variety of fungi, including non-dermatophytic fungi, such as *Scopulariopsis,* among a variety of others. *Tinea unguium* infections begin at the lateral or distal edge of the nail plate and results in paronychial inflammation. As the lesion progresses, the nail becomes thickened and brittle, with accumulation of subungual keratinized debris. The dermatophytes most commonly involved are *T. rubrum* and *T. mentagrophytes*.

Lugo-Somolinos and Sanchez,[174] in a study of 100 consecutive patients with diabetes, found no greater incidence of dermatophyte infections compared with a control population. Dermatophyte infections of one type or another were found in 31% of the patients with diabetes mellitus and in 33% of the control group. Thus, contrary to popular notion, diabetes mellitus apparently does not predispose to dermatophytosis.

Coccidioides species are thermally dimorphic (i.e., produce the yeast or yeast-like form when incubated at 35°C to 37°C. The mold form is produced in the environmental niche of the fungus and produces the infective conidia. Humans and animals become infected predominantly through inhalation of airborne conidia for all dimorphic fungal pathogens, except *Sporothrix schenckii* which predominantly causes infection through direct traumatic implantation. Once in the body (i.e., at a temperature of 35°C to 37°C) the conidia transform into a yeast form, or spherule if the infecting pathogen is a *Coccidioides* spp. Apart from *Sporothrix schenckii,* the dimorphic fungal pathogens are important causes of "deep-seated" infections in humans. The severity of the infection is largely determined by the infecting organism, site of infection and burden of disease, immunologic status of the host, and availability and promptness of antifungal therapy.

The species of medical importance that are reviewed here include the following:

Blastomyces dermatitidis
Coccidioides immitis/C. posadasii
Histoplasma capsulatum
Sporothrix schenckii complex

The colonies from these fungi, with the exception of *Coccidioides* species, typically grow slowly (10 to 30 days) when recovered in primary culture from clinical specimens. In instances wherein the load of fungal organisms in a clinical specimen is high, then growth may be observed within 4 to 7 days. The growth of *Coccidioides* is sufficiently rapid that a fine delicate growth may be observed within 24 to 72 hours on routine blood agar plates incubated at 35°C. This represents a hazard to bacteriologists; therefore, all plates that contain a mold should be handled within a biological safety cabinet. At 30°C, the usual temperature of laboratory incubation, the colonies appear as a mold with a delicate, silky, hairlike, or cobweblike mycelium. In some cases, focal areas may appear yeast-like, particularly during the early phases of growth or if recovered on blood-containing media (Image Plate 21-5A). The colonies typically are gray-white or buff, although a few strains may show light pastel yellow shading into pink pigmentation. Colonies of *S. schenckii* may have a darkened appearance, which increases with extended incubation. The dimorphic fungi as a group also have the capability of growing in culture media containing cycloheximide, a valuable property in separating these fungi from the saprophytic filamentous molds, including their environmental mimics, which grow poorly or not at all.

If mold-to-yeast conversion is attempted (i.e., the temperature of incubation is increased to 35°C to 37°C), a prickly stage of transformation may be observed before the typical yeast-like colonies develop (Image Plate 21-5C). When yeast conversion is complete, the colonies have a pasty, yeast-like appearance, are glabrous, are usually entire, and have an off-white-to-yellow pigmentation (Image Plate 21-5E). Although demonstrable, yeast conversion of mold cultures is rarely attempted in most laboratories; confirmation of suspect

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isolates is usually achieved using commercially available nucleic acid probes or other molecular techniques.

Even prior to the production of conidia, one of the dimorphic molds may be suspected microscopically if very delicate hyaline, septate hyphae is observed and there is growth on cycloheximide-containing media. Commonly, the hyphae line up in parallel bundles. The next step in species-level identifications is made from the microscopic examination of colonies recovered in culture, based on the size, shape, position, and derivation of conidia. Confirmation is usually achieved by the use of a specific nucleic acid probe or another molecule technology (e.g., DNA sequencing). It is also possible to make an identification based on the morphology of the yeast or yeast-like forms as seen in stained tissue sections. The criteria for identification of the individual dimorphic molds will be presented in the paragraphs that follow.

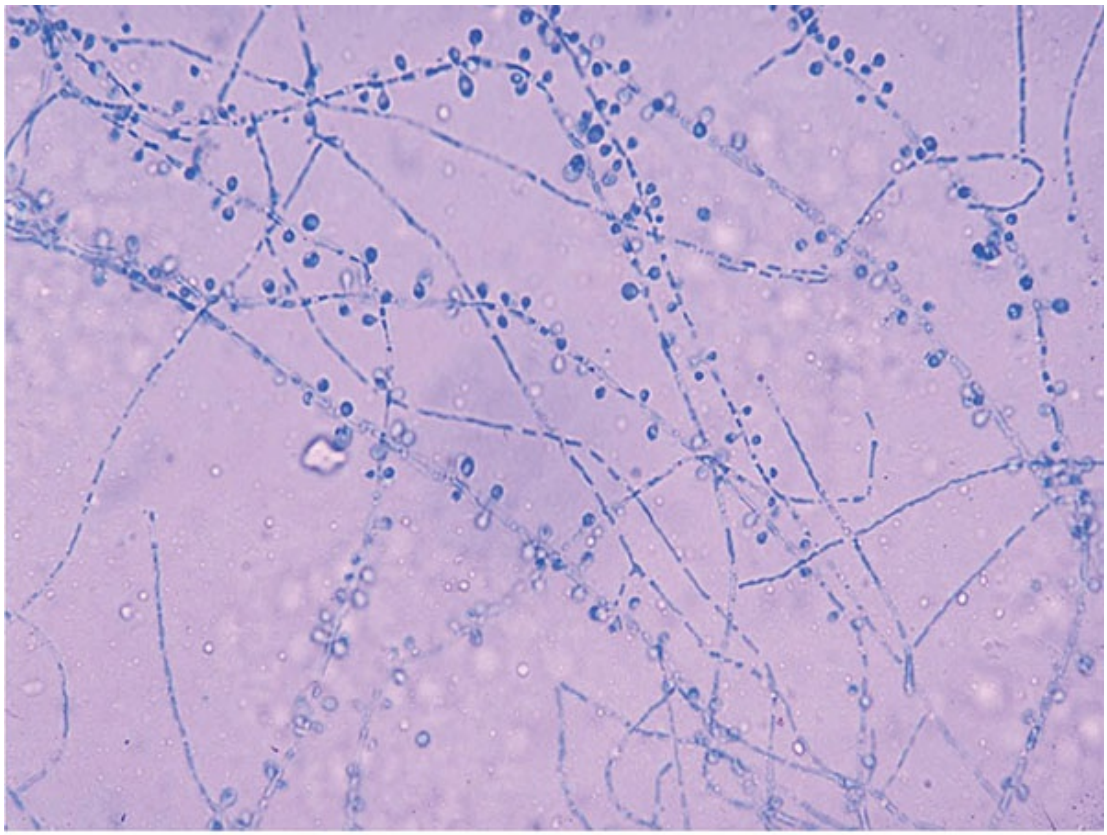
Blastomyces dermatitidis *and Blastomycosis*

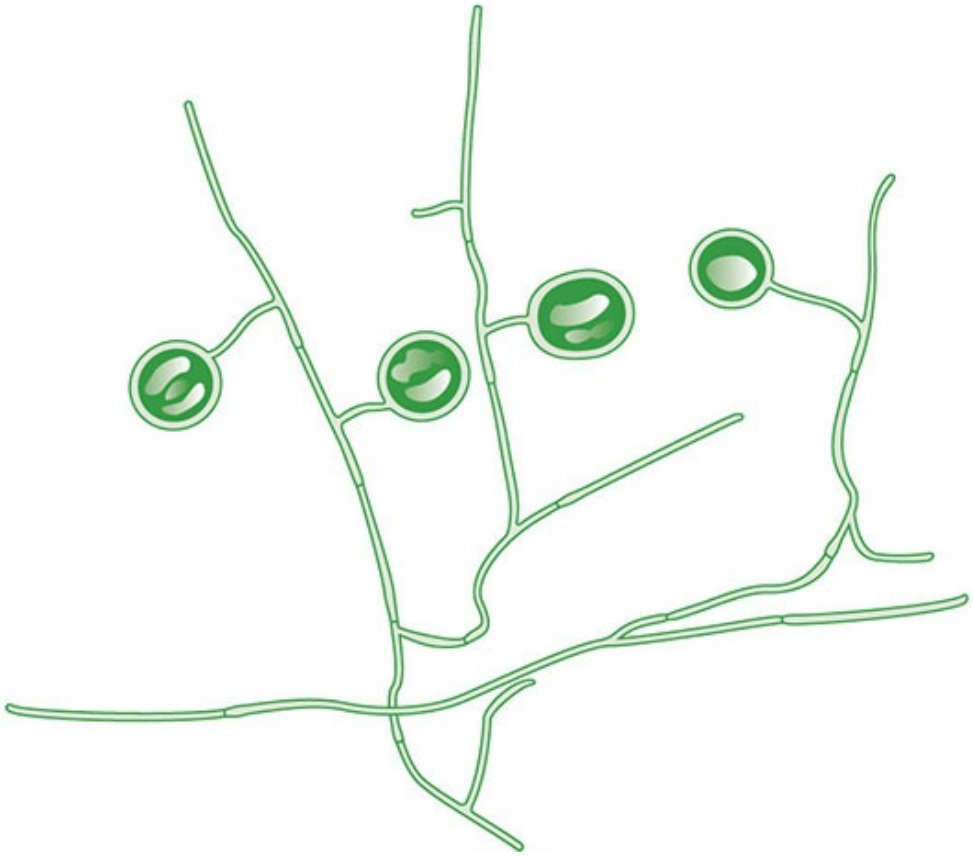
Blastomyces dermatitidis is a mold found in the soil and decaying vegetation. It is endemic in the states adjacent to the Mississippi and Ohio River valleys (Kentucky, Arkansas, Mississippi, North Carolina, Tennessee, Louisiana, Illinois, and Wisconsin).[21,150] In contrast to histoplasmosis, in which the extent of the endemic zone has been clearly mapped based on positive skin tests, the regions of infection with blastomycosis are determined only on the basis of individual case findings because of the lack of a sensitive skin test.[62] Humans are presumed to become infected from inhalation of airborne conidia, possibly secondary to the mechanical disruption of the mold form. One patient contracted blastomycosis after spreading mulch, which presumably harbored the mold. Blastomycosis is also a disease of animals, with a high incidence of infection in dogs.

Laboratory Presentation. The colonies typically grow slowly in primary culture (10 to 30 days), except in cases of heavy infections, wherein growth may be seen within 1 week. At 30°C, the colonies appear as a gray-white to light-buff-colored mold with a delicate, silky, or hairlike mycelium (Image Plate 21-5B). Growth occurs on cycloheximide-containing media, which is an important differentiating feature for molds that may be morphologic mimics for *Blastomyces*. In conversion to the yeast form, a prickly stage may be seen during transformation before the typical yeast-like colonies develop. The colonies become smooth and more yeast-like, except, in some instances, for the aerial protrusion of delicate spikes (Image Plate 21-5C).

Microscopically, in the mold form, the hyphae are delicate (approximately 2 µm in diameter), hyaline, and septate. Spherical to oval conidia measuring 1 to 4 µm in diameter are borne singly at the tips of long or short conidiophores (some believe these resemble "lollipops") (Fig. 21-54). The conidia of the soil saprobe, *Chrysosporium* spp., have a similar appearance microscopically. It is important to recognize that the microscopic examination of very young colonies of *Blastomyces* may contain remnants of the yeast forms that have not completely converted to the mold phase.

The yeast conversion colonies grown at 35°C on enriched agar are small, entire, slightly convex, smooth, and may have an off-yellow or buff pigmentation. Microscopically, the yeast forms are large (10 to 15 µm), and characteristically have a single bud attached by a broad base (Fig. 21-55). These broad-based budding yeast forms may also be observed in tissue sections often associated with a pyogranulomatous inflammatory response (Fig. 21-56, left). These forms may be confused in tissue sections with the endospores and immature spherules of *Coccidioides immitis* that may be lying adjacent to one another. However, the development of endospores within the spherules provides for the true identification of *C. immitis* (Fig. 21-56, right).





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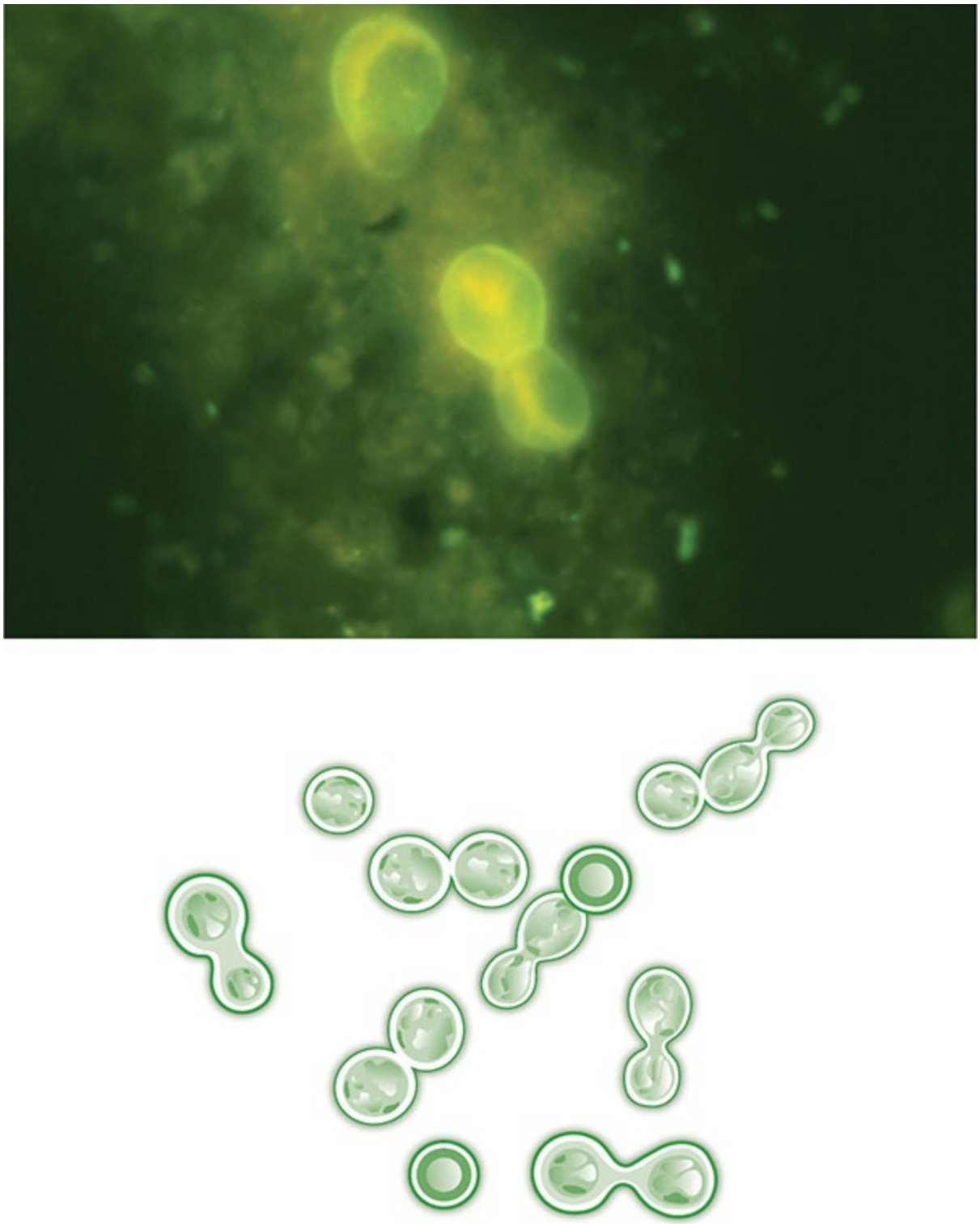
■ **FIGURE 21-54** Photomicrograph and sketch of the small, single conidia of *Blastomyces dermatitidis* attached to the hyphae by delicate, short conidiophores.

Diagnosis Using Nonculture Techniques. Serologic tests for antibody detection alone are generally considered to be inadequate for use in diagnosis of blastomycosis. However, a positive test is useful in a patient wherein blastomycosis is in the differential diagnosis. The immunodiffusion and complement fixation assays are positive in only approximately 25% of known cases. Enzyme immunoassays (EIAs) are more sensitive than immunodiffusion (ID), but less specific. A large common-source outbreak is cited in which the sensitivity of EIA (77%) was higher than that of ID



■ **FIGURE 21-56** **A.** Photomicrograph of a GMS-stained tissue section illustrating loose clusters of black-staining yeast cells of *Blastomyces dermatitidis*, some with a characteristic broad-based bud *(arrow)*. **B.** Photomicrograph of an H&E-stained tissue section, illustrating small empty immature spherules of *Coccidioides immitis*, along with

large endospore-containing, mature forms. Empty spherules lying side by side may simulate the broad-based budding forms of *B. dermatitidis (arrows)*.

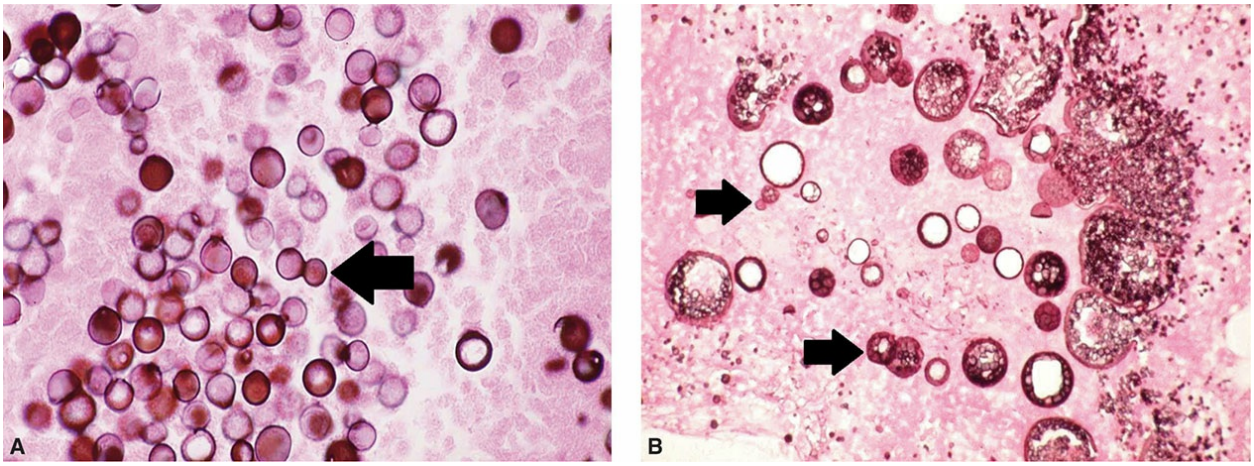
(28%) or complement fixation (CF) (9%), but its specificity was lower, 92%, compared with 100% for ID and CF.[73]

Blastomyces urinary antigen may be used as an ancillary diagnostic test. In one study, it detected antigenuria in 92.9% of patients with blastomycosis, including 89.3% of disseminated and 100% of pulmonary forms of disease.[74] Other body fluids also may be suitable for antigen testing. Antigen has been detected in serum, cerebrospinal fluid (CSF), and bronchoalveolar lavage fluid (BAL). Detection of antigen in BAL may improve the sensitivity for diagnosis in patients with pulmonary disease, particularly in milder cases. Antigen detection in CSF may be helpful in establishing a diagnosis of *Blastomyces* meningitis, as yeast cells are not seen in direct examinations and cultures are negative in approximately 50% of patients. Failure of antigen to clear during a course of therapy may herald treatment failure; a recurrence of illness may be detected if antigen levels rise by two- or threefold.[74] Although Connolly et al.[55] found the assay to have high sensitivity and specificity, they also reported that the *Blastomyces* urinary antigen tests demonstrated cross-reactivity in 95.6% of patients with histoplasmosis. The clinical manifestations of blastomycosis are presented in text in Clinical Correlation Box 21-5.

Coccidioides immitis, Coccidioides posadasii, *and Coccidioidomycosis*

Coccidioides species are endemic in arid and semi-arid areas, such as deserts. There are now two recognized species in the genus *Coccidioides*, *C. immitis*, and *C. posadasii*. *C. immitis* is the species that is endemic in California (e.g., in the San Joaquin Valley), whereas *C. posadasii* is the species found elsewhere (e.g., southern portions of Arizona, and parts of Mexico). These species are morphologically indistinguishable and can be differentiated only using advanced molecular methods. *Coccidioides* is a dimorphic fungus, but it is the one dimorphic pathogen wherein the dimorphism is not due solely to temperature differences (i.e., it is not thermally dimorphic). The mycelial form grows in the hot deserts, in which the elevated subsurface temperatures are ideal for propagation. The hyphae are branched, and fragment into individual arthroconidia when the soil is disturbed. These arthroconidia are tiny, light, and easily windborne in clouds of blowing sand and dust. On inhalation, these arthroconidia escape the mechanical defenses of the upper respiratory tract and reach deeper portions of the bronchial tree. Within the alveoli, the arthroconidia transform into thick-walled spherules, which, on maturity, are filled with endospores. Endospore-containing spherules are the diagnostic forms seen in stained tissue

sections.



■ **FIGURE 21-55** Photomicrograph and sketch of large yeast cells of *Blastomyces dermatitidis*, many producing a broad-based bud.

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CLINICAL CORRELATION BOX 21-5 Blastomycosis

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The clinical presentations of blastomycosis have been categorized by Davies and Sarosi[62,63] as follows:

1. Asymptomatic, usually discovered only in outbreak situations;
2. Flu-like illness of brief duration, resembling other upper respiratory infections;
3. Illness resembling bacterial pneumonia, with acute onset of high fever, lobar infiltrates, and productive cough;
4. Subacute or chronic respiratory illness with a symptom complex simulating tuberculosis or lung cancer, with radiographic presentation of fibronodular infiltrates or mass-like lesions; and
5. Fulminant infectious adult respiratory distress syndrome (ARDS) with high fever, diffuse infiltrates, and progressive respiratory failure.

In clinical settings, blastomycosis almost always begins in the lungs as the primary pulmonary form of the disease, with pneumonia being the most common initial

clinical manifestation.[30] The pneumonia may be short-term, mimicking bacterial pneumonia, or may become more indolent; occasionally, it may be progressive and severe. Most commonly, symptoms resolve spontaneously after a brief flu-like syndrome. Dry cough, low-grade fever, weight loss, night sweats, pleuritic chest pain, and myalgias may be presenting symptoms early in acute infections. Persistent localized chest pain, weight loss, night sweats, and malaise may indicate progression to a chronic form of disease.

Mississippi has been noted to have a high prevalence of blastomycosis.[163] Even in this endemic area of physician awareness, the diagnosis of blastomycosis was difficult to recognize. Blastomycosis was correctly suspected on initial examination in only 18% of 123 patients being evaluated at the University of Mississippi Medical Center in Jackson.[164] Nonspecific pneumonia (40%), malignant tumors (16%), and tuberculosis (14%) were the most frequent misdiagnoses. Only in the presence of cutaneous involvement was the initial suspicion raised to 64%. These misdiagnoses often led to unnecessary surgeries, delays in treatment, or the administration of ineffective antibiotic therapy for months. Immunosuppression (25% of patients) and diabetes mellitus (22% of patients) were the more frequent predisposing conditions.

Rarely pregnant women will contract blastomycosis.[165] Fetal risk exceeded maternal risk in this review. Of 20 babies born from mothers with blastomycosis, only 2 (10%) had transplacental infection and both died. Not only did none of the 18 affected mothers die of the disease, there was no progression with 14 complete cures and considerable postpartum regressions of lesions in the other four women.

Bone is a common site for extrapulmonary infection. From the older literature, a patient with temporal bone osteomyelitis secondary to *B. dermatitidis* manifesting as serous otitis media was reported by Farr et al.[85] In a study of 17 patients by MacDonald et al.,[176] the metaphyses of long bones and small bones were most frequently involved. The metaphyseal lesions tend to be eccentric, well circumscribed, and lytic. More recently, Hadjupavlou et al.[109] reported a patient with blastomycosis of the lumbar spine that caused severe and crippling deformity. The authors emphasize the importance of early, aggressive treatment to prevent deformity and disability.

The genitourinary tract, particularly the prostate gland, epididymis, and kidney; the brain, with local abscess formation; lymph nodes; and the adrenal gland are other extrapulmonary sites of involvement culled by Kwon-Chung and Bennett[160] from the earlier literature. The clinical manifestations commonly are those of nonspecific epididymitis and prostatitis, and the diagnosis must be made by demonstrating organisms in biopsy material, and may be missed in the absence of a high degree of suspicion.

Blastomyces may disseminate to the skin and other sites with squamous epithelium. A report by Hanson et al.[120] of two patients with laryngeal blastomycosis that was misdiagnosed as squamous cell carcinoma is concerning. In one of these patients, this

misdiagnosis led to radiotherapy and laryngectomy. In the second patient, although a clinical diagnosis of glottic squamous cell carcinoma was rendered, the budding yeast forms of blastomycosis were identified in a biopsy specimen. Review of the English-language literature demonstrates that blastomycosis may be misdiagnosed clinically and microscopically as squamous cell carcinoma.

Reder et al.[229] reviewed a large series of blastomycosis diagnosed at the Mayo Clinic. Involvement of the skin and mucous membranes (including the larynx) was quite common, often with clinical and histologic features resembling well-differentiated squamous cell carcinoma. Skin or mucous membrane involvement usually indicates systemic disease, and in many instances these may be the presenting lesions. The diagnosis of blastomycosis should be considered in the differential diagnosis of any patient with nonhealing skin lesions associated with risk factors such as living in an endemic area and having an occupation or vocation involving frequent contact with soil.[16] Single or multiple ulcerating papules or pustules of the skin, usually involving the face, hands, or lower legs, may slowly progress into an ulcerated verrucous granuloma with an advancing serpiginous border. Primary cutaneous lesions occurring at the site of penetrating injuries of the skin do not spread systemically.

Blastomycosis is not considered as one of the AIDS-defining infections; nevertheless, in some practice settings, a distinct increase in incidence and in severity of disease occurring in immunocompromised hosts has been observed.[289] Disease is particularly progressive in patients with AIDS, particularly when the CD4 count drops below 200/mL.[213] Fraser et al.[92] studied in parallel two friends, one with AIDS, who were infected with the

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same strain of *B. dermatitidis* (proven by restriction endonuclease analysis). In the patient with AIDS, severe, progressive fatal pulmonary blastomycosis developed despite aggressive treatment with fluconazole and amphotericin B; his HIV-negative friend responded completely to the same course of therapy. The authors concluded that cellular immunity plays a critical role in the progression of disease in patients with blastomycosis.

Of interest is the review of 123 charts of patients with the diagnosis of blastomycosis at the University of Mississippi Medical Center to determine the role of wet preparation, cytology, histology, and culture in diagnosing this fungal disease.[164] The etiologic agent was detected by cytology in 56.1% of all cases, and in 71.8% of pulmonary cases. Wet preparation was second in sensitivity to detect the fungus (37.4%); histology was the third (32.5%). Cultures were positive in 64.2% of all patients but provided the initial diagnosis in only 3.2% of all patients. There was pulmonary involvement in 87% of patients, cutaneous involvement in 20%, osseous involvement in 15%, and central nervous involvement in 3%. Dissemination with intraocular disease has also been reported.[172]

Rarely, hyphal forms may be found in tissue sections, particularly if the affected site has been exposed to air. Hagman et al.[110] reported five cases of coccidioidomycosis in which hyphae were found in brain tissue or spinal fluid. They theorized that the presence of foreign material in the central nervous system of these patients was associated with morphologic reversion to the saprophytic form.

The incidence of coccidioidomycosis in endemic areas has varied in part based on weather conditions; there were increases in the incidence of disease in 1991 and 1992 during alternating periods wherein heavy rainfall was followed by drought.[38] The disruption of the soil has also been associated with an increase in disease; there was a marked increase in incidence, which also occurred in the decade following the Simi Valley earthquake.[211] Kirkland and Fierer[149] have estimated that this outbreak cost more than $66 million in direct medical expenses and from time lost from work in Kern County, California, alone. In endemic areas, particular attention should be paid to ranchers, farmers, construction workers, and others engaged in outdoor activities requiring intimate exposure to dust and soil. Archeologists participating in excavations in endemic areas are at particularly high risk for infection.

Coccidioidomycosis remains a growing problem in the southwestern United States.[12] Related is the parallel increase in individuals with depressed cellular immunity, particularly those with HIV infection, those who have undergone allogeneic transplantation, and others on immunosuppressive medications.[276] Logan et al.[169] report that coccidioidomycosis remains the most common endemic mycosis in North America, largely related to the increase in solid organ transplants. They cite underlying renal and liver disease, T-lymphocyte suppression from antirejection medication, and activation of immunomodulating viruses, such as cytomegalovirus, among these patients, as factors increasing the risk for coccidioidomycosis. Interestingly, half of all patients that developed coccidioidiomycosis during the first year after transplantation were thought to represent the reactivation of previously acquired coccidioidal infection, rather than the acquisition of a new infection.

Dark-skinned individuals, pregnant women, and individuals of Filipino descent are at higher risk for disseminated disease, in addition to those with compromised immune systems. Individuals who travel to endemic regions are also at risk for infection, which may present after their return to non-endemic regions.[86] Therefore, travel history remains an important part of the clinical history. Patients with acute coccidioidomycosis who come to medical attention are likely to present with pulmonary manifestations, including pneumonia, cavities, and nodules. An old, inactive coccidioma may be detected on routine chest roentgenogram and be excised to exclude malignancy. Thus, coccidioidomycosis is of nationwide importance.

Physicians everywhere must maintain a heightened awareness of this disease and obtain a careful travel history of patients presenting with pulmonary signs and symptoms to avert delays in diagnosis and treatment.

Laboratory Presentation. The colonies of *C. immitis* vary in morphology but usually appear as delicate cobweblike, gray-white mycelium. When grown on blood-containing agar, the colonies may appear dark green-black in areas where hemoglobin pigments have been adsorbed (Image Plate 21-5F). Extreme care should be exercised when examining cultures suspicious for this fungus, since the arthroconidia are readily airborne. It is particularly important that bacteriologists initially examine their plates with the lid in place to assure that a mold is not present, since this fungus grows sufficiently rapidly to be recovered on routine bacteriologic media within the few days these plates are examined.

Microscopically, in the mold form grown at 30°C, the hyphae are delicate and every other cell in the vegetative hyphae becomes a barrel-shaped arthroconidium. The cells between the arthroconidia, dysjunctor cells, are vegetative cells that have died. These appear as empty spaces between the arthroconidia. The alternating pattern of arthroconidium, dysjunctor cell, arthroconidium, etcetera imparts and alternate staining pattern (Fig. 21-57). *Malbranchia* species and *Gymnoascus* species are saprophytic fungi that may appear morphologically similar to *Coccidioides*; these, however, are inhibited by cycloheximide, unlike *Coccidioides*. *Coccidioides* is not thermally dimorphic, so confirmation of the identity of the fungus is usually achieved by genetic probe analysis or another molecular method.

A true yeast form does not exist for *C. immitis*. As mentioned above, the diagnostic forms observed in tissue sections are spherules that range from 20 to 200 µm in diameter and, when mature, are filled with endospores that are 2 to 4 µm in diameterFig. 21-58).

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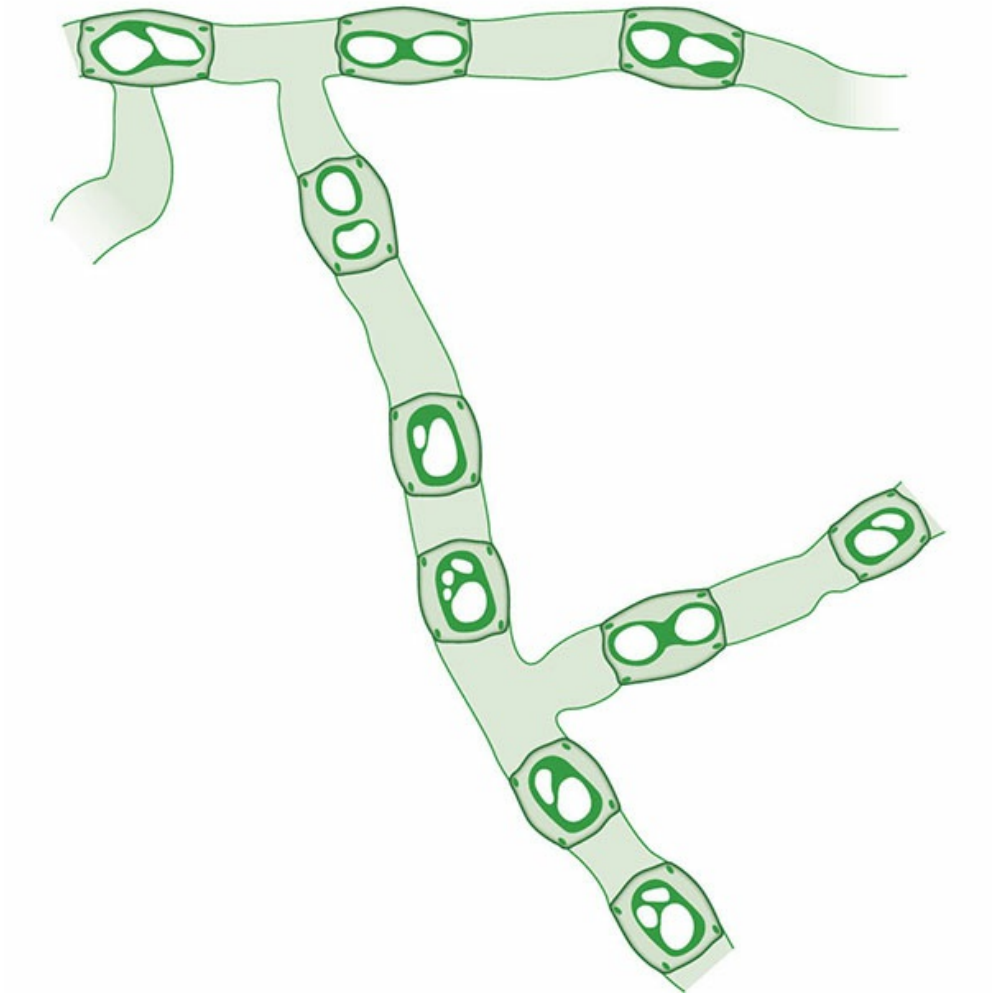
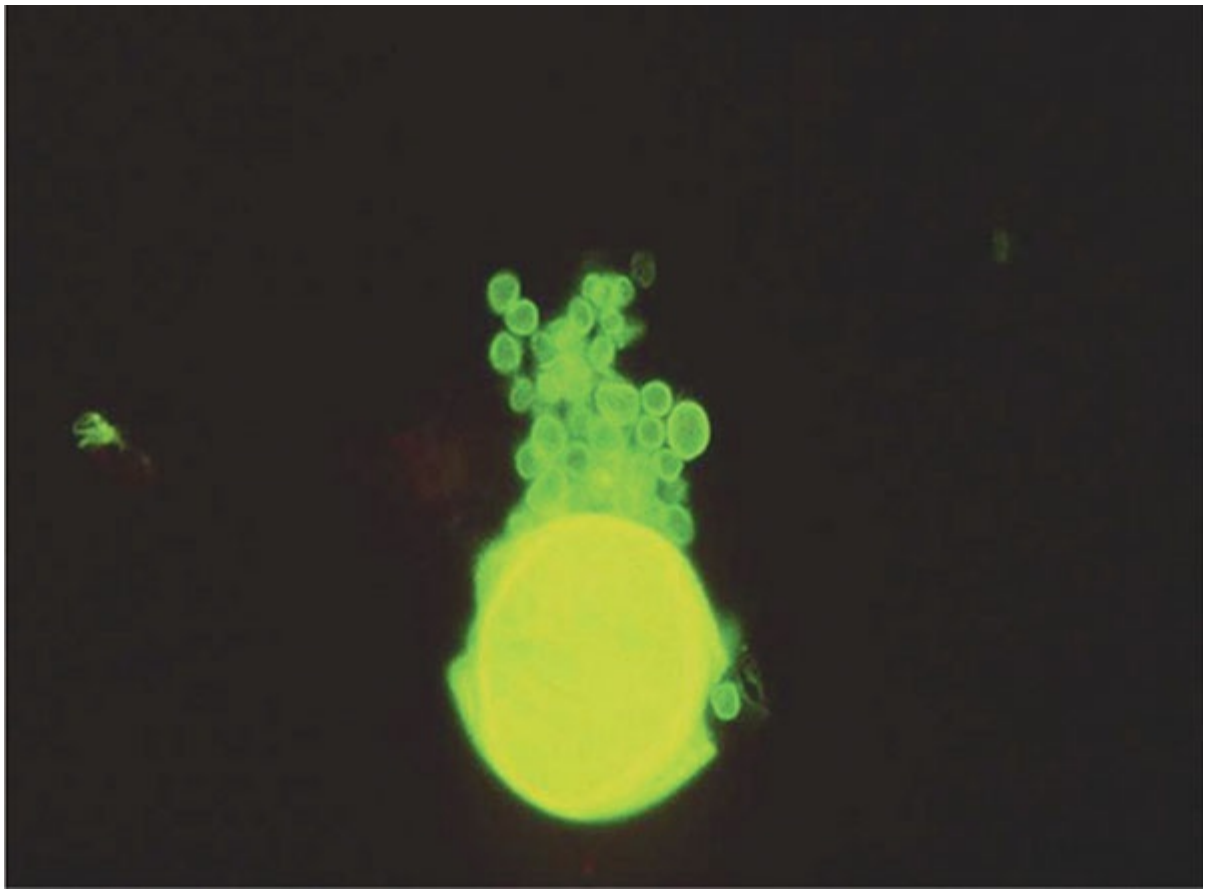


■ **FIGURE 21-57** Photomicrograph and sketch of hyphal forms of *Coccidioides immitis,* illustrating the characteristic barrel-shaped alternating arthroconidia, with individual cells separated by pale-staining empty spaces (i.e., dysjunctor cells).



■ **FIGURE 21-58** Photomicrograph of a spherule of *Coccidioides* discharging its endospores.

The clinical manifestations of coccidioidomycosis are presented in Clinical Correlation Box 21-6.

Histoplasma capsulatum and Histoplasmosis

Histoplasmosis, caused by the dimorphic fungus, *Histoplasma capsulatum*, is a common cause of systemic fungal disease in the United States. It is also the most common fungal infection in patients with AIDS and is an AIDS-defining illness.[188] The major endemic areas of histoplasmosis in North America are the drainage basins of the St. Lawrence, Ohio, Mississippi and Missouri River valleys, where a high percentage of the population is skin-test-positive, indicating past infection.

Histoplasmosis is also found in Central America.

Isolated indigenous cases have been reported from Europe—in Germany, Belgium, Holland, Australia, Italy, and Denmark.[34,54,207] *H. capsulatum* is the dimorphic fungus with the widest geographic distribution.

CLINICAL CORRELATION BOX 21-6 Coccidioidomycosis

Following exposure to *Coccidioides* arthroconidia, an influenza-like syndrome known as "valley fever" develops in the majority of individuals; in most instances, it is confined to the lung and self-limited, resolving over a period of weeks or months without therapy.[96] Sixty percent of infected individuals are asymptomatic; many skin-test-positive individuals do not recall having symptoms. Those who are symptomatic experience an acute, short-term, lower respiratory tract infection with varying degrees of cough, sputum production, chest pain, fever, and arthralgia. A bacterial-pneumonia-type syndrome is unusual, although two such instances have been reported.[171] Chronic pulmonary disease with sequelae ultimately develops in only 2%–5% of infected individuals. Solitary "coin lesions" or granulomas, solid or cavitary, usually located peripherally within the lung parenchyma, are common residual findings.

Based on original work by Fish et al.[89] and later refined by Minamoto and Rosenberg,[188] there are six categories of disease, based on the primary clinical presentation:

Group 1, Focal pulmonary disease—localized alveolar infiltrates; discrete lung nodules, solid or cavitary; hilar adenopathy.

Group 2, Diffuse pulmonary disease—a clinical syndrome simulating *Pneumocystis jirovecii* infection

Group 3, Cutaneous coccidioidomycosis—usually concomitant with pulmonary disease, with papules, pustules, nodules, subcutaneous abscesses, ulcers, and/or verrucous granulomas

Group 4, Meningitis—CSF pleocytosis, chiefly lymphocytes but possibly with increased eosinophils, lowered glucose and elevated protein concentrations.

Group 5, Disseminated disease—extrathoracic lymph node (most commonly inguinal) or liver involvement; distant spread reported most commonly in kidneys, thyroid gland, heart, pituitary gland, esophagus, and pancreas.

Group 6, Positive coccidioidal serology without a clinical focus of infection.

In endemic areas, infection with HIV is also a risk factor for more severe coccidioidomycosis.[12] In a study by Singh et al.[260] of 91 patients coinfected with HIV and *C. immitis*, fever, chills, weight loss, and night sweats were the most common symptoms. The mortality rate in this group was 60%, with diffuse pulmonary disease and a CD4 lymphocyte count of <510/μL, which were independent predictors of death. Coccidioidal serum serology titers were positive in only two thirds (68%) of these patients, with negative results found in 23%. The highest percentage of false-negative serology reactions were in the group of patients with progressive, invasive pulmonary disease.

Antoniskis et al.[15] found that two of eight patients in their study with coccidioidomycosis and HIV infection were repeatedly seronegative, concluding that histopathology and culture remain the most reliable methods for establishing the diagnosis in patients with AIDS. Conversely, Arguinchona et al.[17] found that asymptomatic individuals with positive CF serum serology titers went on to have active coccidioidomycosis. In reference to the accuracy of tissue biopsies, of 54 patients with coccidioidomycosis reported by diTomasso et al.,[68] transbronchial biopsy was 100% sensitive in yielding a rapid diagnosis, in contrast to cytologic examination of bronchial fluid or bronchoalveolar lavage, which provided a diagnosis in only 34% of patients.

The association of increased risk for disseminated coccidioidomycosis during pregnancy remains controversial. Wack et al.,[282] refuting many previous studies in which coccidioidomycosis during pregnancy was considered a devastating disease with high mortality, found only 10 patients among 47,120 pregnancies among women living in Tucson, Arizona. The infection resolved in seven of the women in whom coccidioidomycosis was diagnosed during the first or second trimester; progressive disease developed in two of three women who were diagnosed in the third trimester. Improvement in medical care and the introduction of antifungal therapy relatively early in the course of coccidioidomycosis may account for the lower mortality rate among pregnant women compared with that reported in the past.

Although coccidioidomycosis is primarily a pulmonary disease, Arnold et al.[18] report that disseminated disease affecting skin, subcutaneous tissue, bone, joints, and meninges develops in approximately 0.5%–1.0% of infected individuals. In a review of 47 patients from the medical literature, these authors found that virtually all head and neck manifestations in patients with disseminated coccidioidomycosis involved the skin, with a predilection for the central face. The lesions often tended to be multiple, including life-threatening airway lesions. A report of coccidioidal prostatitis by Truett and Crum[275] serve as a reminder that fungal agents must be included in the differential diagnosis of conditions in which they might be least expected. Persistent sterile pyuria, prostatitis, and granulomatous disease of the prostate were the presenting signs and symptoms in the patients reported.

The mycelial form of *H. capsulatum* is present in warm, moist soil rich in nitrogen and other organic contents, such as that produced by heavy accumulation of bird or bat excreta. Bird roosts, chicken houses, caves, or old buildings frequented by bats are areas that have been associated with infection. Patients with advanced HIV infection (i.e., AIDS) or those who are otherwise severely immunocompromised are counseled to avoid frequenting these areas as much as possible. Disruption of these areas by bulldozing or cleanup efforts may expose humans to large numbers of airborne conidia. Three large outbreaks of histoplasmosis in the Indianapolis, Indiana, and metropolitan area have been cited in 1978 in conjunction with the demolition of an amusement park, in 1980 related to the building of a swimming pool and in 1988 to 1993 during the time of the construction of a large tennis complex.[298]

During one of these outbreaks, Williams et al.[298] detected antigen in 92%, 21%, and 39% of patients with the disseminated, chronic pulmonary, and self-limited forms, respectively. The authors conclude that tests for serum antigen are most useful in patients with clinical findings of disseminated infection or during the first month of illness in cases of severe pulmonary involvement when serologic tests for antibodies may be negative.

Laboratory Presentation. The mold form of *H. capsulatum* is typically the slowest growing of the dimorphic molds taking usually between 10 and 30 days to grow. Rarely, cultures from specimens with a very heavy concentration of organisms may demonstrate growth as early as 5 days of incubation. The colonies are cottony and may develop a delicate cobweblike or hairlike aerial mycelium; these are initially white, but may turn gray or gray-brown with age (Image Plate 21-5D).

Microscopically, the diagnostic structures seen in the mold form are large, roughened/spiked (i.e., tuberculate) macroconidia measuring 10 to 20 µm in diameter

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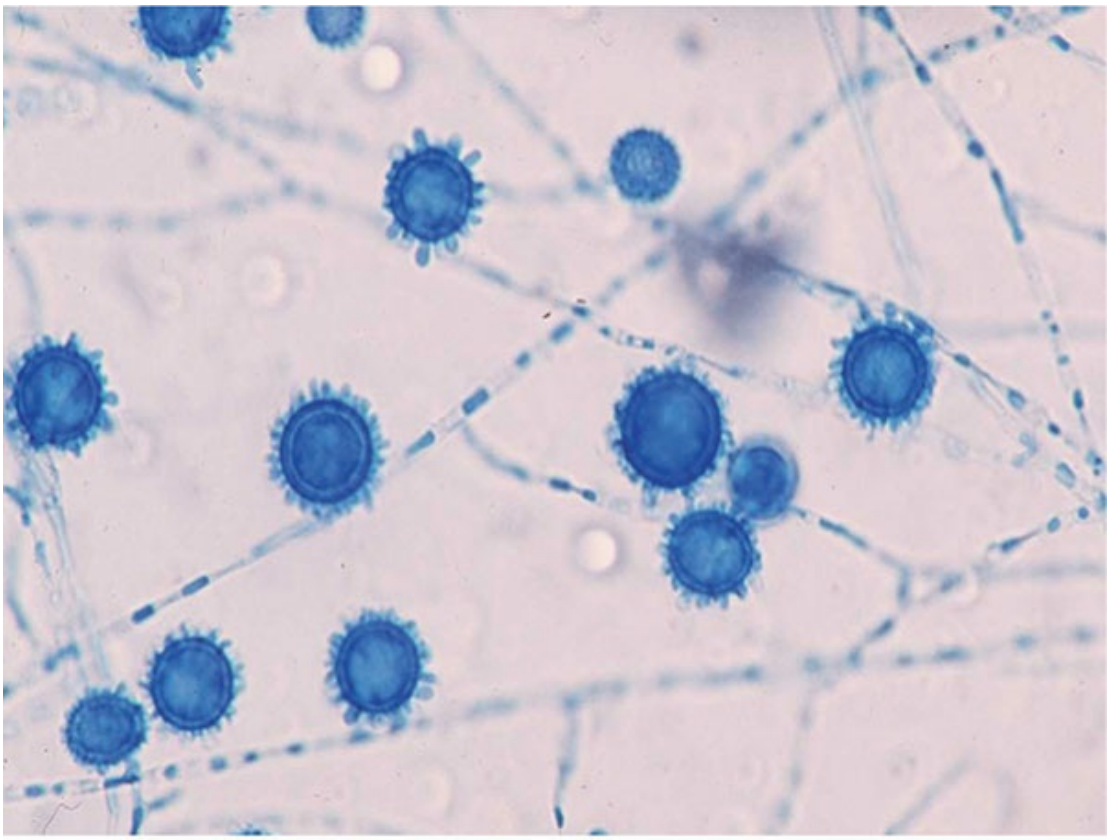
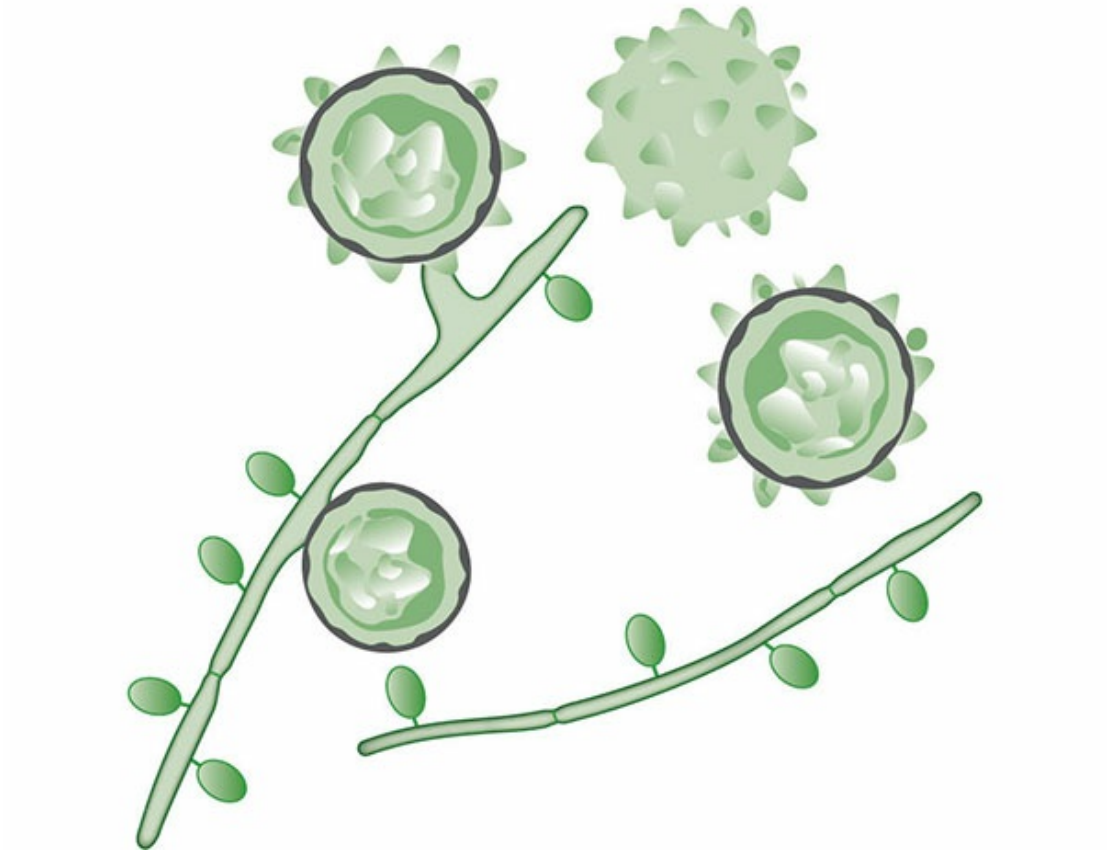


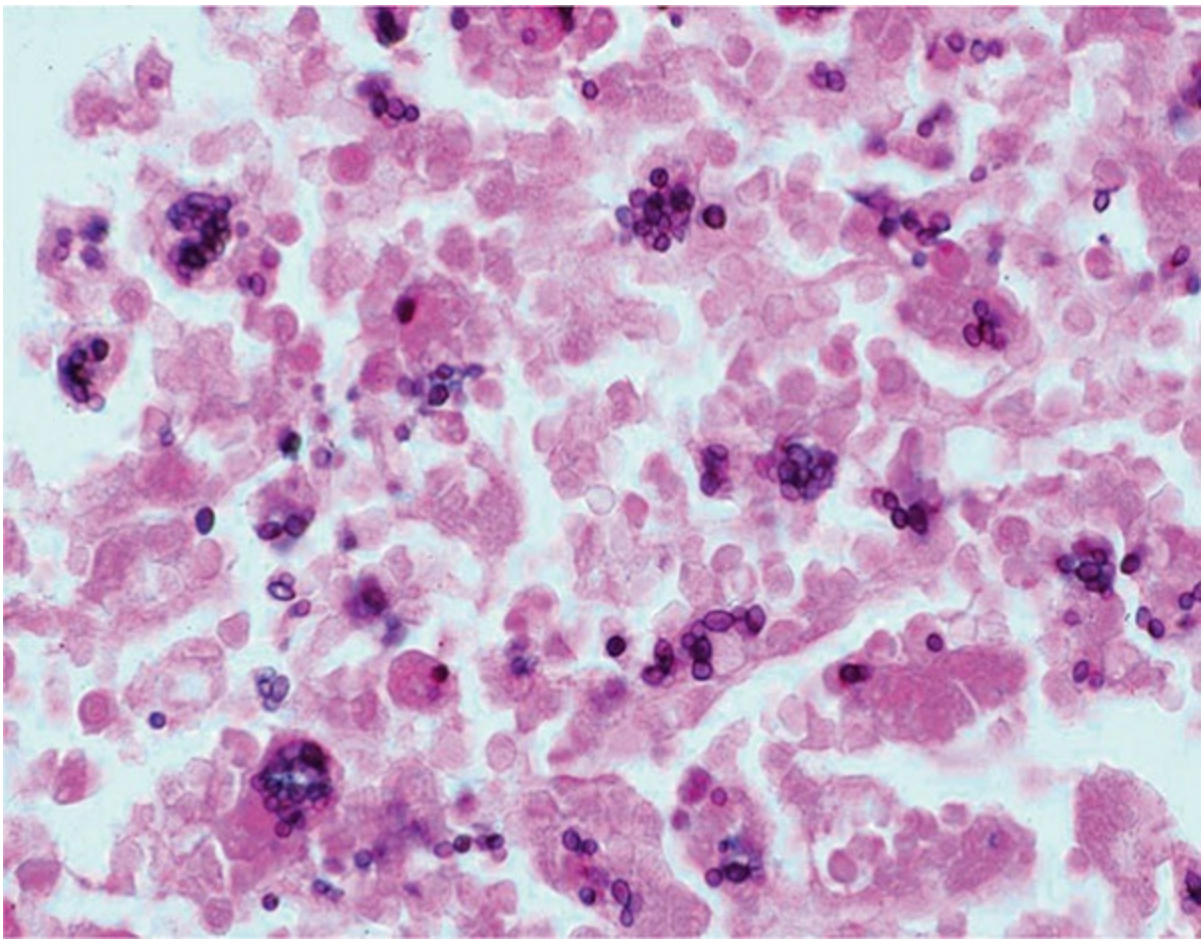


■ **FIGURE 21-59** Illustrated in the photomicrograph and sketch is the mold form of *Histoplasma capsulatum* forming large, spherical tuberculate (i.e., spiked) macroconidia.

(Fig. 21-59). In earlier cultures, small oval microconidia may be seen borne on small conidiophores that simulate those of *B. dermatitidis*. The microscopic features of *Sepedonium* species appear similar, but this species is inhibited by cycloheximide and would be negative in the genetic probe analysis for *H. capsulatum*.

The yeast colonies of *H. capsulatum*, whether grown from primary cultures incubated at 37°C or after conversion from the mold form, are typically smooth, yellow-white, and somewhat glistening, with a pasty consistency (Image Plate 21-5E).

Microscopically, the yeast cells of *H. capsulatum* are small, 2 to 4 μm in diameter, and may show a single unipolar bud. In histologic sections, the yeast cells often appear clustered either within a macrophage or remaining in this configuration after the macrophage has died. In some instances, the yeasts are surrounded by a clear space, which appears to be a capsule (Fig. 21-60). This, in fact, is not a capsule, but rather a retraction artifact due to tissue processing, but is responsible for the species name of this organism. The background inflammatory reaction is granulomatous in the immunocompetent. The granulomas may be necrotizing or non-necrotizing. Cavitation may occur with progressive disease that clinically and radiologically simulates tuberculosis. Patients with profound immunosuppression, particularly T-cell-related immunosuppression, may not be able to generate a granulomatous response. In these patients, the yeasts of *H. capsulatum* appear in histiocytes and macrophages throughout the reticuloendothelial system.



■ **FIGURE 21-60** Photomicrograph of a tissue section revealing macrophages containing loose clusters of intracytoplasmic 2–3 μm diameter, black-staining pseudoencapsulated yeasts forms of *Histoplasma capsulatum* (GMS stain, 400×).

The mold form of *H. capsulatum* is often difficult to convert to the yeast form in culture. For this reason, the nucleic acid probe test or other molecular methods are recommended to confirm the final identification. The nucleic acid probe test is relatively easy to perform and requires minimal growth mass (i.e., it may be accurately within 1 day after the appearance of initial growth in culture). The cultures need not be mature or have developed conidia for probe testing to be performed. Huffnagle and Gander[132] evaluated 95 mold-phase fungi, including 41 isolates of *H. capsulatum*, and demonstrated 100% specificity and sensitivity. Padhye et al.[209] correctly identified 103 of 105 *H. capsulatum* cultures using a chemiluminescent, acridinium ester-labeled, single-stranded DNA probe complementary to the rRNA of *H. capsulatum* mold forms (AccuProbe; Gene Probe, San Diego, CA). Similarly, Hall et al.[114] correctly identified 53 of 54 isolates of *H. capsulatum* and Stockman et al.[264] demonstrated 100% sensitivity and specificity in the study of 86 strains of *H. capsulatum* and 154 other

nontarget fungi also using the AccuProbe assay. The age of the culture, medium for isolation, and morphologic state did not affect the results, which indicates that an identification can be made before characteristic spores are produced.

The diagnosis of histoplasmosis may be made or supported by identifying the small, intracellular budding yeast cells in tissue sections or stained smears. Blumenfeld et al.[25] were able to identify intracytoplasmic organisms in both Diff-Quik and Papanicolaou-stained smears of bronchoalveolar lavage fluid. Follow-up studies of tissue sections stained with methenamine silver revealed budding yeasts in an intracellular location, confirming that they were *H. capsulatum.* Caution, however, is recommended as other yeasts,

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such as *C. glabrata,* have the same morphologic appearance. In such instances, the type of inflammatory response present is useful in suggesting the most likely pathogen. *C. glabrata* elicits a neutrophilic response, whereas *Histoplasma* elicits granulomas in the immunocompetent and will be present in histiocytes in the profoundly immunocompromised.

Diagnosis Using Nonculture Techniques. Molecular methods, particularly commercially available nucleic acid probes, remain a common method for the confirmation of isolates as *H. capsulatum.*[294] Some have used the probes on all suspect isolates prior to sporulation, in an effort to reduce the time to identification. Broad-range fungal PCR with DNA sequencing and species-specific rapid cycle PCR are alternate molecular methods that may be used for the confirmation of suspect isolates. These have also been used successfully for the detection of *Histoplasma* directly in clinical specimens. Serologic tests for anti- *Histoplasma* antibodies are useful studies in patients with suspected histoplasmosis. Serologic studies are positive in over 90% of patients with cavitary or disseminated forms of histoplasmosis. These serologic tests, however, are less sensitive in immunosuppressed patients (50% to 80%), many of whom cannot produce antibodies. Tests for antibody also have the disadvantage of a 4- to 6-week delay before a sufficiently high titer of antibody is achieved to afford detected. *Histoplasma* antibodies may also be elevated in patients with blastomycosis, coccidioidomycosis, and paracoccidioidomycosis.[293]

The CF test using yeast and mycelial antigens is more sensitive than the ID test. In acute pulmonary histoplasmosis, the CF test is positive at titers of at least 1:8 in 90% of patients, whereas in the immunodiffusion test, M bands are present in about 76%, and H bands in 23% of patients.[73] Titers in the range of 1:8 to 1:16 may be residual from past infection. A single titer of 1:32 or higher or a fourfold increase in titer is diagnostic of active histoplasmosis. Immunodiffusion is sufficiently insensitive that

other serologic tests are recommended.

Radioimmunoassay and enzyme immunoassay also cannot be recommended, although antibodies may be detected sooner and in a higher proportion of patients. These assays are poorly standardized and difficult to quantitate and interpret. Specificity is also low, with relatively high rates of false positivity in persons with other fungal or mycobacterial infections.[293]

Antigen detection provides a useful test in the diagnosis of histoplasmosis, particularly disseminated histoplasmosis in immunocompromised patients. The assessment for this antigen in the urine of patients with histoplasmosis has better sensitivity compared with the assessment of a serum. Therefore, urine is the preferred specimen for testing. In a study of 226 patients 18 years old or younger in whom *H. capsulatum* antigen was detected in urine specimens by radioimmunoassay, Fojtasek et al.[90]found that 85% had disseminated disease and 15% had self-limited pulmonary disease, when at least one other corroborating standard test was positive. The use of this test on other specimen types, such as bronchoalveolar lavage specimens has also been studied. *H. capsulatum* antigen was detected in bronchoalveolar lavage fluid in 19 of 27 cases (70.3%) of pulmonary histoplasmosis studied by Wheat et al.[291] *Histoplasma* urinary antigen testing is now commonly used in patients suspected to have histoplasmosis. The several clinical manifestations of histoplasmosis are presented in Clinical Correlation Box 21-7.

CLINICAL CORRELATION BOX 21-7 Histoplasmosis

Most patients with histoplasmosis resolve after an acute pulmonary illness of varying degrees of severity, characterized by fever, headache, chills, cough, and chest pain. In most instances, patients have an influenza-like illness that resolves without therapy. Progression to pneumonia and enlargement of the mediastinal lymph nodes may occur. In less than 1% of patients, a chronic pulmonary form may develop, characterized by persistent cough, low-grade fever, and occasional episodes of hemoptysis. Cavitary lesions may develop in adults, or one or more thick, laminated, calcified "histoplasmomas" may be seen by chest roentgenogram examination. Mediastinal granuloma formation followed by fibrosing mediastinitis has been reported, but the etiologic nature of *H. capsulatum* in this disease remains in contention.[181] Dyspnea, hemoptysis, post-obstructive pneumonia, and superior vena cava obstruction are associated complications. The intense fibrosis found in these cases renders surgery difficult. A rare complication of pleural effusion and pericardial fibrosis has been reported by Kilburn and McKinsey.[147] Constrictive pericarditis later developed in the reported patient, confirmed at autopsy. Because *H. capsulatum* is an obligate intracellular organism residing in macrophages of the

reticuloendothelial system, varying degrees of hepatomegaly, splenomegaly, and lymphadenopathy may be seen in cases of acute and chronic disseminated disease, which usually occurs in immunosuppressed hosts.

Progressive Disseminated Histoplasmosis

Progressive disseminated histoplasmosis is an AIDS-defining illness, being the first manifestation in 50%–75% of AIDS patients with histoplasmosis.[245,292] The clinical manifestations are fever, fatigue, and weight loss, the latter often presenting as a wasting disease.[112] Nightingale et al.[199] found a 4% prevalence of histoplasmosis among 980 patients with AIDS seen in Dallas, Texas; examination of the peripheral smear and bone marrow established the diagnosis in 88% of these patients. Currently, the histoplasma urinary antigen test would be used and has a very high sensitivity in this setting (i.e., disseminated disease). In addition, Huang et al.[131] report five patients with disseminated histoplasmosis in patients with AIDS; all had fungemia, and three died within 4 weeks after the diagnosis was established. The use of the Isolator lysis-centrifugation system is recommended for blood cultures for patients with suspected *H. capsulatum* fungemia.

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The presence of AIDS typically renders histoplasmosis refractory to therapy. Kurtin et al.[158] reviewed bone marrow and peripheral blood specimens in 13 patients with AIDS and disseminated histoplasmosis. Anemia, leukopenia, and thrombocytopenia were found in 12, 10, and 7 patients, respectively. Circulating organisms in blood smears or buffy-coat preparations were seen in five patients, usually associated with the presence of circulating normoblasts and severe absolute monocytopenia. The marrow specimens revealed one of four morphologic patterns: (1) no morphologic evidence of infection; (2) discrete granulomas; (3) lymphohistiocytic aggregates; or (4) diffuse macrophage infiltrates.

Lysis centrifugation for recovery of organism from blood cultures and cultures for bone marrow aspirates or respiratory specimens, in conjunction with morphologic examination of the bone marrow, peripheral blood smear, and respiratory secretions usually confirm the diagnosis.[194]

Other organ systems involved with histoplasmosis in patients with AIDS include the central nervous system, which may take the form of chronic meningitis, cerebral or spinal cord mass lesions simulating neoplasms, or encephalitis and an unusual instance of sinusitis reported by Butt and Carreon.[13,33,290]

Wheat et al.[290] found CNS manifestations in 10%–20% of patients with disseminated histoplasmosis, and that the organism may be the cause of chronic meningitis with no other evidence of dissemination in select patients. Cerebral or spinal cord mass lesions resembling neoplasms or abscesses and encephalitis were the most common forms of presentation in this series.

Gastrointestinal Tract

Histoplasmosis of the gastrointestinal tract, usually of the small bowel, may present as ulcers or as a mass, often mimicking inflammatory bowel disease or carcinoma.[42] The gastrointestinal tract is secondarily involved in 70%–90% of cases of disseminated histoplasmosis, although primary involvement of the gastrointestinal tract is infrequently encountered.[266] Jain et al.[136] report a 67-year-old man who presented with chronic diarrhea that did not respond to the conventional treatment. He was found to have isolated colonic histoplasmosis infection, which was treated with itraconazole.

Patients with histoplasmosis of the oropharynx have also been reported, in which lesions may be mistaken for carcinoma on initial presentation,[126] particularly when the vocal cords are involved. Sataloff et al.,[246] in a review of the literature, found fewer than 100 cases of histoplasmosis presenting as laryngeal carcinoma, since the first case was reported in 1952. Perhaps of more significance, Economopoulou et al.[75] reviewed 20 case reports from the literature of oral histoplasmosis in HIV-infected patients, in many instances, the oral lesions appeared to be the primary or only manifestation of disease.

Although disseminated histoplasmosis came to the attention of the medical community early in the HIV epidemic, prior to the use of highly active antiretroviral therapy (HAART), it is less commonly seen in resource-rich countries that can provide the necessary therapy for patients with HIV. The new risk for disseminated histoplasmosis is secondary to the approved and widespread use of immunomodulating agents (i.e., so-called "biologics). These agents, which are used in patients with rheumatoid arthritis, inflammatory bowel disease, and psoriasis, include etanercept, adalimumab, and infliximab. The modulation of the immune system in these patients, in addition to aiding in the control of their underlying disease, increases their risk for disseminated fungal infections and tuberculosis.[276]

Cutaneous Histoplasmosis

Cutaneous lesions may be the initial presentation for histoplasmosis in about 10% of patients with disseminated disease. Erythematous or hyperpigmented papules, pustules, folliculitis, eczematous changes, erythema multiforme, and rosacea-like rashes are the more common skin manifestations.[289] Chalub et al.[39] reported four patients with disseminated histoplasmosis in patients with AIDS. Multiple small (up to 3 mm in diameter) erythematous maculopapules on the extremities, face, and trunk, often centered around hair follicles were observed. These appear similar to the lesions caused by molluscum contagiosum. Histologically, perivascular infiltrates with conspicuous leukocytoclasis, the lack of a macrophage response, and absence of granulomas and organisms free in the dermis, intraneurally and in skin appendages, were seen.

Genitourinary Histoplasmosis

Although genitourinary tract fungal infections are more commonly seen in blastomycosis, genitourinary involvement in histoplasmosis has been reported. Kahn and Thommes[139] reported a patient with massive granulomatous orchitis and epididymitis with caseous necrosis. Two additional patients have been reported by Friskel et al.[93]

Ocular Histoplasmosis

The ocular histoplasmosis syndrome has been reviewed by Ciulla et al.[46] The classic clinical triad includes discrete atrophic choroidal scars in the macula or mid-periphery known as histo spots, peripapillary atrophy, and choroidal neovascularization. Severe loss of central vision is a major complication.

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Septicemia

Although septicemia with fungal agents is uncommon, the opportunity to establish an early diagnosis may be missed if the blood culture techniques being used are inadequate. Paya et al.[215] found that the use of the lysis centrifuged blood culture tubes was the optimal technique for recovering *H. capsulatum* yeast forms from the blood in suspected cases of disseminated disease, significantly increasing the yield of positive cultures and shortening the time of recovery. The superiority of this system is also supported by the observations of Murray,[194] who in a study of 182 fungal isolates of all types, found that *H. capsulatum* was recovered only in the lysis-centrifugation system. Early diagnosis is important, as dissemination can be rapidly progressive and fatal in immunocompromised patients.

Sporothrix schenckii Complex and Sporotrichosis

Nucleic acid sequence analysis has demonstrated that the fungi traditionally considered as *Sporothrix schenckii* is actually a group of fungi that are morphologically similar.[179] The differentiation of these organisms is not necessary for the treatment of disease, so the use of the term "complex" is acceptable, until a time it is demonstrated that differentiation is needed. Members of the *Sporothrix schenckii* complex (hereafter simply *S. schenckii*) are fungi that are found worldwide in soil and in vegetative matter, particularly sphagnum moss, wood splinters, and rosebush thorns. These fungi, when they find their way into a subcutaneous location, usually through traumatic inoculation, are the causative agent of sporotrichosis, a chronic infection of humans, and wild and domestic animals. Humans become infected when the organism penetrates the skin of individuals handling contaminated substances. The infection is characterized by the development of nodular lesions of the cutaneous and subcutaneous tissues, and if untreated, the development of lymphatic involvement (lymphocutaneous disease). Draining sinuses from foci on subcutaneous infection may form in chronic infections. Dissemination is rare, but may occur in immunocompromised patients, with systemic spread to the bone, muscle, central nervous system, and lungs. Also rare is solitary pulmonary involvement, but this has occurred after inhalation of conidia from highly contaminated substances.

Certain occupations place workers at increased risk for infection. Hajjeh et al.[113] reported an outbreak of sporotrichosis in workers at a tree nursery in Florida. Lymphocutaneous sporotrichosis developed in 9 of 65 (14%) of workers involved in the production of sphagnum moss topiaries. The risk was directly related to the duration of working with sphagnum moss, in particular with the task of filling topiaries, and in individuals with less gardening experience. Those wearing gloves were protected.

An interesting outbreak was reported by Dooley et al.[71] Within a 5-week period, five patients living in Oklahoma presented with cutaneous sporotrichosis, four of whom had maintained hay bales in a Halloween haunted house. A fifth patient apparently contracted the disease while visiting the house. Thus, contact with hay may represent yet another risk factor.

A high area of endemicity for sporotrichosis has been reported by Pappas et al.[212] In a remote area of the south central highlands of Peru, 238 patients with culture-proven sporotrichosis were observed in a period of 3 years. The incidence of sporotrichosis in this region was calculated at between 48 and 60 cases per 100,000 persons, with the highest incidence among children aged 7 to 14 years. The skin of the face was the most commonly affected site. The disease was clinically confined to the skin and subcutaneous tissue in all patients.

Laboratory Presentation. The growth of *Sporothrix schenckii* in culture is usually within 3 to 5 days at 30°C, although smooth, yeast-like colonies may develop as early as at 36 to 48 hours on 5% sheep blood agar incubated at 35°C. In some instances, the initial growth that occurs at 30°C may appear yeast-like, as full yeast-to-mycelial conversion has not yet occurred. These yeast cells may be elongated with somewhat pointed end, resembling the corn smut *Ustilago.* The colonial morphology varies depending on the media used and the presence or absence of blood in the media. Colonies are usually smooth and may become tough and wrinkled or folded. A delicate, dark, fuzzy, aerial mycelium develops when the colonies mature. The color is initially gray-white, but may turn buff, brown or brown-black as the colony matures (Image Plate 21-5G). The colonies become distinctly yeast-like when incubated at 35°C to 37°C, and may turn dark brown or black on maturity (Image Plate 21-5H).

The identification can be made microscopically by observing in the mold form delicate, septate hyphae from which are borne oval, smooth conidia measuring 2 to 4 µm in diameter. The hyphae and conidia are often hyaline, but some variants may increase the pigmentation with time. The conidia are attached by a delicate, hairlike structure (i.e., a denticle) to the conidiophore. By exacting focus of the microscope and using an oil immersion objective, it is possible to observe the characteristic hairlike attachments between the conidia and the conidiophore (from which the species name is derived). The publication by Sigler et al.[255] includes excellent photomicrographs illustrating this microscopic feature. Conidia of *S. schenckii* are arranged either laterally along the side of the hyphae or in daisylike clusters from the tip of a straight, delicate conidiophore (i.e., a fleurette morphology) (Fig. 21-61).

The yeast forms of *S. schenckii* are 2 to 4 µm in diameter and may be spherical, oval, or elliptical, often with a single bud (Fig. 21-62). These may be observed in the histologic assessment of a skin biopsy, the direct examination (i.e., KOH/calcofluor white) or following mycelial-to-yeast thermal conversion. Most helpful in making the microscopic identification of *S. schenckii* yeast forms is the observation of elongated oval forms (i.e., cigar-bodies) that measure 3 × 10 µm. These yeast forms may be difficult to detect in routine H&E-stained sections of human tissue, so if infection is suspected based on the inflammatory response present, then a methenamine silver stain should be ordered. This will demonstrate a mixture of spherical yeast with the more characteristic elliptical yeast cells (Fig. 21-63).

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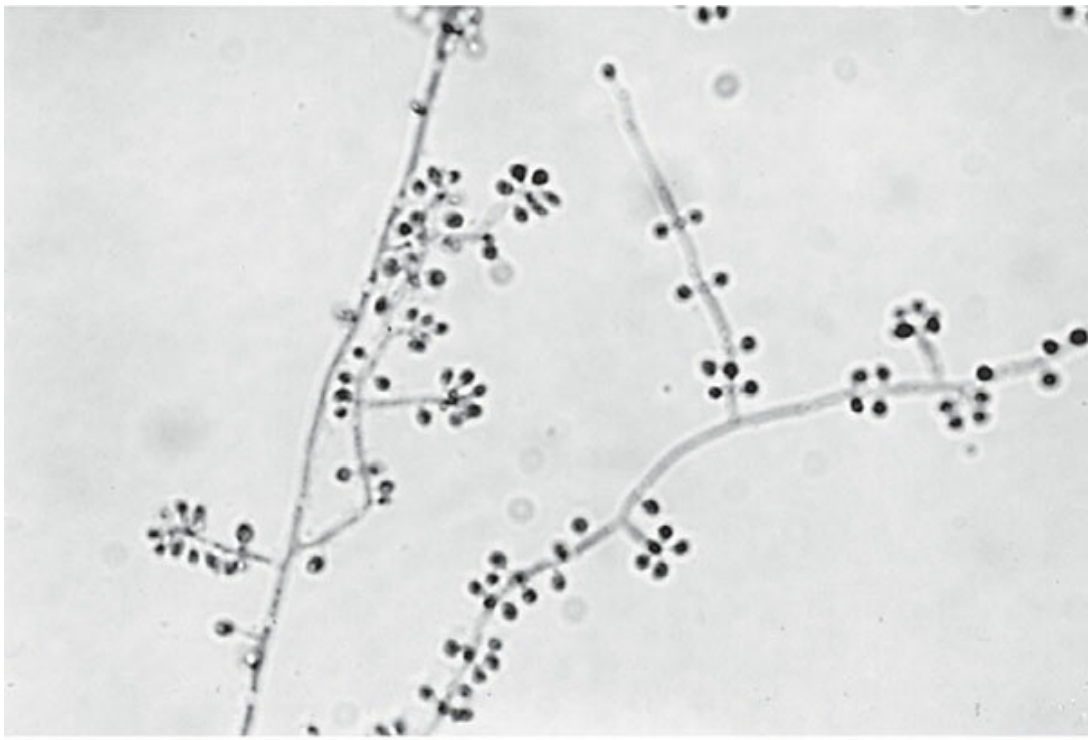
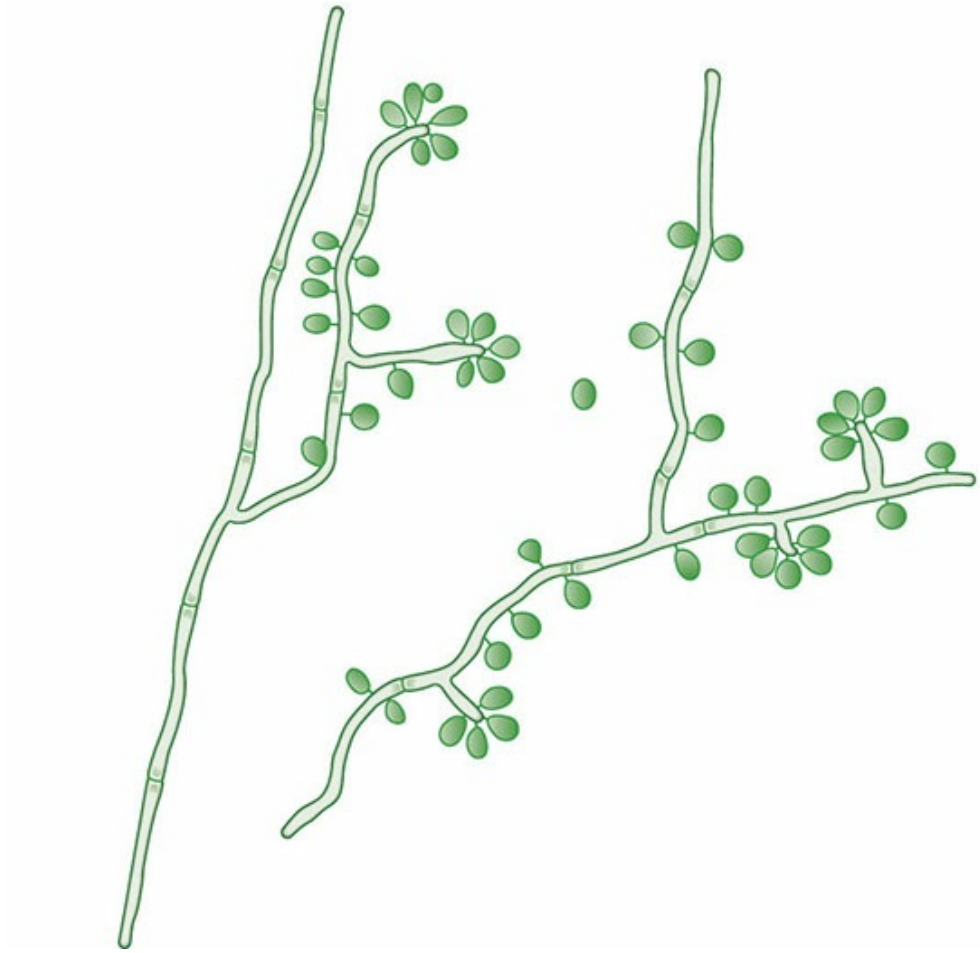
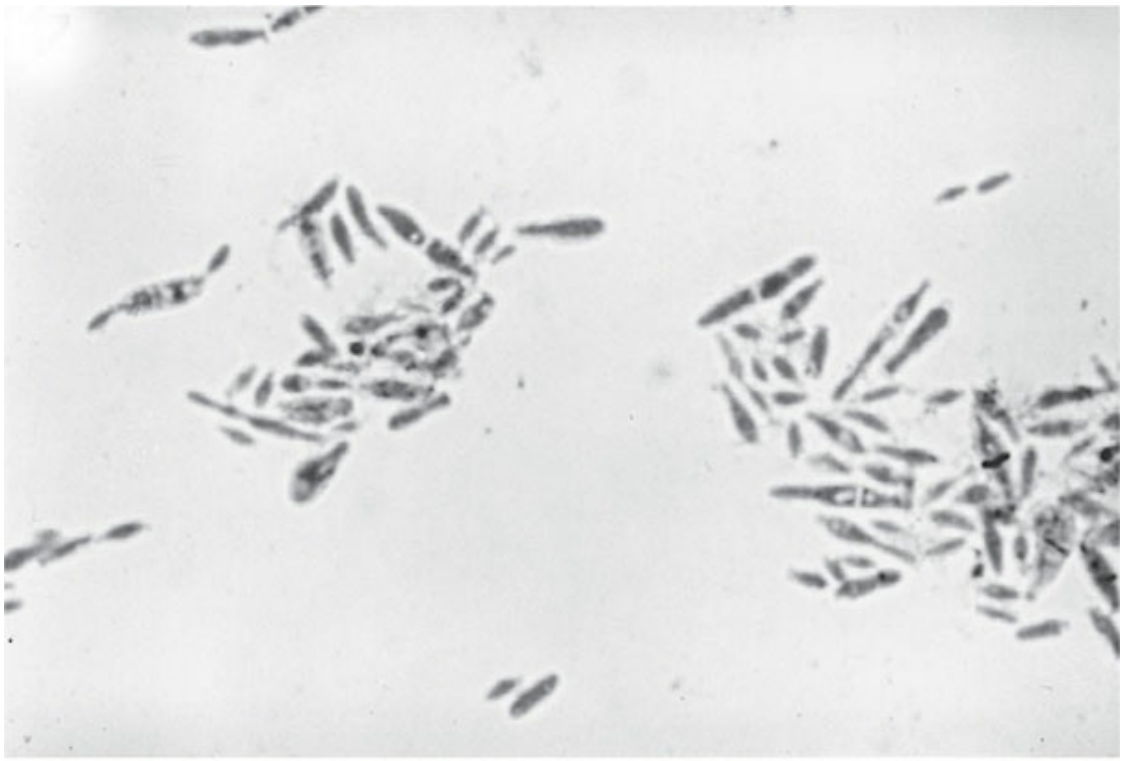


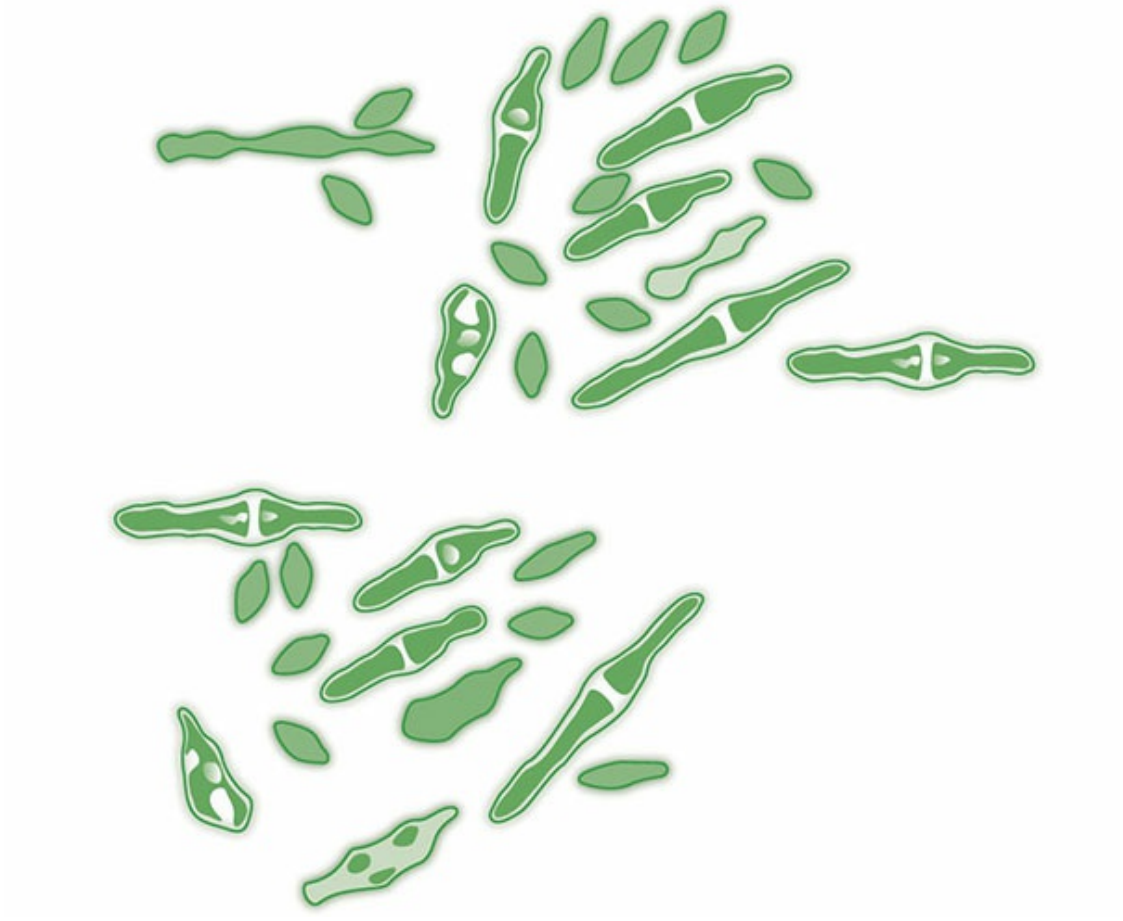


■ **FIGURE 21-61** Photomicrograph and sketch of the mold form of *Sporothrix schenckii,* illustrating the delicate hyphae and daisylike clustering of small, oval conidia from the sides or the tips of straight conidiophores.

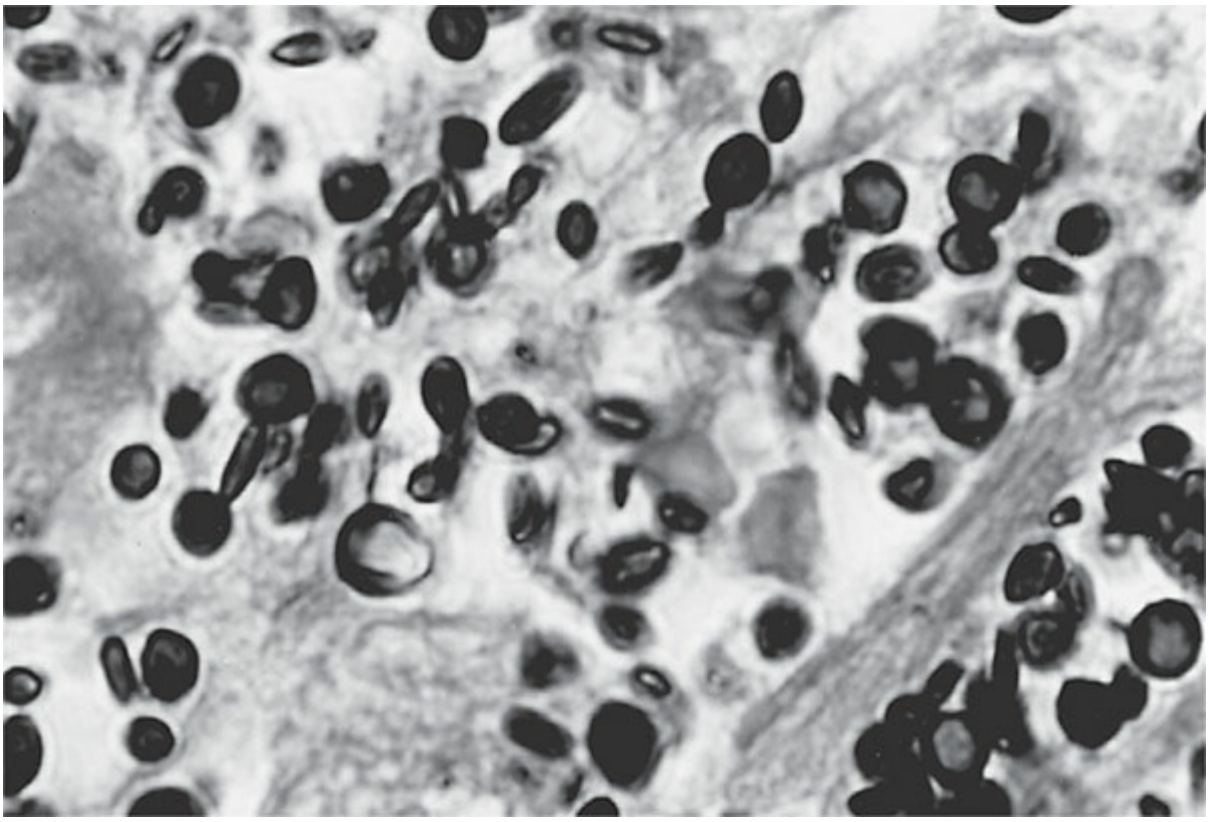
Diagnosis Using Nonculture Techniques. Nonculture techniques are not routinely employed for the diagnosis of sporotrichosis. Although molecular assays have been developed, they are not used on a routine basis. The application of serologic techniques has proven to be of minimal benefit in the diagnosis of sporotrichosis. Latex agglutination tests have been used in the past with limited success, particularly in the diagnosis of primary skin infections, in which results are usually negative. Agglutination titers of 1:80 or higher have been helpful in the diagnosis of active, extracutaneous infections.[61]

The clinical manifestations of sporotrichosis are presented in Clinical Correlation Box 21-8.





■ **FIGURE 21-62** Photomicrograph and sketch of the yeast form of *Sporothrix schenckii,* revealing elongated, two-celled yeast cells, many with a "cigar body" form.



■ **FIGURE 21-63** Photomicrograph of a cutaneous biopsy from a patient with sporotrichosis revealing numerous spherical to oval yeast cells of *Sporothrix schenckii*, some of which show elongated, two-celled cigar-shaped buds (high-power, methenamine silver stain).

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CLINICAL CORRELATION BOX 21-8 Sporotrichosis

In the United States, subcutaneous fungal infections are most commonly caused by a member of the *Sporothrix schenckii* complex.[11] DNA sequencing studies have revealed that a number of morphologically similar species make up what has been termed *"Sporothrix schenckii."*[188] Although differentiation of these is possible, the term "complex" is preferred, since they all produce a similar disease and there is no

difference in treatment.

The most common form of sporotrichosis is lymphocutaneous. The appearance of a small, red, painless pustule on an extremity, together with the appearance of multiple, linearly placed secondary pustular or ulcerating lesions along the proximal lymphatics is sufficient to suspect the diagnosis. The primary pustule may slowly enlarge, ulcerate, and discharge a small amount of serosanguineous exudate. Varying degrees of cellulitis with swelling and redness of the surrounding subcutaneous tissue may be observed. The secondary satellite lesions present initially as verrucous, erythematoid plaques, or scaly patches, often developing into ulcers that also exude purulent material. Diagnosis is often delayed because associated secondary bacterial colonization may be mistaken for the primary infectious agent. Bacterial superinfections may also confuse the gross appearance.

In patients with progressive disease, the infection may spread to joints, tendon sheaths, bursae, bone, and muscle.[302] Purvis et al.[228] report a patient with bilateral polyarticular arthritis of the wrists and elbows. Although arthritis is an uncommon manifestation of sporotrichosis, this report points to the broadened spectrum of disease that may be caused by this fungus.[43]

Sporotrichal arthritis is an uncommon disease. Howell and Toohey[129] report the findings of 13 patients with sporotrichal arthritis who had been treated in Wichita-area hospitals. The typical presentation was of an afebrile patient with a mildly swollen warm joint without erythema. Seventeen joints were involved, including 10 knees, 3 interphalangeal joints, 1 elbow, 1 midtarsal, 1 intercarpal, and 1 metatarsophalangeal joint. Most of the patients were middle-aged men, and significant alcohol intake was noted in 77% of the patients. Wang et al.[284] report a patient with a prepatellar bursa infected with *Sporothrix schenckii*. The infection persisted despite itraconazole therapy, and cure was achieved only after surgical excision of the bursa.

Primary pulmonary sporotrichosis is rare. However, England and Hochholzer[80] reported primary pulmonary sporotrichosis in eight patients following inhalation of airborne conidia, an otherwise rare occurrence. Cough, sputum production, and low-grade fever are the usual presenting symptoms.

In immunosuppressed patients, sporotrichosis may have atypical presentations, with the disease tending to become widespread and disseminated. Cutaneous disease beginning as localized lesions may disseminate to other skin sites, with dissemination to the meninges in one reported patient, which is a presentation this author has also seen.[70] Diagnosis may be difficult when sporotrichosis initially presents as fungemia and disseminated disease.[9] Although localized sporotrichosis is an innocuous disorder that responds well to therapy, these authors found in immunocompromised hosts that the disease is potentially life-threatening and often is difficult to treat, requiring prolonged therapy with potentially toxic medications such as amphotericin B. Reversal of immunosuppression in conjunction with antifungal therapy is the proscribed treatment.

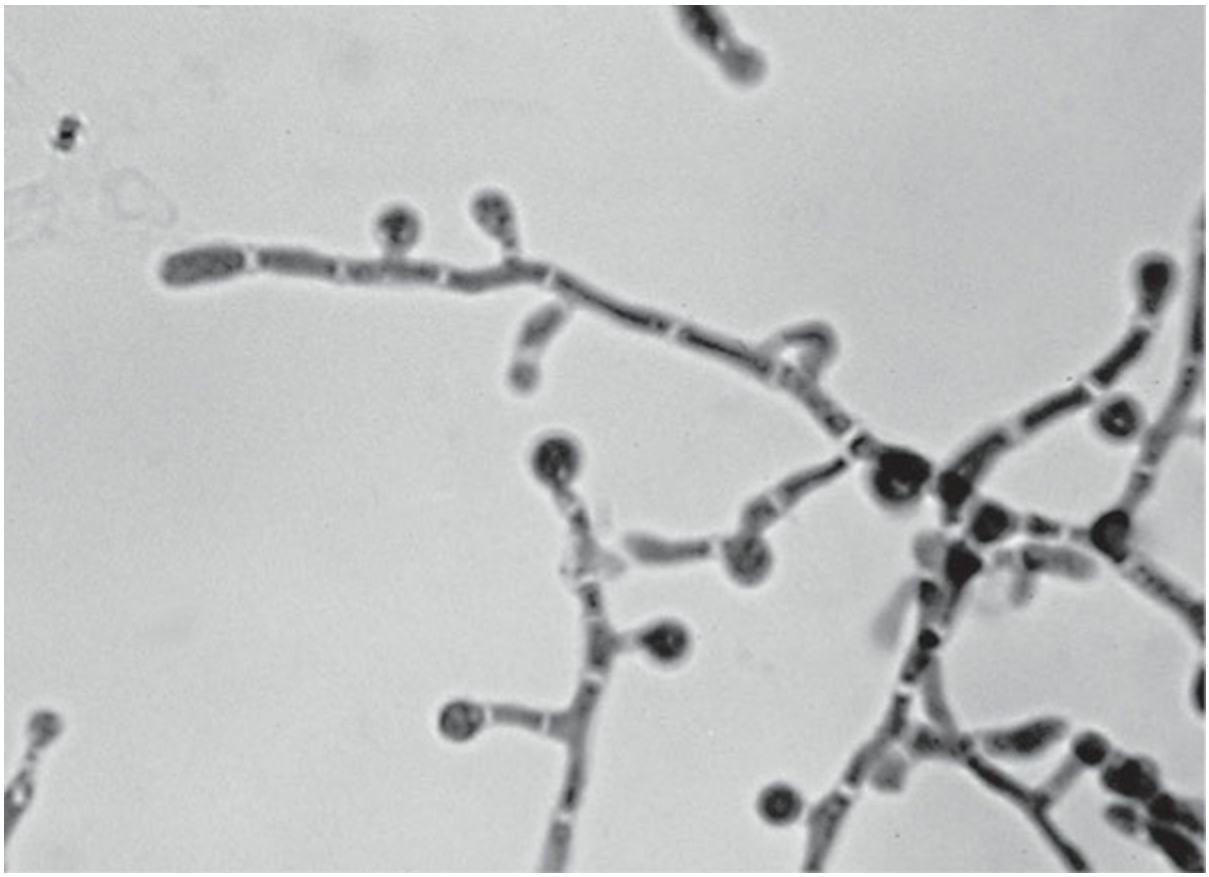
Paracoccidioides brasiliensis and Paracoccidioidomycosis

Paracoccidioidomycosis is a progressive subacute to chronic systemic granulomatous fungal infection caused by the thermally dimorphic fungus, *Paracoccidioides brasiliensis*. The mycelial form of this fungus resides in the soil, and pulmonary infection develops in humans by inhalation of the small (4 μm diameter) conidia. Skin and mucocutaneous infections occur by direct inoculation of spore-contaminated material. In South America, 80% of infections have been reported from Brazil; followed in order of incidence by case reports from Colombia and Venezuela; endemic areas also exist in southern Mexico and in all countries in Central America except Belize and Nicaragua.[32]

The overall prevalence rate of paracoccidioidomycosis is difficult to derive, since infections often occur in resource poor countries with a limited public health infrastructure. Although estimates of a million or more infected patients have been made, many patients either are not reported or remain undiagnosed. Botteon et al.[28] studied two groups of blood donors. One group consisted of donors living in a rural area where paracoccidioidomycosis is endemic, whereas the other from a group of urban residents residing in Sao Paulo. Serologic studies showed that 21% of 700 patients from the endemic rural areas had evidence of current or remote disease, whereas only 0.9% of 350 urban patients were serologically positive. Thus, with a population of approximately 169 million, even if the lower urban prevalence rate of paracoccidioidomycosis is used, well over 1.5 million residents of Brazil alone have been infected.

Countries in the Caribbean islands, the Guyanas, and Chile have been free of reported disease. In endemic areas, most infections occur in and around humid forested regions. The disease most frequently involves adults over 30 years of age, is rare in children, and occurs in males more often than in females, with an overall ratio of 15:1. Although the majority of patients are agriculturists, infections have been reported in individuals with rare direct exposure to soil and vegetation as indicated by the report cited above. Whites are more prone to infections than Native Americans; more severe infections tend to develop in immigrants coming to endemic areas.[32]

1378



■ **FIGURE 21-64** Photomicrograph of the mold form of *Paracoccidioides brasiliensis,* illustrating the small, smooth, spherical conidia borne laterally and terminally from short conidiophores derived from septate hyphae.

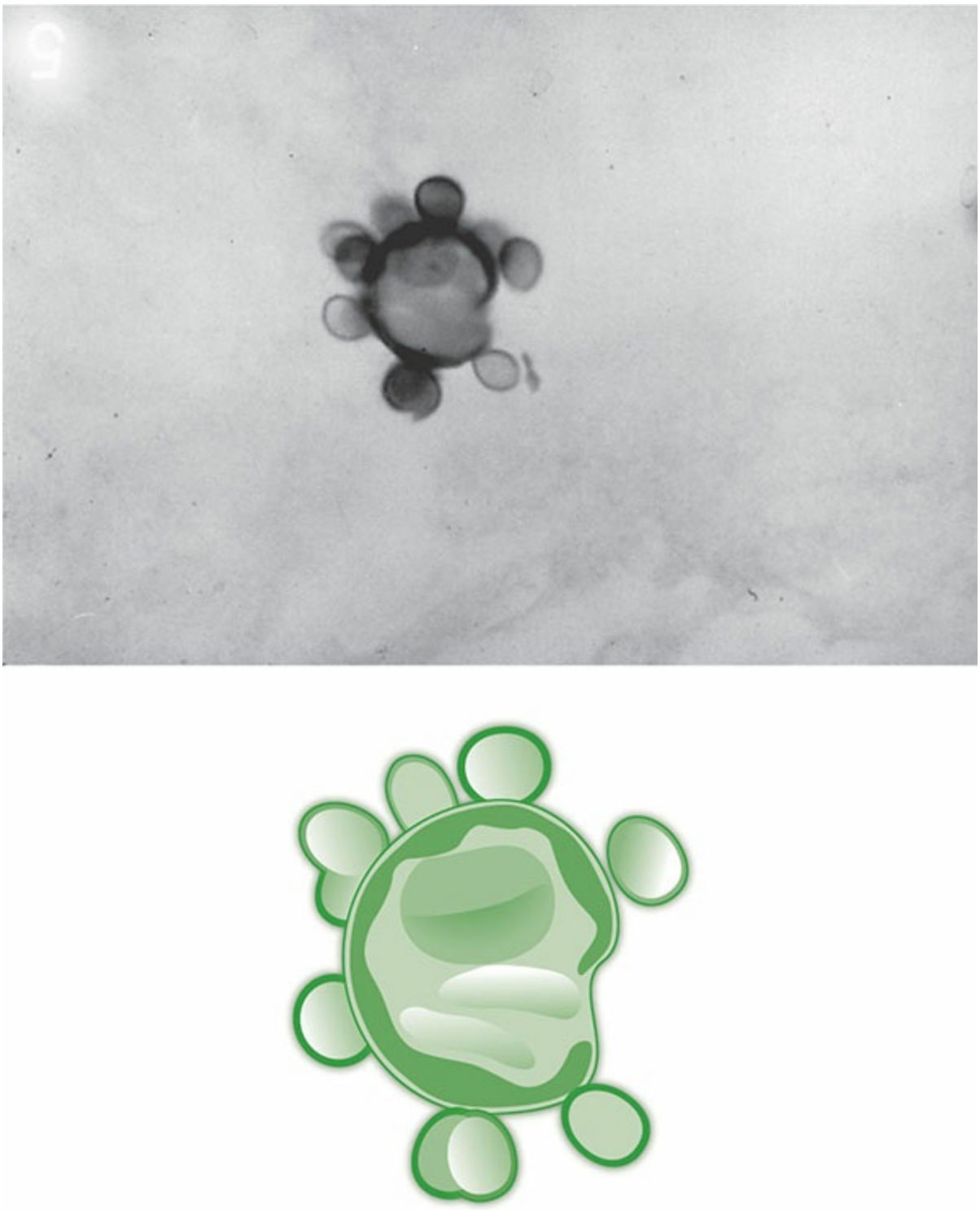
Laboratory Presentation. The colonies are similar in appearance to those of *Blastomyces dermatitidis*. They are slow-growing at 30°C, requiring 10 to 20 days to mature, are gray-white, and have a delicate silky or hairlike mycelium.

When incubated at 25°C to 30°C, *P. brasiliensis* grows very slowly over 10 to 30 days as a silky, white to gray-tan mold. The identification and differentiation from other similar appearing molds can be made by microscopically observing the delicate, hyaline septate hyphae that make up the background mycelium. Oval, nonpigmented, 2 to 4 µm conidia are borne singly from short, slender conidiophores directly from the hyphae, in a "lollipop" fashion, similar in appearance to those produced by *Blastomyces dermatitidis* (Fig. 21-64).

The identification can be confirmed by converting the mold to the yeast form, which is accomplished by inoculating a small portion of the mold colony to enriched brain-heart infusion agar. Conversion to the yeast form, however, is slow. The mother yeast cells of *P. brasiliensis* observed in culture are usually 6 to 15 µm in diameter, but may grow larger. These are differentiated from the similarly-sized *B. dermatitidis*,

by the presence of multiple narrow-necked buds (i.e., in contrast to the single, broad-based budding forms of *B. dermatitidis*). Although a genetic probe is not commercially available for *P. brasiliensis*, the commercially available probe for *B. dermatitidis* cross-reacts with *P. brasiliensis*, and can be used in conjunction with other findings to secure the identification.

The same yeast form is present in the tissues of infected patients. Therefore, the diagnosis of paracoccidioidomycosis can be most quickly established by demonstrating directly in clinical materials the characteristic 10 to 30 μm spherical, thick-walled yeast cells, with multiple daughter buds, each attached by a narrow-necked bud, which has been likened to a "mariner's wheel" (Fig. 21-65). These yeast forms may be best observed in KOH/calcofluor white preparations. In some cases, the budding daughter cells may form short chains. In tissue sections, the background inflammation is usually granulomatous-to-pyogranulomatous (i.e., granulomatous inflammation that is admixed with varying concentrations of polymorphonuclear leukocytes). The yeast forms are best seen in PAS- or GMS-stained sections.



■ **FIGURE 21-65** Photomicrograph and sketch of the yeast form of *Paracoccidioides brasiliensis,* illustrating the large central yeast cell that is giving rise to multiple buds, forming a "mariner's wheel."

Diagnosis Using Nonculture Techniques. The exoantigen test, refined by Camargo et al.,[35] is a means for confirming the culture identification of unknown isolates. Indirect immunofluorescence techniques, immunoenzymatic assays, and molecular methods have been used by investigators to establish the diagnosis of paracoccidioidomycosis. The immunodiagnostic approaches to the diagnosis of paracoccidioidomycosis have been reviewed by Brummer et al. and de Camargo.[32,65] Serum IgG levels remain elevated for at least 1 year after infection.

Mendes-Giannini et al.[184] demonstrated IgG, IgA, and IgM-specific antibodies to a 43-kDa antigen of *Paracoccidioides brasiliensis* in patients with paracoccidioidomycosis, and further demonstrated that a lowering of the titers during a course of treatment was correlated with an improvement in symptoms. Gomez et al.,[102] using a monoclonal antibody directed against the yeast form of *Paracoccidioides brasiliensis*, were able to develop an inhibition ELISA test capable of detecting small quantities of circulating antigen in the serum of patients with active disease. Of 46 patients with paracoccidioidomycosis, 37 tested positive (80.4%).

Antigen-detection assays are being used for the diagnosis of paracoccidioidomycosis. The detection of the gp43 and gp70 antigens of *Paracoccidioides* is the target of these assays.[65] In addition to detecting these in blood, they have been

assessed in cerebrospinal fluid and bronchoalveolar lavage fluid. The quantitative or semiquantitative nature of these assays allows for trend analysis of antigen levels; antigen decline and clearance should occur with successful therapy.

Molecular methods have also been employed to diagnose paracoccidioidomycosis. Sandu et al.[242] have developed a 14-base DNA probe specific for the 5′ terminus of fungal 28S ribosomal gene sequences of *P. brasiliensis*. Applying a uniform diagnostic protocol, consisting of common cell lysis and DNA purification procedures followed by PCR amplification, these authors demonstrated selective identification of *P. brasiliensis* from 47 other species of fungi representing 25 genera. PCR and other molecular methods provide alternate means to establish the diagnosis of paracoccidioidomycosis.

The clinical manifestations of paracoccidioidomycosis are presented in Clinical Correlation Box 21-9.

CLINICAL CORRELATION BOX 21-9 Paracoccidioidomycosis

Upon inhalation of spore-contaminated dust, the small (4 μm diameter) conidia reach the distal portion of the pulmonary parenchyma, where they develop into yeast cells that may be confined locally, or where they may propagate and disseminate to distal organs in patients with progressive disseminated disease. In an immunocompetent host, local growth is retarded and the infection may end without development of a lesion. When symptomatic, Brummer et al.[32]have divided paracoccidioidomycosis into two general categories: (1) juvenile form, acute or subacute, and (2) chronic form, adult-type.

Juvenile Form, Acute or Subacute

The juvenile form of disease includes only 3%–5% of all infections, primarily involving children or young adults, the majority of whom are immunosuppressed. The course of disease is relatively rapid (weeks to months), marked by diffuse involvement of the reticuloendothelial system. Hepatosplenomegaly, lymphadenopathy, and bone marrow hyperplasia are common clinical presentations. Dysfunction of the bone marrow may be so severe as to simulate a lymphoproliferative disorder.[170] Biopsies of the involved organs or tissues often reveal a large number of actively proliferating yeast cells, in the absence of granuloma formation. In this form of the disease, the lungs are seldom involved, although organisms may be detected in respiratory secretions. Mesenteric lymph nodes may on occasion become so enlarged as to cause bowel obstruction. Prognosis of the juvenile form of the disease is poor.

Chronic Form, Adult-Type

Approximately 90% of patients have this form of disease, which occurs most commonly in adult men. Interestingly, the high level of estrogen in pre-menopausal women acts to inhibit the mycelial-to-yeast conversion that is necessary for infection; this, in part, explains the differences noted in the incidence of infection between men and women. The disease is slowly progressive, tends to remain confined primarily to the lungs, and may take months or years to become fully established. The lungs are involved in over 90% of infected patients, and in many instances the lungs are the sole site of infection. The pulmonary lesions as seen on chest roentgenogram may be nodular, infiltrative, fibrotic, or cavitary and are preferentially localized to the lower lobes of the lungs.[170] Cough, expectoration of sputum, and shortness of breath are the common symptoms, often accompanied by low-grade intermittent fever, weight loss, and anorexia. The clinical and radiologic findings often mimic tuberculosis. The chronic disease may be mild, moderate, or severe, depending on the patient's general condition and immune status; severe fibrosis leading to chronic obstructive pulmonary disease and *cor pulmonale* are the fatal sequelae.

Brummer et al.[32] cite several examples of extrapulmonary involvement, including the infection of the eyes, the central nervous system, bone, and the genital tract. Sant' Anna et al.[243] review the findings from seven patients with laryngeal disease. All these

patients were middle-aged males, predominantly farm workers. Dysphonia, dysphagia, dyspnea, and cough were the main presenting symptoms. The first diagnostic impression was carcinoma in each of these patients.

These and other dimorphic fungal infections may also involve the mouth.[8,100] Almeida et al.[8] report that oral fungal infections (mycoses) have come into particular prominence since the advent of HIV infections, as well as the phenomenal increase in world travel, with increased exposure to infections endemic in the tropics. Almeida et al. further report on 21 Argentinian patients living in the province of Corrientes who presented with oral manifestations of *Paracoccidioides brasiliensis*. All patients except one had detectable pulmonary involvement. The long-term administration of itraconazole provided cures for these patients. The authors emphasize the need for early diagnosis and adequate therapy to prevent extensive tissue destruction. Long-term follow-up also is mandatory, as the recurrence rate is high.

The clinical manifestation encountered among the 27 patients with AIDS reported by Goldani and Sugar[101] ranged from indolent infection to a rapidly progressive course. Disseminated disease was the most common form, with lung, skin, and lymph nodes being the most common sites of infection. Prolonged fever, lymphadenopathy, hepatosplenomegaly, and cutaneous lesions were most commonly encountered. The diagnosis is best made by direct examination and culture of sputum specimens, skin biopsies, and lymph node aspirates. As

mentioned before, PCR assays of these specimens promises to greatly enhance the ability to make a diagnosis. About half of the patients in this series died despite aggressive antifungal therapy.

Severo et al.[252] report involvement of the male genital tract in a native of Brazil, and review of 18 additional patients gathered from the Brazilian literature. Manns et al.[177] discuss a 59-year-old man who presented with paracoccidioidomycosis more than 15 years after leaving South America. Practitioners throughout the world must keep this infection in mind when evaluating a patient with appropriate travel history who has pulmonary, mucosal, or cutaneous manifestations suggestive of this disease, along with weight loss and other constitutional symptoms.

Dematiaceous Fungi

Agents of Phaeohyphomycosis

In contrast to the hyaline group of fungi that have nonpigmented cells walls, there is a second large and genetically heterogeneous group of saprophytic molds that produce

melanin and melanin-like pigments in their cell walls. This results in the production of darkly pigmented hyphae. Both the reverse and the surface of the colonies are visibly dark, appearing gray, brown, or black. The examination of the reverse of the colony gives the best indication of the hyaline or dematiaceous nature of a mold, since some hyaline molds, such as *Aspergillus niger,* produce black spores yet are hyaline molds.

The term phaeohyphomyces (Greek *phaeo* = dusky, gray) has been applied to fungal infections caused by this dematiaceous group of fungi. The concept of "phaeohyphomycosis" was first proposed by Ajello et al.[6] in 1986 to "cover all infections of a cutaneous, subcutaneous and systemic nature caused by hyphomycetous fungi that develop in the host tissues in the form of dark-walled, dematiaceous septate mycelial elements." Thus, as originally described, *phaeohyphomycosis* was a term used to describe a histopathologic entity rather than representing any particular clinical disease or fungal species as identified in laboratory cultures.[66,234] It has come to be used as the dematiaceous equivalent of hyalohyphomycosis (i.e., a generic term for an invasive fungal infection caused by a dematiaceous mold). Chromoblastomycosis and mycetoma are distinctive clinical entities different from phaeohyphomycosis. Chromoblastomycosis is caused by select dematiaceous fungi, whereas mycetomas may be caused by bacteria, hyaline molds, or dematiaceous molds. Select etiologic agents of phaeohyphomycosis, chromoblastomycosis, and mycetoma will be covered here.

Laboratory Presentation. This dematiaceous fungus, in the broadest terms, produces either dark gray, brown, or black wooly colonies or colonies that have a velvety surface. As denoted above, the reverse of the colony is darkly pigmented, as this represents the dematiaceous hyphal mat. Although the colony morphology is not distinctive among this group of fungi and microscopic examination is necessary to make a genus/species identification, the type of colony (i.e., wooly vs. velvety) provides a clue to the likely possibilities. For example, *Alternaria*, *Bipolaris*, and *Curvularia* produce wooly colonies, whereas *Fonsecaea, Phialophora*, and *Cladosporium/Cladophialophora* produce velvety colonies. Representative colonies of the more rapidly growing, dematiaceous molds are shown in Image Plate 21-3A–D. In general, the agents of chromoblastomycosis and mycetoma are usually slower-growing, small, and velvety colonies.

Microscopically, the dematiaceous molds have a dark brown mycelium composed of uniform hyphae with parallel walls and distinct septations. Pigment may not be obvious in some species in the earliest phases of growth, but will be developed with further incubation. The following genera of fungi are rapidly growing molds (i.e., wooly colonies) that produce darkly staining, multicellular macroconidia: *Alternaria*, *Ulocladium*, *Stemphylium*, *Epicoccum*, *Bipolaris*, *Drechslera*, *Curvularia*, and *Exserohilum*.

The identification of these fungi can be simplified by first separating these into one of two categories based on the morphology of the conidia: 1. Macroconidia (i.e., multicelled conidia) that have septations that are both longitudinal and perpendicular (i.e., transverse septa) with respect to the longitudinal axis of the conidium. These conidia often also have oblique septations and are commonly referred to as muriform. 2. Macroconidia that contain only perpendicular (i.e., transverse) septations with respect to the longitudinal axis of the conidium.

Macroconidia with Transverse and Longitudinal Septa (Muriform)

The genera of fungi of medical importance included in this group are:

- *Alternaria* species
- *Ulocladium* species
- *Stemphylium* species
- *Epicoccum* species

***Alternaria* Species.** The formation of short chains of large, smooth-walled, multicelled, macroconidia separated by both cross and longitudinal septa (muriform) are characteristic of *Alternaria* spp. The macroconidia are shaped like drumsticks, with the elongated beak of one conidium butting against the rounded, blunt end of the next (Fig. 21-66). The fungi are most commonly contaminants and only rarely cause human infections. In fact, they are so infrequently the causes of disease that the assurance of corresponding histopathologic evidence of infection is strongly recommend prior to considering the initiation of therapy.

***Ulocladium* Species.** *Ulocladium* spp. also produce muriform macroconidia; however, the conidia from these fungi are more spherical than those of *Alternaria* spp. They do not form in chains, but rather are borne from short, twisted "bent knee," or **geniculate,** conidiophores (Fig. 21-67). These conidiophores are formed circumferentially (i.e., around), which is a **sympodial derivation**. This sympodial, geniculate conidiation is a type of conidiogenesis that will be encountered in other dematiaceous fungi.

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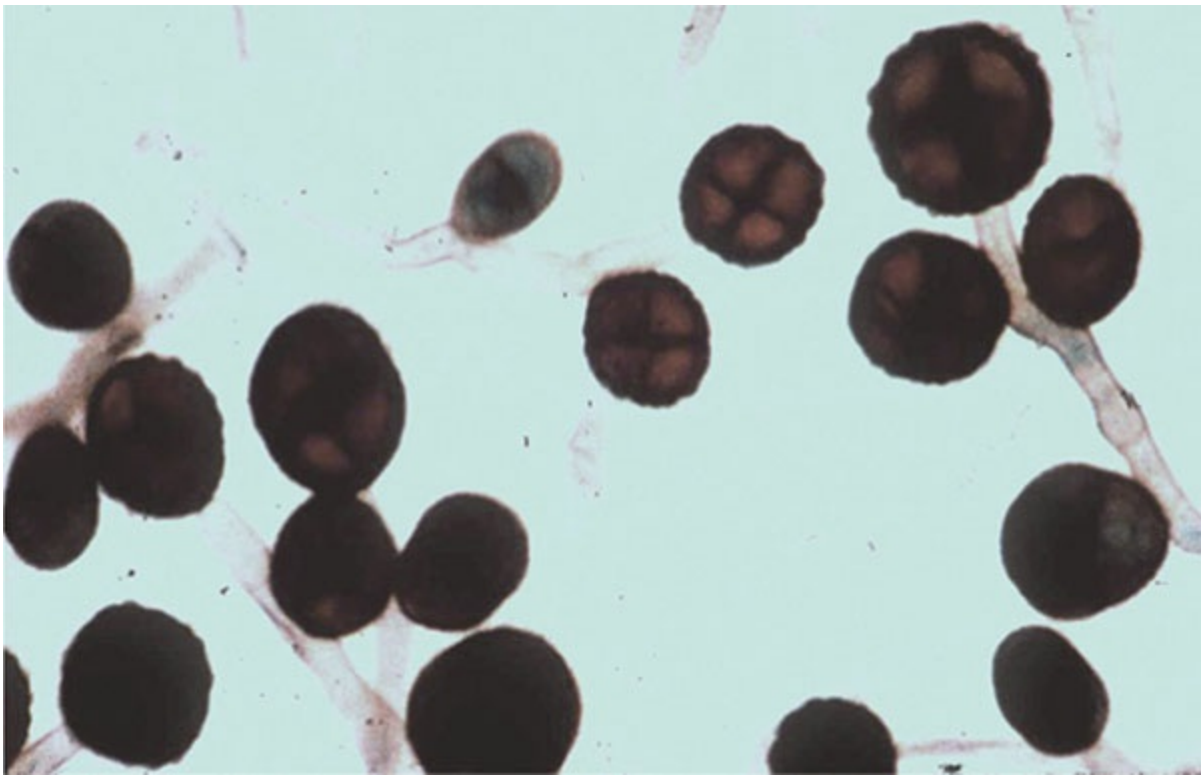
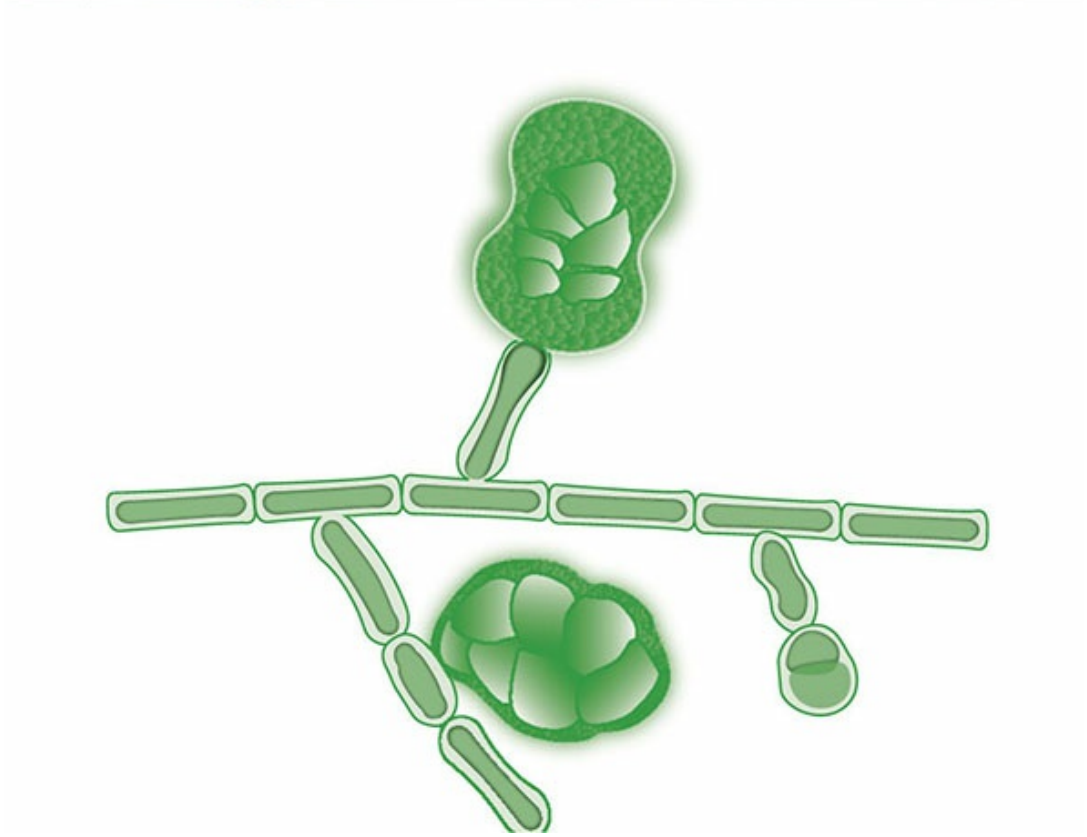
■ **FIGURE 21-66** Photomicrograph of a short chain of dark-staining, muriform macroconidia characteristic of *Alternaria* species.



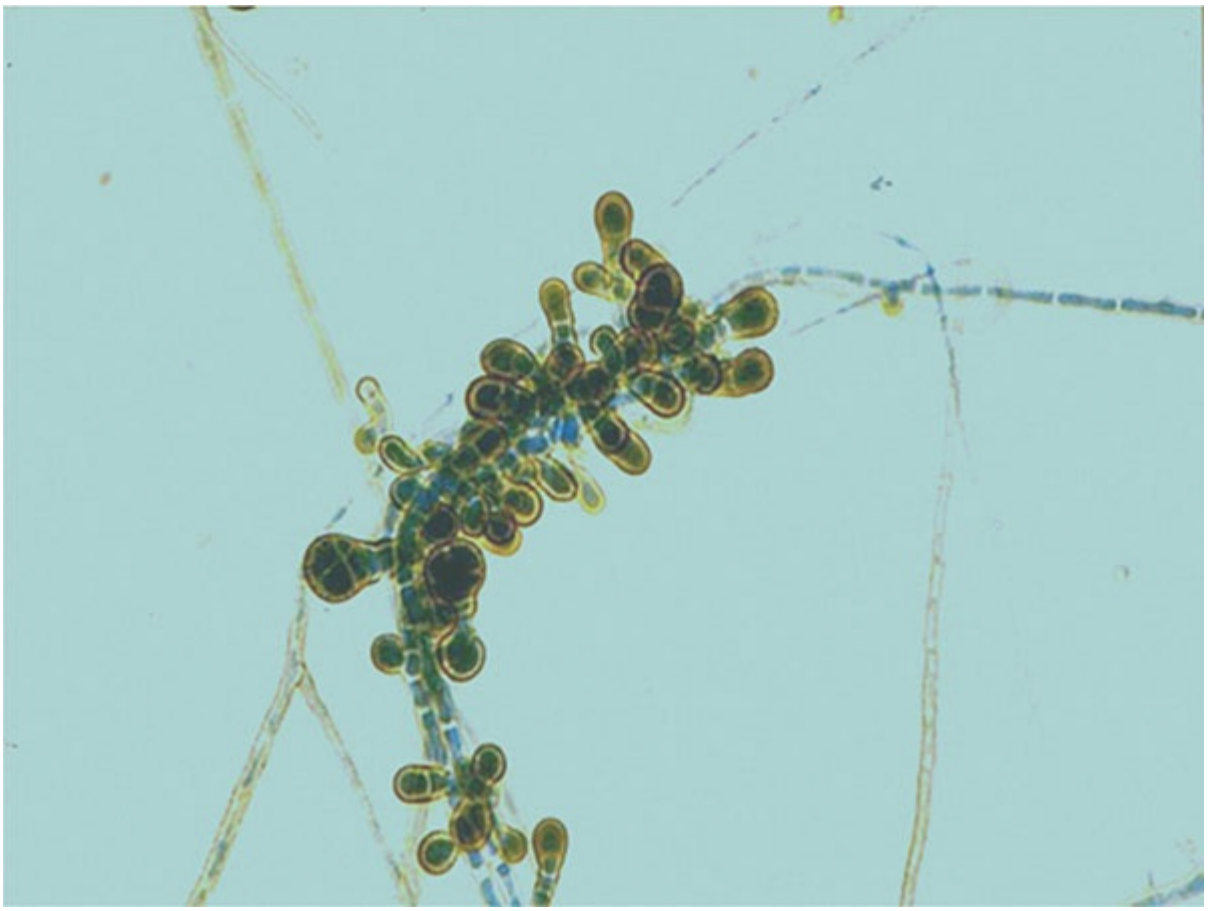
■ **FIGURE 21-67** Photomicrograph of dark-staining, muriform macroconidia derived from geniculate conidiophores, characteristic of *Ulocladium* species.

***Stemphylium* Species.** The muriform macroconidia produced by *Stemphylium* species is superficially similar to that of *Ulocladium* spp. Closer inspection, however, will disclose that they are slightly constricted in the mid-aspect of the conidium, which gives the conidium a "Figure 8" appearance. In addition, these are borne singly at the apex of a short, straight conidiophore (i.e., the sympodial geniculate conidiation of *Ulocladium* is absent). The terminal aspect of the conidiophore is swollen and is often more darkly pigmented than the rest of the conidiophore (Fig. 21-68).

***Epicoccum* Species.** The hyphae of *Epicoccum* typically form focal repeated branching and rebranching in the foci of conidiation, forming masses known as **sporodochia**. Short, club-shaped conidiophores arise from these masses, bearing multicelled, muriform, spherical to slightly club-shaped macroconidia, which may be roughened on the surface, giving a blackish wartlike appearance (Fig. 21-69). An important colonial characteristic is the production of a yellow pigment that diffuses into the agar.



■ **FIGURE 21-68** Photomicrograph and sketch of muriform macroconidia characteristically supported on the tip of a short, straight conidiophore, "bale of cotton on a stick," characteristic of *Stemphylium* species.



■ **FIGURE 21-69** Photomicrograph of immature and mature muriform macroconidia of *Epicoccum* species, characteristically borne in loose clusters termed a sporodochium.

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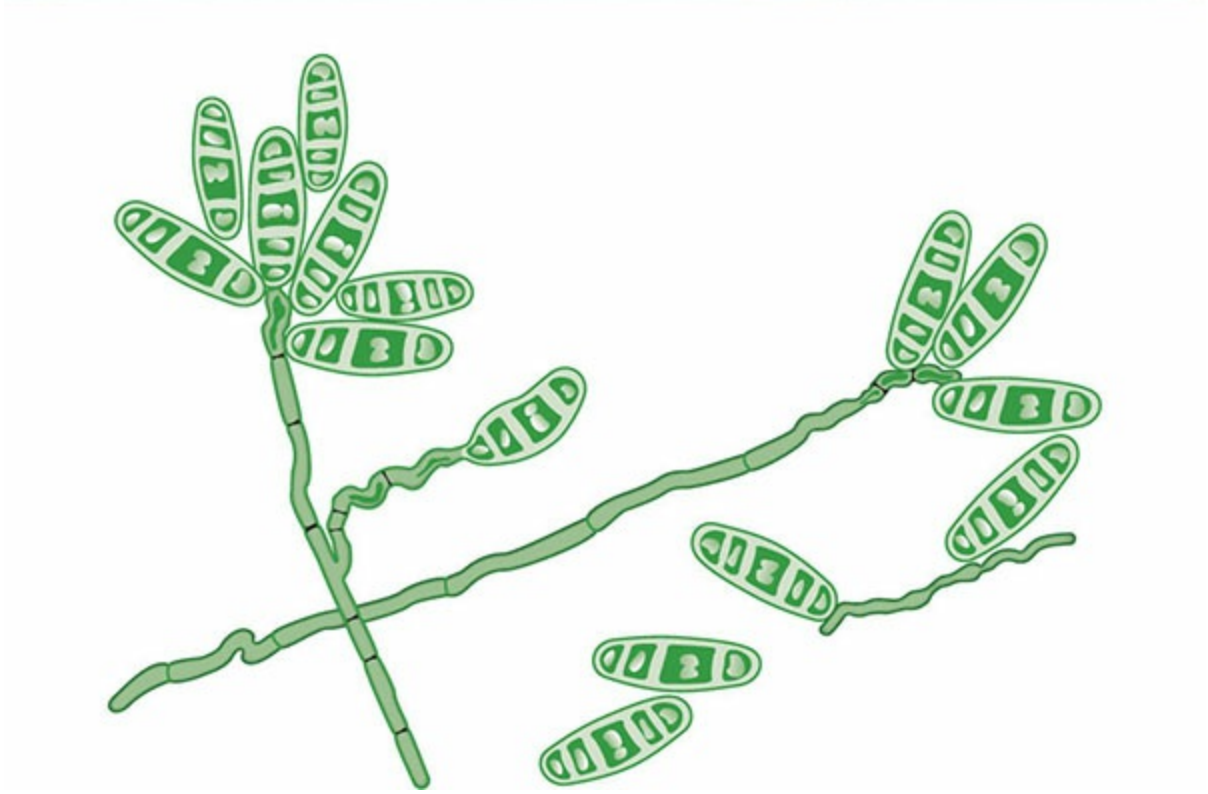

■ **FIGURE 21-70** Photomicrograph and sketch of cylindrical, smooth-walled, multicelled macroconidia characteristic of *Bipolaris* species.

Macroconidia with Transverse Septa

***Bipolaris* Species.** The distinguishing feature of *Bipolaris* spp. is the production of multicelled, elliptical to oval, thick-walled macroconidia with smooth surfaces; these are produced by sympodial, geniculate conidiation, as described above (Fig. 21-70). Close inspection of these conidia in preparations using a stain, such as lactophenol cotton blue, will disclose that the septations between the cells of the conidium are incomplete. These are termed distoseptae and when present, one can often visualized the continuity of cytoplasm from one cell in the conidium to the adjacent cell. The designation *Bipolaris* is derived from the property of producing germ tubes that **arise from both (i.e., bi) end (i.e., polar) cells of the macroconidia**, which grow in parallel with the long axis of the cell. These can be demonstrated when examined in direct water or saline mounts of the conidia that have been incubated at 25°C for 8 to 24 hours.

Bipolaris species are important causes of fungal keratitis following an eye injury and may cause phaeohyphomycosis in the immunocompromised host.

***Dreshslera* Species.** *Drechslera* spp. also produce cylindrical, multicelled macroconidia from sympodial geniculate conidiation that are indistinguishable from those of *Bipolaris* spp. The conidia have non-protruding, rounded contours at the base cell. The germ tube test is used to differentiate this fungus from *Bipolaris*. In saline mounts, a germ tube may be produced by any of the cells of the multicellular conidium. A microscopic search of the germinating conidia will demonstrate some wherein the germ tube is arising from an internal cell, rather than only from the terminal cells. These germ tubes are readily recognized, since they extend **at right angles to the longitudinal axis** of the conidium.

Drechslera spp. are very infrequent in clinical practice. *Drechslera* is not an important cause of human disease and is presented here because of the morphological similarities of the conidia to *Bipolaris*. The vast majority of fungal isolates that produce conidia that are morphologically consistent with *Bipolaris* or *Drechslera* are determined to belong to the genus *Bipolaris*.

***Curvularia* Species.** The macroconidia of *Curvularia* spp. are easy to recognize, having four to five cells separated by transverse septa, borne through sympodial geniculate conidiation. The second cell from the terminal end (i.e., non-hilar end) of the conidium continues to grow after the others have stopped, which imparts a curved shape to the conidium, which some believes resembles a boomerang (Fig. 21-71). *Curvularia* is an important caused of phaeohyphomycosis and may cause allergic

fungal sinusitis.

***Exserohilum* Species.** The conidia of *Exserohilum* spp. are similar to those of *Bipolaris* spp., except that they are longer, have more cells/conidium and characteristically have an **extended, prominent protruding hilum** from the hilar cell (Fig. 21-72). Although *Exserohilum* is an infrequent cause of human disease, in 2014 there was significant contamination of an injectable methylprednisolone with environmentally-derived *Exserohilum*. This product was nationally distributed and was the source of infection in many patients.[154]

Miscellaneous Moderately to Rapidly Growing Dematiaceous Molds

There are three genera of the molds fungi that will be considered here largely because they are not well categorized elsewhere. These are *Nigrospora* species, *Phoma* species, and *Chaetomium* species. The latter two fungi may be confused with one another, so careful attention to differentiating characteristics is important.

***Nigrospora* Species.** The colonies of *Nigrospora* spp. are rapidly growing and cottony; initially they are dirty white, but turn gray with maturity as the vegetative hyphae become pigmented. The reverse of the colony also becomes darkly pigmented. Microscopically, the conidia are solitary, large, subglobose, smooth, and jet black and are borne from the tips of inflated, urn-shaped conidiophores (Fig. 21-73). The conidia of *Nigrospora,* to some, resemble a jet-black rugby ball or North American Football.

***Phoma* Species.** The colonies of *Phoma* spp. are buff to dark brown, spreading over the surface with an indistinct outer margin. The consistency is more glabrous than cottony because aerial hyphae develop poorly. Microscopically, large dark brown to black, smooth-walled, leathery, saclike structures called **pycnidia** are characteristically seen (Fig. 21-74).

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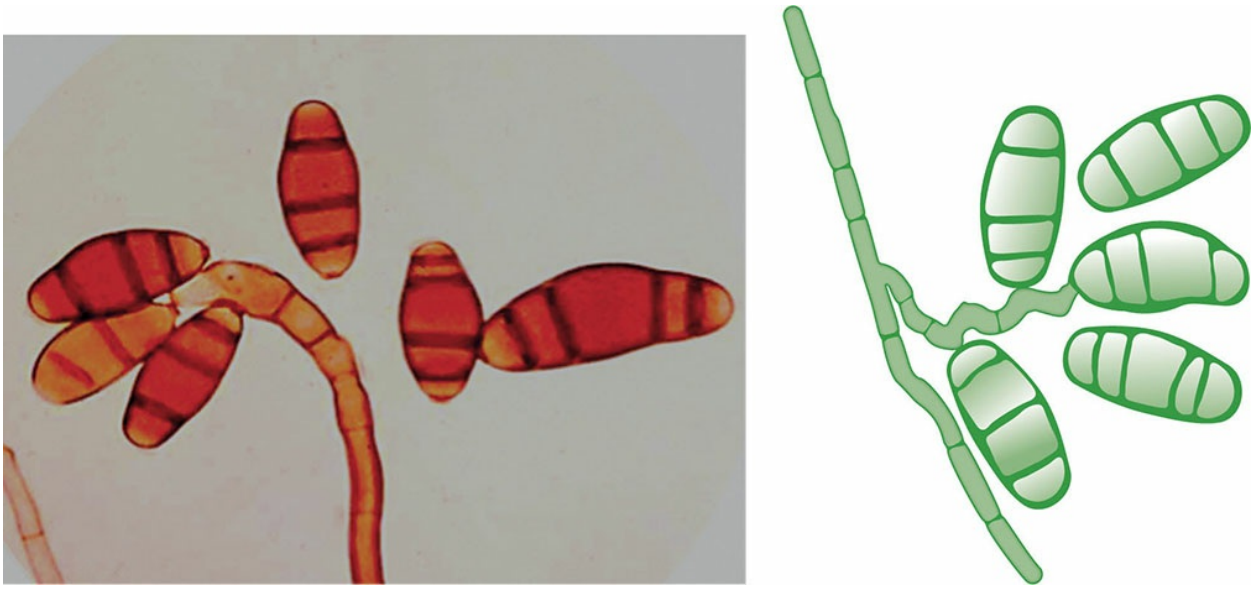
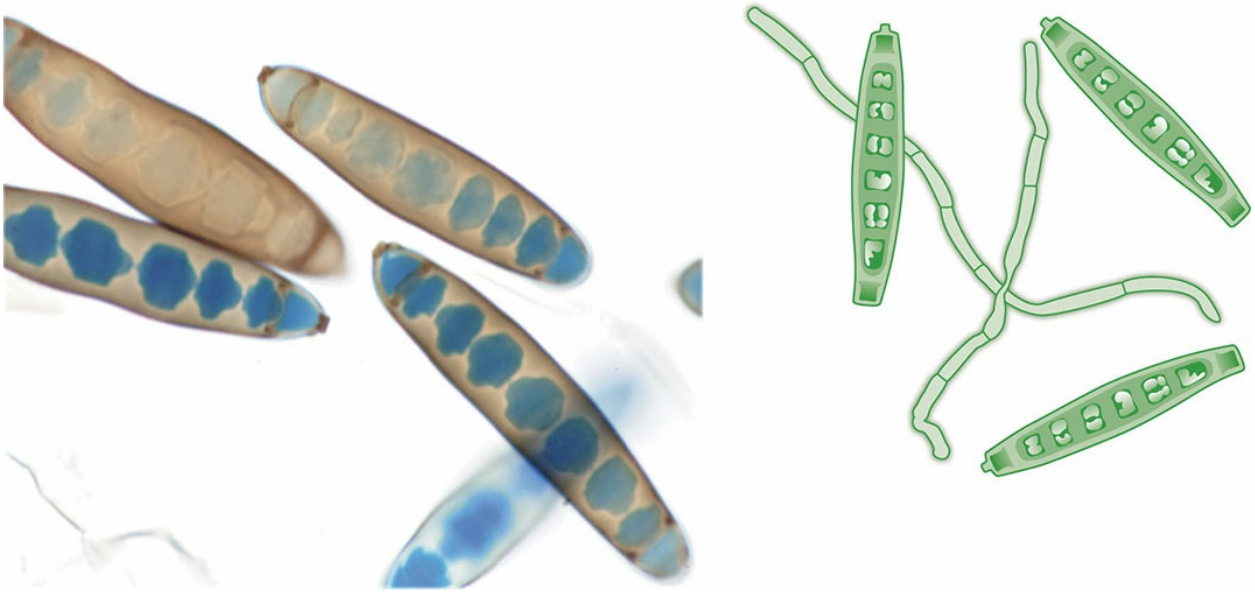
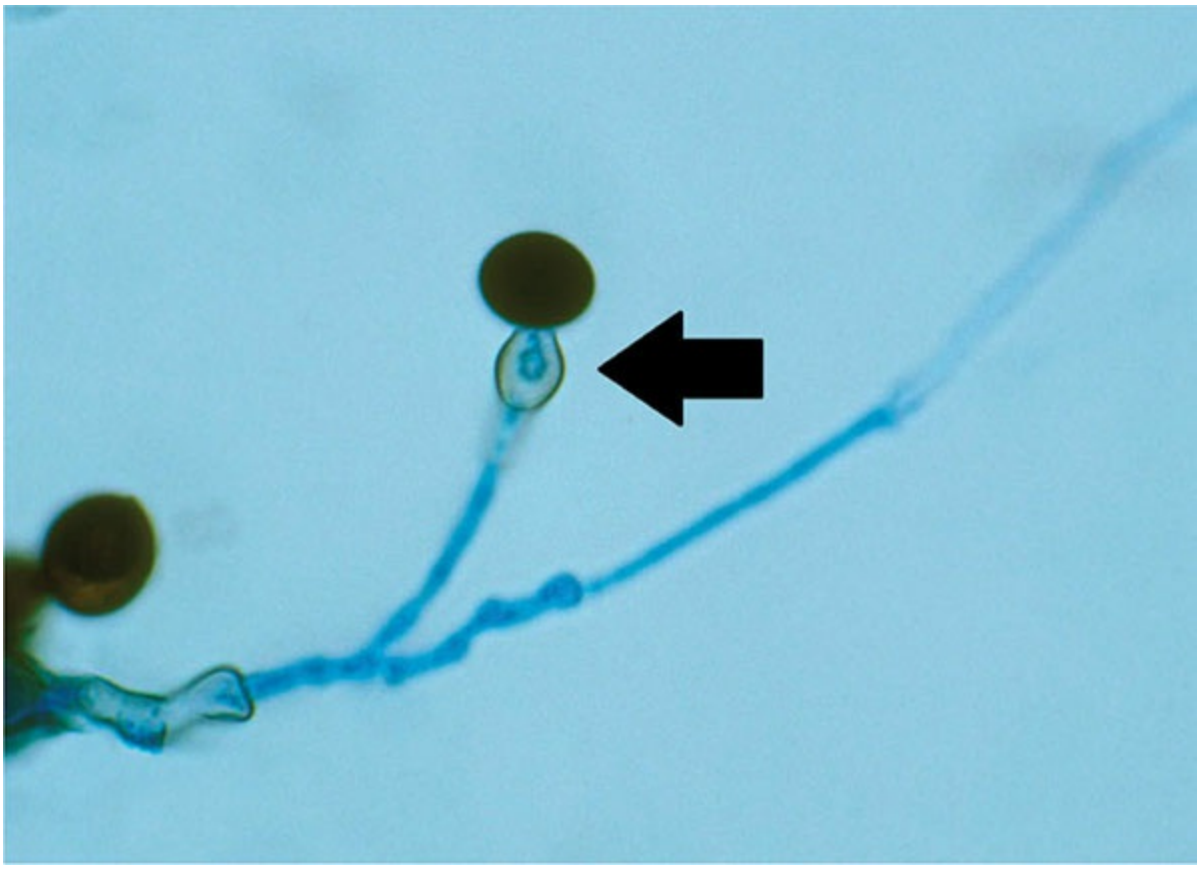


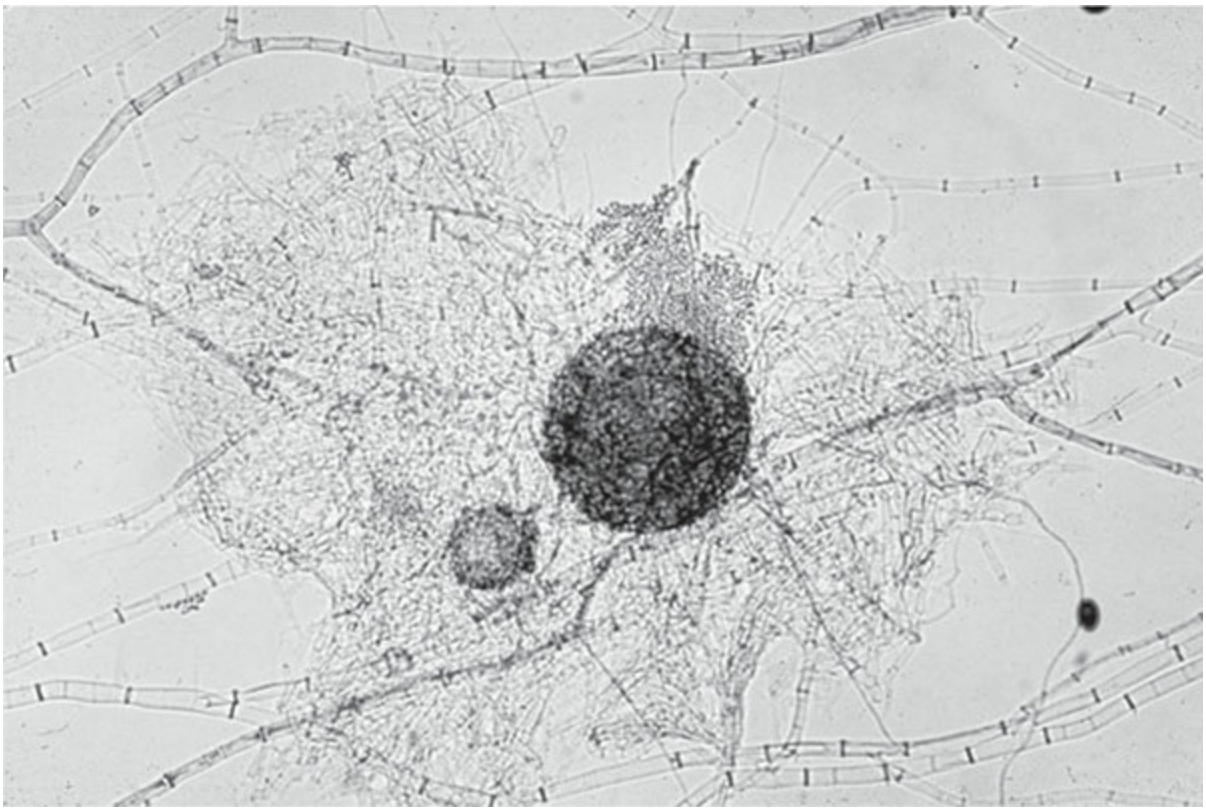
■ **FIGURE 21-71** Photomicrograph and sketch of dark-staining, multicelled macroconidia divided by transverse septa and bent due to overgrowth of next-to-terminal cell, characteristic of *Curvularia* species.



■ **FIGURE 21-72** Photomicrograph and sketch of long, pencil-shaped macroconidia of *Exserohilum* species, characterized by a terminal protrusion from the hilar cell.



■ **FIGURE 21-73** This photomicrograph of *Nigrospora* demonstrates a jet-black conidium supported by a bulbous conidiophore.



■ **FIGURE 21-74** Photomicrograph of a large saclike pycnidium of a *Phoma* species, with scattered tiny, hyaline conidia observed in the background.

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Within the pycnidia are borne myriad cylindrical, spindle-shaped or spherical, hyaline, one-celled conidia, which are asexually derived and released through a hole or ostiole. Because pycnidia may appear similar to the cleistothecia produced by *Aspergillus* spp., *Chaetomium* spp., and other telomorphic fungi, one must determine whether the spores contained are conidia, as is the case with *Phoma* spp., or are the larger, darker-staining ascospores characteristic of the ascosporogenous fungi. This differentiation can be made in cellophane tape mounts by gently pressing the surface of the tape with the tip of a pencil over the unknown baglike structure, while visualizing it under the scanning objective of a microscope until it ruptures. The release of the one-celled hyaline conidia as described above confirms the identification of *Phoma* spp.

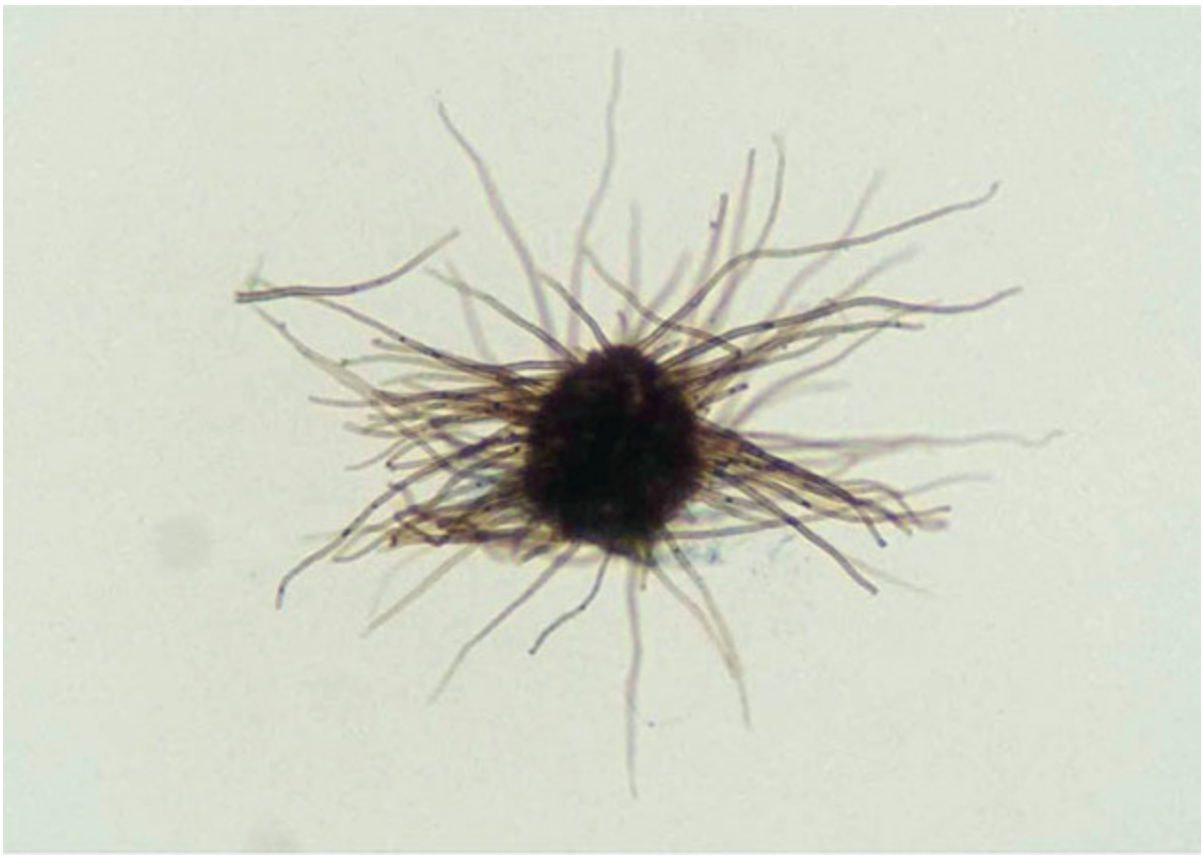
***Chaetomium* Species.** The colonies of *Chaetomium* spp. are initially white, but may become yellow, yellow-green, or copper-colored on maturity. Dark dots appear with the formation of cleistothecia. Microscopically, septate hyphae are formed, in addition to large, setae (i.e., spiked hyphae-like elements) may be observed extending from the wall of saclike cleistothecia. The word "setae" is derived from the Latin term for

bristle; these structures may be straight, coiled, or curved. Some believe the combination of these structures simulates the extending legs of a spider (Fig. 21-75).

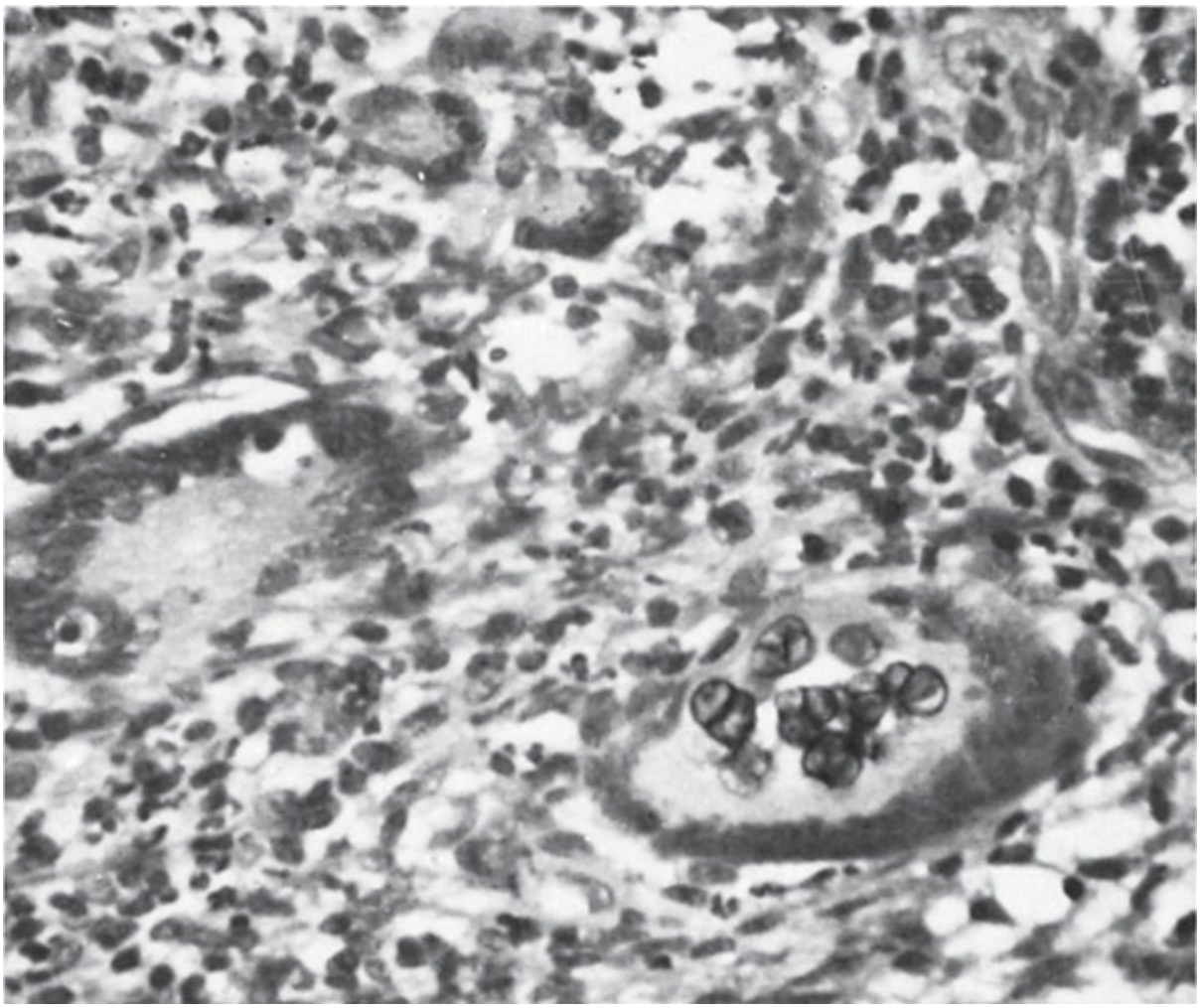
Slowly Growing Dematiaceous Fungi

The slowly growing dematiaceous molds are responsible for a variety of types of infections, including phaeohyphomycosis, chromoblastomycosis, and mycetoma. Phaeohyphomycosis has been described above, so a brief review of chromoblastomycosis and mycetoma will be given.

Chromoblastomycosis is an infection of the skin and subcutis. This is clinically characterized by the formation of elevated, roughened multicolored verrucous vegetations, most commonly spreading over the dorsal surfaces of the feet and lower leg. These agents gain entrance to the skin through traumatic wounds and penetrating injuries. The diagnostic forms are muriform, light-yellow-to-brown oval-to-spherical bodies that are grouped in clusters. Internal septations are seen in some of these yeast-like forms; however, budding does not occur. These are termed sclerotic bodies or more colloquial as copper pennies (Fig. 21-76). Microabscesses and granulomatous nodules (i.e., a pyogranulomatous response), extreme acanthosis, and pseudoepitheliomatous hyperplasia with varying degrees of fibrosis and scarring are the common histologic changes.



■ **FIGURE 21-75** Photomicrograph of the spiderlike pycnidium of a *Chaetomium* species.



■ **FIGURE 21-76** Photomicrograph of a tissue section from a patient with subcutaneous chromoblastomycosis. Note the inclusion of several spherical sclerotic bodies within the giant cell located in the lower right quadrant in the field of view (H&E, 400×).

Chromoblastomycosis is caused by a group of slow-growing, dematiaceous fungi belonging to the genera *Cladophialophora/Cladosporium*, *Phialophora*, and *Fonseceae*.[77] Mycetoma is an infection that is located primarily in the subcutaneous tissue, forming an abscess or granulomatous mass with the formation of sinus tracts that reach the surface of the skin. A mycetoma is a tumifactive mass that damages tissues through erosion and pressure, rather than invasion. Mycetomas may be caused by bacteria (i.e., eubacterial mycetoma) or fungi (i.e., eumycotic mycetoma). Purulent material containing grains or granules of microbial elements are often discharged from these sinus tracts, and are also observed in direct examinations and histologic sections. The grains are white if the eumycotic mycetoma is caused by a hyaline mold, whereas the grains are brown or black if the eumycotic mycetoma is caused by a dematiaceous mold.

The following species of slow-growing dematiaceous fungi are the most common causes of chromoblastomycosis, mycetoma, and other deep-seated mycotic infections:

- *Cladophialophora (Cladosporium) carrionii*
- *Cladophialophora (Xylohypha) bantiana*
- *Phialophora verrucosa*
- *Pleurostomophora (Phialophora) richardsiae*
- *Fonsecaea pedrosoi*
- *Exophiala* species

The differentiation between each of the species listed above depends on the observation of key differences in the mode of conidiation, the structure of the conidiophores and phialides or annellides, and the morphology and arrangement of the conidia.

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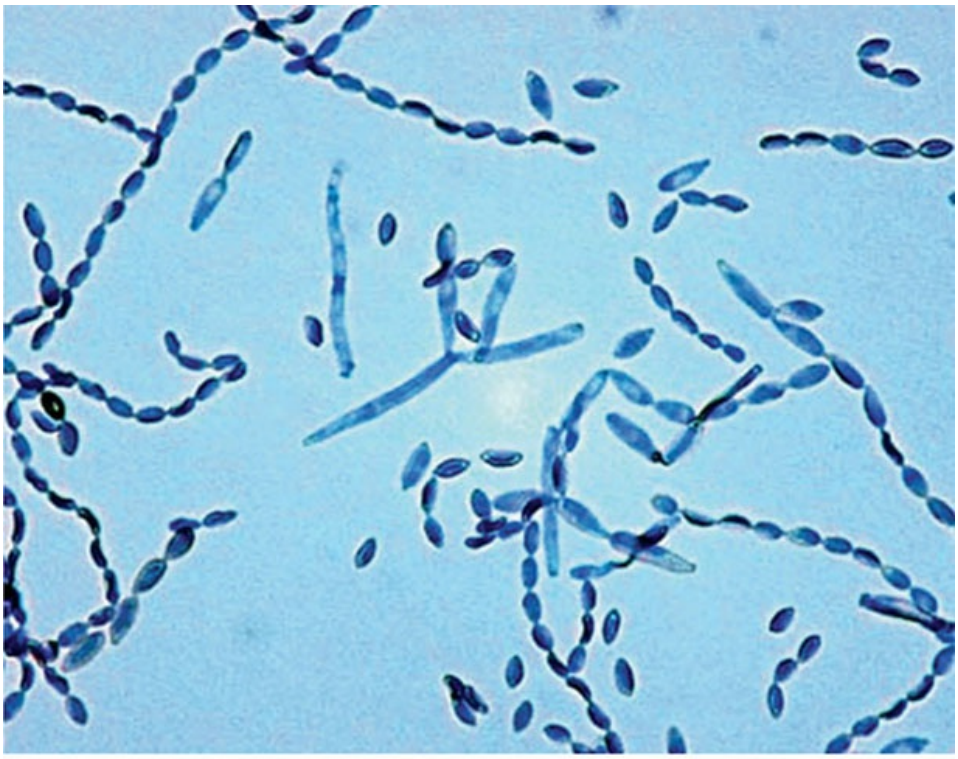
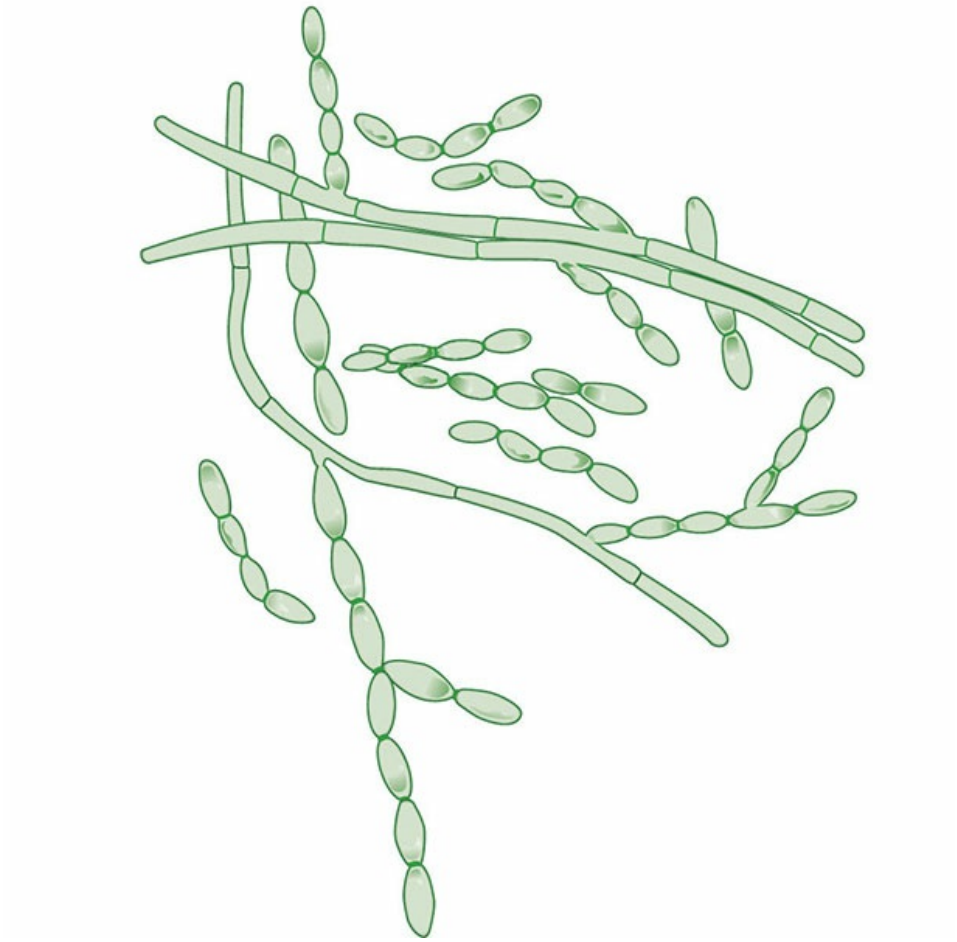
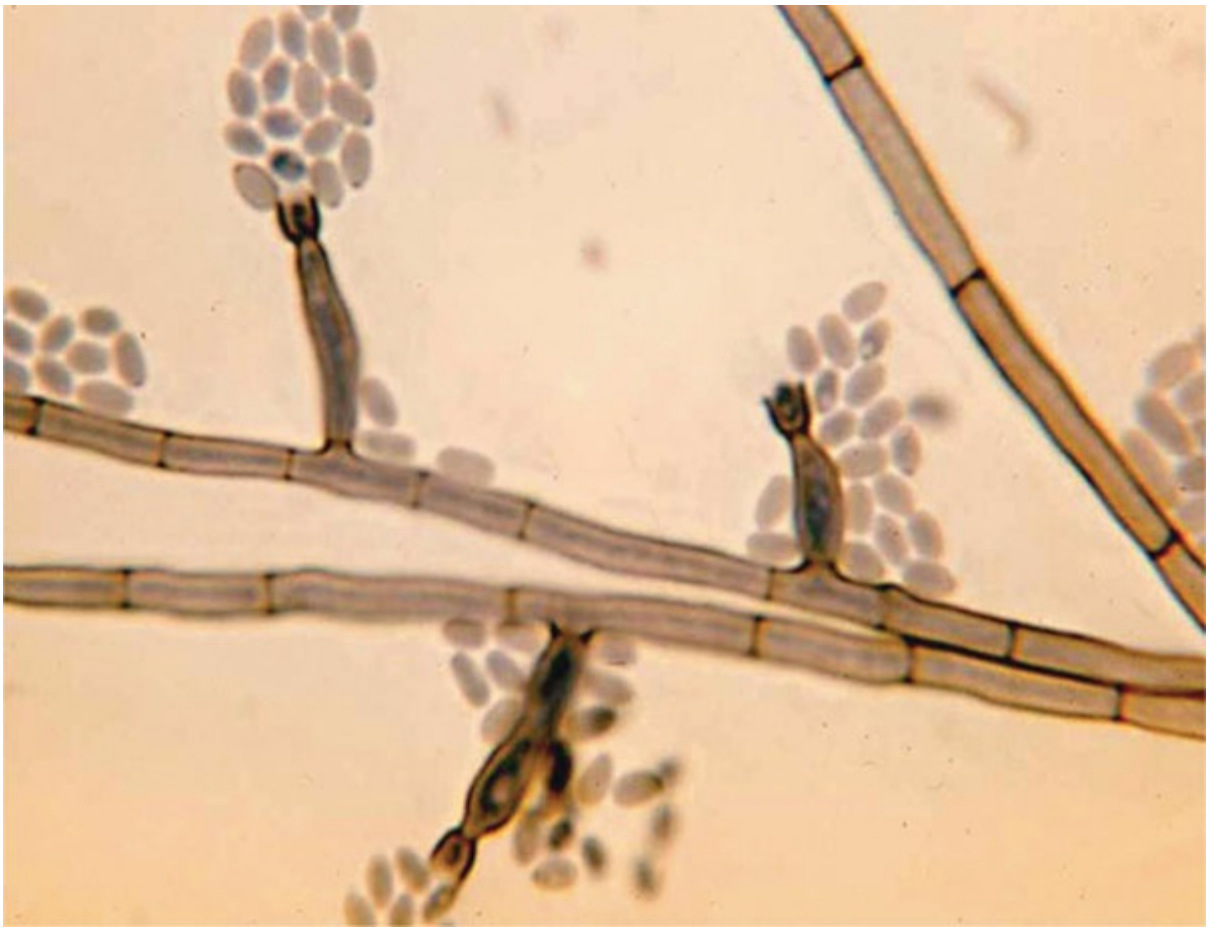
The causative agents of chromoblastomycosis include *Cladophialophora*, which reproduces with typical *Cladosporium*-type conidiation, which may be complemented by the production of phialides. In the *Cladosporium*-type sporulation, elliptical, dark-staining conidia are produced in long, branching chains, each separated by a delicate scar known as a dysjunctor (Fig. 21-77). *Phialophora verrucosa* is another cause of chromoblastomycosis and reproduces with *Phialophora*-type sporulation. This is characterized by the production of single phialides that are either urn-shaped or long and tapered. Phialides, which range in length from 4 to 7 μm in length, are borne directly from the sides of the hyphae. The terminal portion of the phialide is characteristically elongated, simulating the top of a soda bottle. Spherical to oval-shaped, yellow-pigmented conidia are produced from within each phialide, and aggregate in ball-like clusters at the terminal opening. These conidia may dislodge and aggregate along the sides of the phialides and along the adjacent supporting hyphae (Fig. 21-78). In the acrotheca-type sporulation, sympodial branches of conidiophores are produced from the sides of the hyphae, simulating the prongs of a coat rack. Short chains of conidia are borne from these clusters (Fig. 21-79). Although *Rhinocladiella* species are not a cause of chromoblastomycosis, the *Rhinocladiella*-type sporulation may be produced by *Fonsecaea* species. This is a variant of acrotheca-type sporulation in which the conidia are produced singly, directly, and laterally from the sides of the hyphae (Fig. 21-80). It is important to note that in addition to the typical *Fonsecaea*-type sporulation, that *Fonsecaea* species may also produce *Cladosporium*-type, *Phialophora*- type, and *Rhinocladiella*- type sporulation. These have been termed polymorphic fungi, because they produce a variety of morphologic forms. The conidia in a related, less commonly encountered species, *Fonsecaea compacta*, are

arranged in tight clusters.





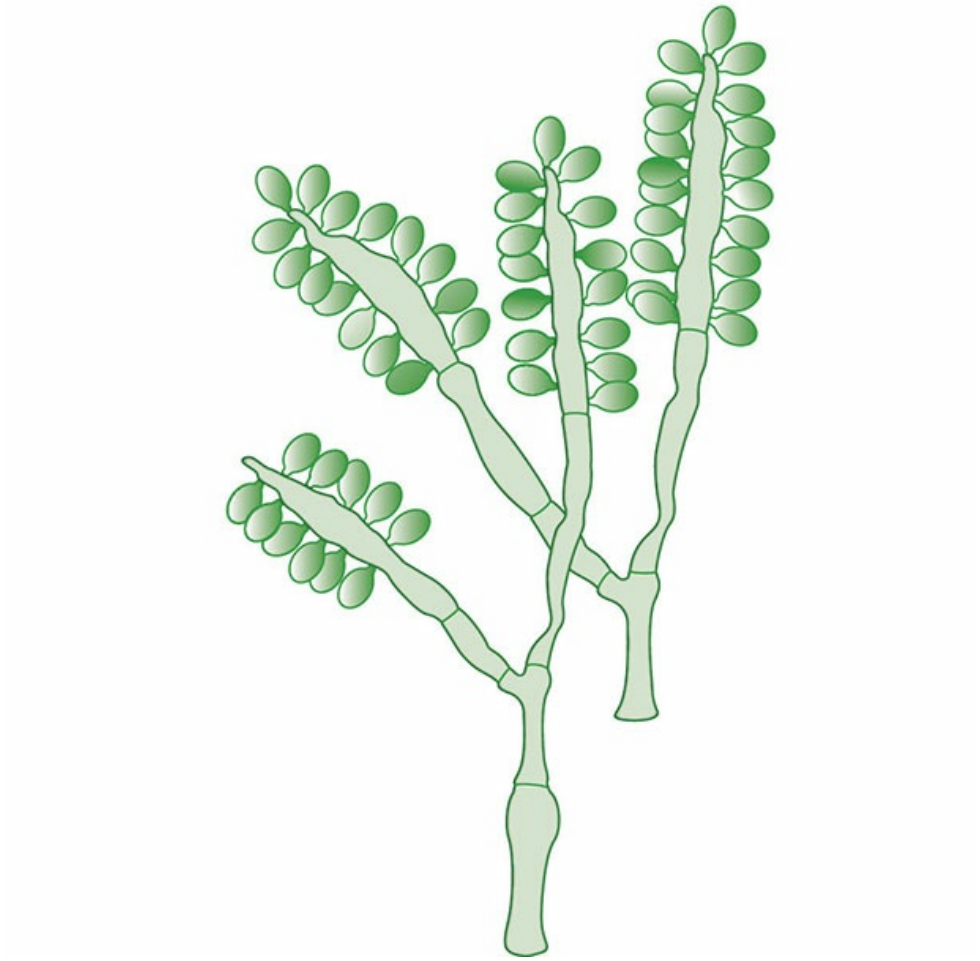
■ **FIGURE 21-77** Photomicrograph and sketch illustrating cladosporium-type sporulation, in which chains of elliptical conidia are connected by dark-staining dysjunctors.



■ **FIGURE 21-78** Photomicrograph of phialophora-type sporulation, illustrating short urn-shaped conidiophores, each with a narrow soda-bottle-like mouth from which spherical conidia are produced, arranging in loose clusters at the tip.

■ **FIGURE 21-79** Photomicrograph of acrotheca-type sporulation, in which short chains of elliptical conidia are produced sympodially from the ends of conidiophores.

1386



■ **FIGURE 21-80** Photomicrograph and sketch of rhinocladiella-type sporulation in which elliptical conidia are borne directly and laterally in compact rows from the sides of the conidiophore.

***Cladophialophora/Cladosporium* Species.** There are a variety of *Cladosporium* and *Cladophialophora* species. The two most common or of the most medical importance will be highlighted. *Cladosporium carrionii* produces freely branching hyphae that give rise to long chains of dark-staining, elliptical conidia. The so-called "shield cells" are the points wherein branching occurs. The conidia and shield cells often show scars or dysjunctors that are more darkly pigmented that the rest of the hyphae or conidia and represent the points of previous attachment (Fig. 21-77).

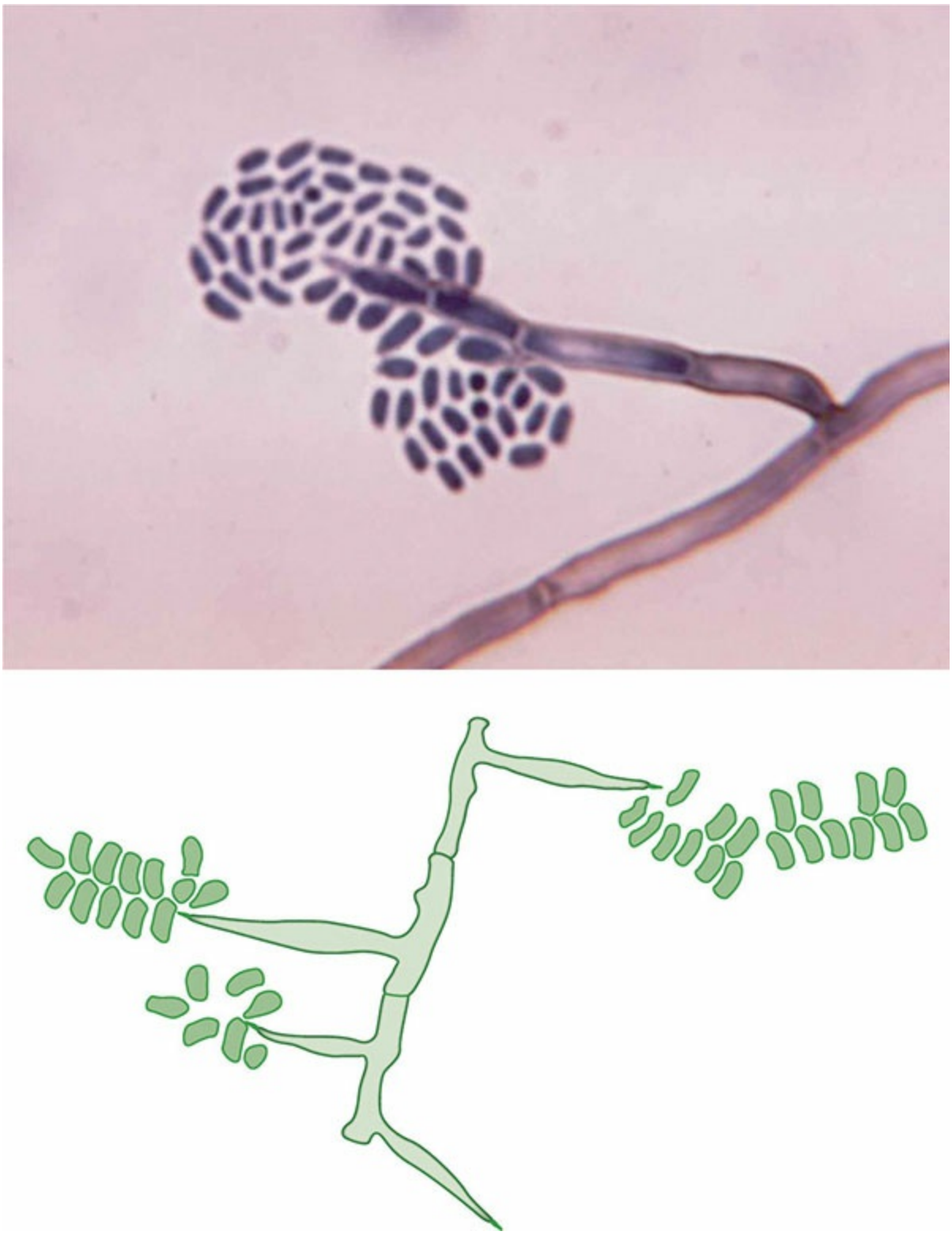
***Cladophialophora bantiana* (formerly *Xylohypha* or *Phialophora bantianum*).** This is a closely related species that also produces conidia of the *Cladosporium*-type; however, the chains of conidia are very long, with as many as 30 cells each and producing nonpigmented conidia without dysjunctors. *Cladophialophora bantiana* grows at 42°C and does not liquefy gelatin, which are two additional characteristics by which it can be differentiated from *Cladophialophora carrionii,* which does not possess these properties.

■ **FIGURE 21-81** Photomicrograph of the long conidiophores of *Pleurostomophora richardsiae*, illustrating the characteristic flat saucerlike terminal ending of the phialide.

Pleurostomophora (Phialophora) richardsiae. In contrast to the narrow bottlelike phialides of *Phialophora verrucosum*, those of *Pleurostomophora richardsiae* are flat and saucer-shaped (Fig. 21-81). *Pleurostomophora richardsiae* is a common agent recovered from phaeohyphomycotic cysts and may cause invasive phaeohyphomycosis. Spherical to elliptical, hyaline conidia are borne in tight clusters at the tips of these phialides, held together by a mucinous material. These conidia may also dislodge and aggregate along the sides of the phialides.

Scedosporium prolificans. *Scedosporium prolificans (inflatum)* is a rarely encountered, but important cause of phaeohyphomycosis in humans, particularly those with immunocompromising conditions. Microscopically, this may be mistaken for members of the *P. boydii* complex. The colony morphology of this organism is more distinctly dematiaceous with a surface that is often an olive-drab green, in distinct contrast to the house-mouse gray of the *P. boydii* complex (Image Plate 21-2G).

***Exophiala* Species.** There are a number of *Exophiala* species. Many of these have phenotypic characteristics of *E. jeanselmei,* so will be included in the *E. jeanselmei* complex. The other *Exophiala* species, which is morphologically identical but may be differentiated by biophysical characteristics, is *E. dermatitidis,* formerly known as *Wangiella dermatitidis.* Both of these species often produce wet-to-mucoid, yeast-like colonies upon initial isolation. Cursory microscopic examination discloses yeast-like cells. However, upon closer examination it is apparent that true budding is absent and instead these are single-celled forms that are reproducing through annelloconidiation (i.e., annellides rather than phialides are produced). The colonies transform from wet and yeast-like to a velvety mold with extended incubation. Long, tapered conidiophores are produced that extend from the hyphae in right or obtuse angles. The tips of the conidiophores appear to be sharply pointed from the production of progressive annellide formation (Fig. 21-82). Rings or annellides may be



■ **FIGURE 21-82** Photomicrograph and sketch of *Exophiala* species, illustrating the long, tapered conidiophores from the tips of which are released elongated annelloconidia that arrange in loose clusters.

observed beneath the tapered tip of the phialide with exacting focusing of the microscope and diminution of the condenser light.[160] The clinical manifestations of phaeohyphomycosis, chromoblastomycosis, and mycetoma are presented in Clinical Correlation Box 21-10.

The Laboratory Identification of Yeasts

The monomorphic yeast species of medical importance that are most commonly encountered in clinical microbiology laboratories include:

- *C. albicans* and other *Candida* species
- *Trichosporon* species
- *Rhodotorula* species
- *Saccharomyces* species
- *Wickerhamomyces (Hansenula/Pichia) anomalus*
- *Malassezia furfur*

Candida Species and Candidiasis

Candida species are the yeasts most commonly isolated from clinical specimens. From data obtained from 180 U.S. hospitals participating in the National Nosocomial Infections Surveillance (NNIS) system, 27,200 fungal isolates associated with nosocomial infections were reported in the United States between January 1980 and April 1990. Of these, *Candida* spp. accounted for 19,621 (72.1%).[137] Factors predisposing to this high frequency of yeast infections include:

CLINICAL CORRELATION BOX 21-10 Phaeohyphomycosis, Chromoblastomycosis, and Eumycotic Mycetoma

Brandt and Warnock[31] from the CDC have published a review covering the epidemiology, clinical manifestations, and therapy for infections caused by these dematiaceous fungi. They list *Alternaria* species, *Bipolaris* species, *Cladophialophora bantiana*, *Curvularia* species, *Exophiala* species, *Fonsecaea pedrosoi*, *Madurella* species, *Phialophora* species, *Scedosporium prolificans*, *Scytalidium dimidiatum*, and *Exophiala dermatitidis* as the more important human pathogens within this group. They describe these fungi as being widespread in the environment, and found in soil, wood, and decomposing plant debris. The cutaneous,

subcutaneous, and corneal infections associated with these dematiaceous fungi occur worldwide, but are more common in tropical and subtropical climates. Infection most commonly results from traumatic implantation into the skin or other tissue. Most progressive cases occur in immunocompetent individuals. They also cite the dematiaceous molds as being important causes of invasive sinusitis and allergic fungal sinusitis, most commonly following inhalation of infective conidia.

Phaeohyphomycosis

An invasive fungal infection caused by a phaeoid or dematiaceous mold is a phaeohyphomycosis. Although cerebral infection is the most important form of systemic phaeohyphomycosis, other localized deep forms of the disease, such as arthritis, and endocarditis, have been reported. Disseminated infection is uncommon, but the incidence is higher among immunocompromised individuals. *Scedosporium prolificans* is an important cause of disseminated infection in the immunocompromised and is difficult to eradicate, as it is multidrug resistant. Literally hundreds of isolated cases of fungal infections caused by the rapidly growing, saprophytic species of dematiaceous molds have been reported in the recent medical literature. Space here allows for only a few examples. The three more common clinical manifestations of phaeohyphomycosis caused by the saprophytic fungi are keratitis, sinusitis, and onychomycosis.

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As an example of keratitis, Wilhelmus and Jones[297] reported their laboratory experience with the isolation and identification of *Curvularia* species as a cause of keratitis over a 30-year period. The records for 32 individuals were available for analysis. It was determined that trauma, usually with plants or dirt, was the risk factor in half the cases. The majority of patients (69%) were infected during the hot, humid summer months along the U.S. Gulf Coast. Presenting signs varied from superficial, feathery infiltrates of the central cornea to suppurative ulceration of the peripheral cornea.

Sinusitis is also a common site of infection with dematiaceous fungi. From the older literature, Adam et al.[2] reported nine patients with sinusitis presenting as allergic rhinitis or nasal polyposis, caused by *Bipolaris* and *Exserohilum* species. Fernandez et al.[87] reported on the findings derived from a search of the literature for pediatric *Curvularia* infections, and of the 16 cases identified, 13 had allergic sinusitis, a unique form of sinusitis in which a history of asthma or nasal polyps were predisposing conditions. The patients in this review were usually immunocompetent teenagers who had a prolonged duration of symptoms, usually >3weeks.

Onychomycosis caused by *Alternaria* species is being reported with increasing frequency, particularly in patients with immunodeficiency. Romano et al.[237] reported nine cases of onychomycosis caused by *Alternaria alternata* observed in Tuscany,

Italy. Diagnosis was made on the basis of repeated direct microscopic mycologic examination and culture. In most of the patients, dystrophy and distal subungual hyperkeratosis of one or two nails of the feet or hands were the primary clinical manifestations. Seven cases were treated with oral itraconazole; this treatment was successful in six cases. Importantly, chronic nail infections may serve as the nidus for dissemination non-dermatophytic fungi in immunocompromised patients.

A number of dematiaceous fungi are neurotropic, including *Cladophialophora bantiana*, *Ramichloridium* (*Rhinocladiella*) *mackenziei*, *Ochroconis gallopava*, and *Exophiala dermatitidis*. Although infections have occurred in immunocompromised persons, cerebral phaeohyphomycosis is most common in immunocompetent individuals who may or may not have obvious risk factors.[69] Revankar et al.[230] reviewed 101 cases of culture-proven primary central nervous system phaeohyphomycosis reported in the English-language literature from 1966 to 2002. They found that the most frequently isolated species was *Cladophialophora bantiana*. The mortality rates were high among these reported cases, regardless of immune status. Therapy has not been standardized, although the combination of amphotericin B and the new triazoles, such as posaconazole, along with an aggressive medical and surgical approach may improve survival rates.

Chromoblastomycosis

Chromoblastomycosis is widespread in tropical South America, occurring most commonly in male farm and plantation workers. Silva et al.[256] reviewed 325 cases reported in the Amazon river drainage region of Brazil. The data obtained revealed a mean age range of affected individuals between 41 and 70 years, 93.2% of whom were males. Agricultural workers comprised 86.1% of the total. Lesions on the lower limbs (feet and legs) were found in 80.7%. In 24% of patients (78 cases), the etiologic agent was isolated and identified through culture. *Fonsecaea pedrosoi* was present in 77 patients and *Phialophora verrucosa* in only one case. Similar results were reported by Minotto et al.[189] in a study of 100 patients residing in Rio Grande do Sul, Brazil. Again, male patients predominated (4:1); the majority were white farmers ranging in age from 50 to 59 years. Most commonly, severe verrucous lesions appeared on their lower limbs, with the average time between the appearance of the disease and medical diagnosis being 14 years. Thorn wounds were associated with the disease in 16% of the cases. Statistical analysis showed recrudescence of the disease in 43% of cases despite the treatment used. *Fonsecaea pedrosoi* was recovered from 96% of the cases; *Phialophora verrucosa* in 4%.

As noted, these fungi most commonly gain entry through traumatic inoculation. For example, Wortman[304] reported a patient with chromoblastomycosis caused concurrently by *Fonsecaea pedrosoi* and *Nocardia brasiliensis*, complicating a traumatic penetrating injury.

Mycetoma

Mycetoma refers to a subcutaneous infection in which the tissue is markedly swollen with the formation of deeply penetrating sinus tracts that erode through the superficial skin and discharge purulent material. The feet (Madura foot) and hands are most commonly involved, becoming markedly swollen and deformed in serious infections. Mycetomas have two primary causes—bacteria belonging to the family Actinomycetales (*Actinomyces*, *Nocardia*, and *Streptomyces* species), and the true fungi (eumycotic mycetomas), primarily the dematiaceous fungus *Exophiala jeanselmei* and the hyaline mold *Pseudallescheria boydii* in sporadic cases encountered in the United States.

Mycetoma is more commonly seen in other parts of the world, particularly western and eastern Africa. A history of prior injury is common. The lower extremities tend to be involved in the majority of cases, with the upper extremities and other sites such as the gluteal region, the lumbar area, submandibular area, and face less commonly.

The description many years ago of the presentation of a mycetoma by Thammayya et al.[273] of a patient who also lived in India remains classic for the condition: "The right lower leg and foot were irregularly swollen, firm,

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painless, and nontender and had many small nodules and sinuses all over the swelling. The lower end of the tibia and bones of the foot were affected.

The discharge from the sinuses and nodules contained black-brown, soft, vermicular, crescent-shaped to irregular granules, measuring 0.5–2.00 mm and composed mostly of swollen spherical cells, 4–8 μm diameter, and a few hyphae, 2.5–3.0 μm diameter."

A relatively high incidence has also been encountered in Japan. Murayama et al.[191] report a 34-year-old woman with SLE who developed a mycetoma involving the lower leg. In an accompanying literature review of *Exophiala jeanselmei* infections in Japan, these authors found reference to 54 cases (24 in men and 30 in women). Fifty of these infections (21 men and 29 women, an interesting shift in the male:female ratio) were caused by dematiaceous fungi. About half of these patients had underlying diseases, and the sites of the lesions were mainly on the extremities.

- Immunosuppressive conditions associated with chemotherapy, and stem cell and solid organ transplantations
- Prolonged hospitalization
- Vascular catheterizations
- Prolonged administration of broad-spectrum antibacterial agents

Wright and Wenzel,[305] in a review of patients with nosocomial candidiasis, cite burns,

artificial respiratory assistance, colonization with *Candida* species, and repeated transfusions as additional risk factors. These authors cite a 1996 nationwide study of participating hospitals, conducted under the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) program that *Candida* species has been established behind *Staphylococcus* species as the most frequent cause of septicemia in hospitalized patients.

Patients with cancer, particularly those with leukemia lymphoproliferative disorders, in whom the peripheral white blood count is depressed, are also at increased risk. Kalin and Petrini[141] cite an increase in mortality in patients with leukemia caused by fungal infections from a low frequency of 5% prior to the antibiotic era, to a current frequency of 40%. *Candida* species were recovered most frequently as the primary agents. Advanced age, although not directly related to yeast infections, nonetheless contributes to increased morbidity and mortality. For example, mucocutaneous candidiasis in the aged, including thrush and denture stomatitis, was commonly associated with local mechanical irritation of the oral mucosa.[124]

At the opposite end of the age spectrum, critically ill newborn babies also are at high risk for acquiring nosocomial yeast infections, with particular involvement of the central nervous system. Other conditions predisposing to yeast infections include altered host defenses due to compromised protein stores or antibody production, suppression of the normal bacterial flora, pregnancy, and diabetes mellitus or other chronic metabolic diseases.

Candida species are identified by a variety of methods that include germ tube testing, the use of chromogenic media, biochemical/assimilation testing, cornmeal agar morphology and increasingly by molecular methods and mass spectrometry.

In vitro antifungal susceptibility testing has been standardized through the efforts of the CLSI. This has made it possible to determine the degree of resistance of yeast isolates.[220,231] Applications of the E test have also made available to clinical laboratories the ability to determine select antifungal susceptibility profiles.[221,222] Key clinical manifestations of candidiasis are presented in Clinical Correlation Box 21-11 (*C. albicans*) and Clinical Correlation Box 21-12 (*Candida* spp., non- *albicans*).

CLINICAL CORRELATION BOX 21-11 Candidiasis (*Candida albicans*)

Human candidiasis is most commonly caused by *Candida albicans*. The rapid germination in tissues after seeding from the bloodstream; the production of proteases, adhesins to extracellular matrix proteins, and complement-binding

receptors; and phenotypic switching are the most important virulence factors contributing to its increased infectivity.[57,202] The history, taxonomy, epidemiology, and virulence factors for *Candida albicans* have been reviewed by McCullough[182] and by Pfaller.[219] The clinical manifestations of *Candida albicans* are primarily of three types: mucocutaneous, cutaneous, and systemic.

Mucocutaneous Candidiasis

Candidiasis of the mucous membranes most commonly involves the oral cavity and the vaginal canal. Oral candidiasis, a condition known as thrush (a term derived from the Scandinavian word trosk), is one of the most common clinical manifestation of candidiasis in humans. The infection is manifested as white patches or plaques on the buccal mucosa and tongue,[20] which, in more serious infections, may coalesce into a membrane. These adhere firmly to the epithelium, revealing a reddened, edematous base when removed. The diagnosis can be made by observing microscopically the characteristic pseudohyphae and blastoconidia in Gram-stained preparations of

smears prepared from some of the exudate (Fig. 21-89). Alteration of the normal flora after prolonged antibiotic therapy, low pH of the salivary secretions in newborns, hypertrophy of the papillae of the tongue ("black hairy tongue"), and chronic glossitis are predisposing causes.[25] Oral candidiasis is now recognized as an AIDS-defining condition, seen in virtually 100% of patients with AIDS.[270]

Although classically caused by *Candida albicans*, the closely related species, *Candida dubliniensis*, may also cause thrush.[180,203,267] The mucous membranes of the trachea and bronchi, and virtually any part of the alimentary canal may harbor *Candida* infections, most commonly representing extensions of oropharyngeal disease. Cases of esophagitis, gastritis, enteritis, and perianal disease have been reported. The gastroesophageal junction in particular is a common site for *Candida* infections. A low-pH environment may explain this predisposition, particularly in patients with hematologic malignancy. Dysphagia, retrosternal pain, upper gastrointestinal bleeding, and nausea are associated symptoms. Esophageal candidiasis may also occur as an extension of oropharyngeal thrush, particularly in newborns.

Cutaneous Candidiasis

Infections of the skin commonly involve the moist, intertriginous areas, such as in the webs of the fingers and toes, beneath the female breasts, in the armpits, and in the folds of the groin. Infection of the nails proper is known as onychomycosis, or paronychia if the folds of skin encasing the nails are involved. Diaper rash infection of neonates is also a common manifestation. Chronic mucocutaneous candidiasis is an opportunistic infection of skin and mucous membranes associated with several genetic defects involving compromised leukocyte function, or of the endocrine

system. Thymic dysplasia with and without hypogammaglobulinemia, hypoparathyroidism, and chronic granulomatous disease, the latter involving a myeloperoxidase defect of phagocytes precluding post-phagocytic killing of yeast forms, are among the predisposing conditions.[235]

Disseminated Candidiasis

Systemic candidiasis is a relatively rare condition, occurring primarily as a terminal event in patients with debilitating, neoplastic (e.g., blast crisis of leukemia), immunosuppressive diseases and following organ transplantation, particularly during an acute rejection syndrome. The conditions underlying hematogenous candidiasis is reviewed by Abi-Said.[1]

Candidiasis of the Urinary Tract

This presentation is relatively rare, manifesting as cystitis (more commonly caused by *Candida glabrata*) and pyelonephritis, either ascending from a bladder infection or from hematogenous spread from a distant primary site of infection.

Aggregates of pseudohyphae and blastoconidia may be seen histologically in glomeruli, presumably in a microenvironment conducive to growth because of the lowered pH from Na^+ and H^+ ion exchanges. Currently, *Candida glabrata* ranks second or third as the causative agent of superficial (oral, esophageal, vaginal, or urinary) or systemic *Candida* infections, as reported by Fidel et al.[88] The emergence of *C. glabrata* as a nosocomial pathogen may be related to partial or complete resistance to fluconazole, which has been effectively used in the treatment of other yeast infections. Zmierczak et al.[310] report a patient with chronic, recurrent *C. glabrata* arthritis initially involving the right ankle. One year after successful treatment, the infection recurred in the left knee; this was thought to represent hematogenous dissemination.

Fungemia and Endocarditis

Candida albicans is the most common cause of fungemia, accounting for usually somewhere between 50% and 60% of isolates. The remainders are predominant non-*albicans Candida* species, with the most frequent among these being *C. glabrata.* Most instances of fungemia are associated with an intravascular access device. *Candida* endocarditis is most often seen in people with preexisting valvular disease, particularly following episodes of septicemia associated with the use of indwelling catheters, prolonged intravenous infusions, and IV drug abuse. Hogevik and Alestig[128] present a review of seven patients with endocarditis occurring in western Sweden. In four patients, the infections were associated with prosthetic-valve placements; in three patients the native valves were involved. Because of high mortality, the authors stress the need for early diagnosis, immediate antifungal therapy, and emergency surgery if sonography reveals a lack of response.

Most strains of *C. albicans* can be recovered from blood cultures in most

commercially available blood culture bottles, Marcelis et al.[178] report that culture media containing resin (specifically, BACTEC PLUS high-blood-volume resin was used in their study) may enhance recovery, particularly in patients receiving antifungal agents.

Candida Meningitis

This rare condition is secondary to dissemination from sites of infection in the gastrointestinal or respiratory tracts, from septic emboli released from infected heart valves, from trauma, or as a complication of neurosurgery. In a retrospective review of 21 patients with *Candida* species isolated from cerebrospinal fluid following neurosurgery, Geers and Gordon[97] found that 86% of these patients had indwelling cerebrospinal devices (shunts). Gelfand et al.[98]reported instances of superinfection following acute bacterial meningitis in adults who

incurred trauma to the central nervous system or surgical intervention. They counsel that any patient with bacterial meningitis who does not improve with antimicrobial chemotherapy should be examined for *Candida* superinfection, particularly patients with indwelling catheters.

CLINICAL CORRELATION BOX 21-12 Infections with *Candida* Species, Non- *albicans*

Species of *Candida* other than *C. albicans* are also normal microbiota of cutaneous and mucocutaneous surfaces. Wingard[301] found in a review of 1,591 *Candida* infections published in 37 reports showed that non- *Candida albicans* species were the causative agents in 46% of systemic infections. *Candida tropicalis* accounted for 25% of the infections, *C. glabrata* for 8%, *C. parapsilosis* for 7%, and *C. krusei* for 4%. From these reports, patients with leukemia were more likely to be infected with *C. albicans* or *C. tropicalis*, whereas bone marrow transplant recipients were more likely to be infected with *C. krusei* or *C. lusitaniae*.

In a similar study, Wright and Wenzel[305] found that *Candida albicans* was recovered from 58% of nosocomial infections, *C. tropicalis* in 25%, *C. parapsilosis* in 15%, *C. glabrata* in 6%, and *C. lusitaniae* in 2%. *C. tropicalis* and *C. krusei* were more frequently recovered from patients with neutropenia who had lymphoma or leukemia, *C. parapsilosis* from neonates receiving hyperalimentation fluids, and *C.*

glabrata in postsurgical patients who had solid tumors removed. The non- *albicans Candida* species are commonly encountered, so it is important that laboratories maintain competency in the ability to accurately identify these isolates.[52]

In an extensive review of new and emerging yeast pathogens, Hazen evaluated what at the time were considered 30 emerging yeast pathogens with 168 references.[123] Although among the non- *C. albicans* species, *C. parapsilosis*, *C. glabrata* (particularly isolates in blood cultures), *C. krusei*, *C. guilliermondii*, *C. lipolytica*, and *C. kefyr* (formerly *C. pseudotropicalis*) were on the increase, Hazen also mentions the emergence and significant increase in incidence of *Malassezia*, *Rhodotorula*, *Wickerhamomyces (Hansenula/Pichia) anomalus,* and *Trichosporon* species as non- *Candida* species isolates from clinical materials, with several citations to specific case reports of infections caused by these agents.

Several causes have been proposed for the sudden emergence of species of yeasts as agents of invasive infections. Included are the use of broad-spectrum antibiotics and antineoplastic agents, the widespread administration of vancomycin, intravenous catheterization, and the increased number of patients with neutropenia and immunosuppression. The widespread use of fluconazole may account for the relative decrease in recovery of *C. albicans* from blood cultures relative to other *Candida* species.

Cross-contamination by hospital personnel may also account for increases in yeast infections in certain environments. For example, a survey of hospital personnel by Strausbaugh et al.[265] revealed that 70% of nurses and non-nursing hospital personnel carried yeasts on their hands, with *Rhodotorula* spp. and *C. parapsilosis* being the most frequently recovered. In this regard, newborn infants, who are intimately handled by nursing personnel, represent another population that is susceptible to candidiasis.[145] See Weems for a full review of the clinical pathology of *C. parapsilosis*.[285]

Display 21-7 provides a guide to the laboratory identification of these yeasts. Most clinically significant yeast isolates grow within 36 to 72 hours in culture on sheep blood agar, potato dextrose, and most other nonselective primary isolation media. The colonies are typically white or yellow-white with a smooth or pasty consistency (Image Plate 21-6A). When an isolate presents with colonies consistent with one of the monomorphic yeasts, traditionally the first step is to determine whether the isolate has the ability to produce a germ tube. If a germ tube is produced in the germ tube test, then a presumptive identification of *C. albicans* can be reported, and further workup is usually not necessary. It is acknowledged that *C. dubliniensis* can also produce a germ tube. If one desires to distinguish between *C. albicans* and *C. dubliniensis*, then additional testing is necessary, but in most instances germ tube producing isolates are simply reported as *C. albicans.*

Germ Tube. A germ tube is defined as a filamentous extension from a yeast cell that is about half the width and three to four times the length of the parent cell (Fig. 21-83). The true germ tube produced by *C. albicans* has no constriction at the neck (i.e., the base wherein the hyphal extension connects with the parent cell). The germ tube is the initial phase of true hyphal production and does not have the constriction at the base characteristic of pseudohyphae. *C. tropicalis* is known to produce a pseudogerm tube, which can be mistaken for a germ tube, if attention is not given to base of the hyphal extension. A description of the germ tube procedure is included in Box 21-6. As mentioned before, a presumptive identification of *C. albicans* can be made if germ tubes are produced. Not all strains of *C. albicans* produce germ tubes; therefore, if a germ tube is not produced, then the culture should be submitted for biochemical testing and inoculated to a cornmeal agar plate. At the end of the incubation period, the cornmeal preparation is observed microscopically for the presence of chlamydospores and regularly spaced blastoconidia, which, if present, also allow for the presumptive identification of *C. albicans* (Fig. 21-84).

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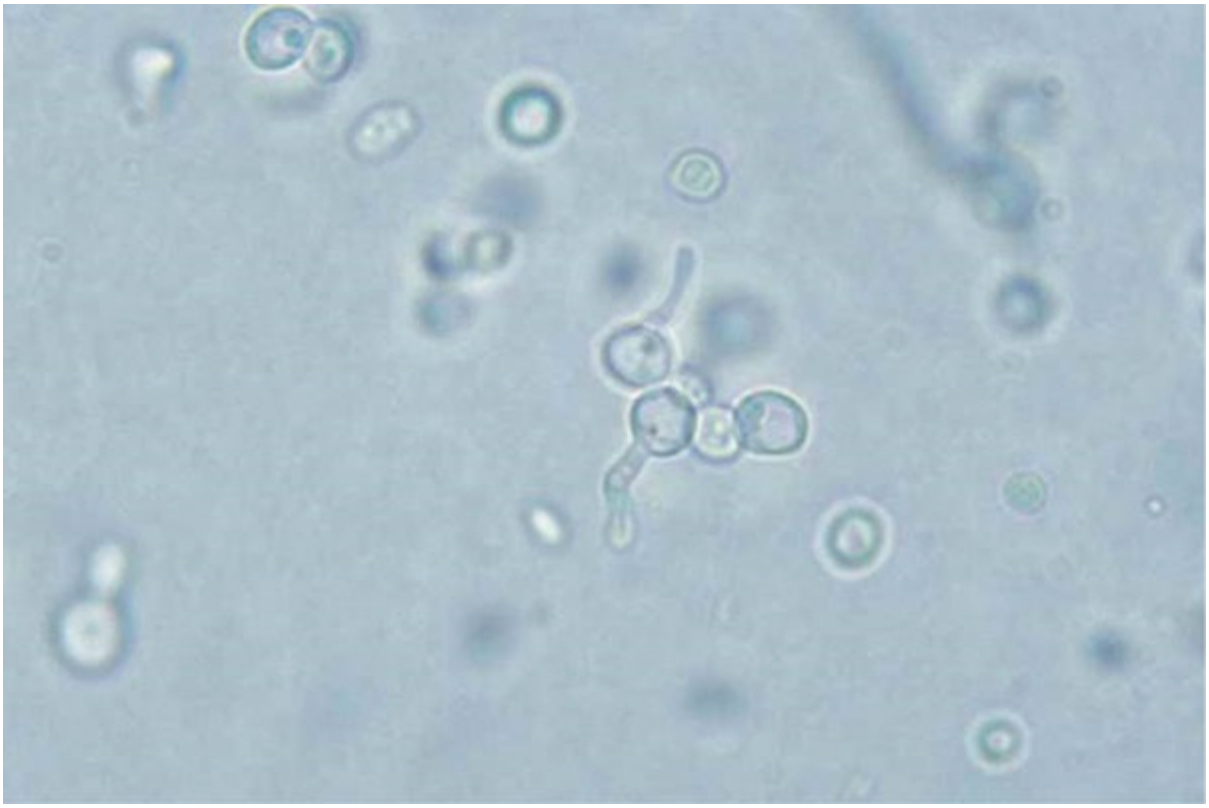
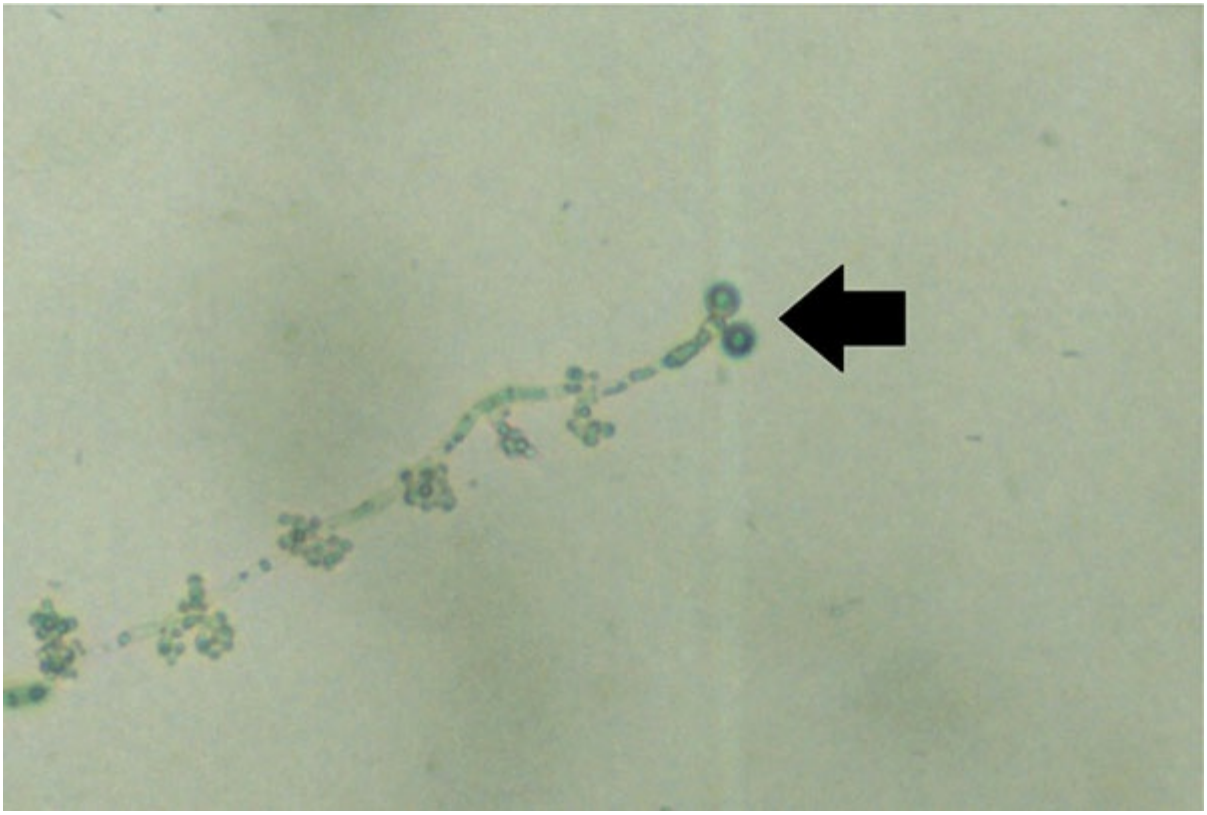


■ **FIGURE 21-83** Photomicrograph of a germ tube, characteristic of *Candida albicans* (oil immersion).

BOX 21-6

Procedure: Germ Tube Test

1. A small portion of an isolate colony of the yeast to be tested is suspended in a test tube containing 0.5 mL of rabbit or human plasma or serum.
2. The test tube is inoculated at 35°C for no longer than 2 hours.
3. A drop of the yeast-serum suspension is placed on a microscope slide, overlaid with a coverslip, and examined microscopically for the presence of germ tubes (Fig. 21-83).
4. The test is not valid if examined after 2 hours.



■ **FIGURE 21-84** This cornmeal preparation of *C. albicans* demonstrates chlamydospores *(arrow)* and regular clustering of blastoconidia.

Cornmeal Agar Preparations. The preparation of the cornmeal agar mount is described in Box 21-7.

Again follow Display 21-7. If pseudohyphae and blastoconidia are present, a

presumptive identification of *Candida* species can be made. The different patterns of growth in the cornmeal agar preparations are often sufficiently distinctive to establish presumptive species identification.

BOX 21-7

Procedure for Inoculation of Cornmeal-Tween 80 Agar

1. Make three parallel streaks 1/4 in., or 1 cm, apart into the surface of cornmeal agar, holding the inoculating wire at about a 45-degree angle.
2. Lay a coverslip on the surface of the agar, covering a portion of the inoculated streaks.
3. Incubate the inoculated plates at 30°C for 24–48 hours in a closed, moisturized chamber. At the end of the incubation period, examine microscopically through the coverslip and observe the pattern of growth.

Growth Patterns of Yeasts on Cornmeal Agar. When observing the growth in a cornmeal agar preparation, determine at the onset whether pseudohyphae are present. The observation of pseudohyphae and blastoconidia put an unknown isolate into the genus *Candida*. Most human infections are caused by *C. albicans*. However, with the increase in immunosuppression related to organ transplantation and other related conditions, and the emergence of opportunistic fungal pathogens, the recovery of non-*albicans Candida* species in pure or predominant culture from blood or other sterile body sites cannot be ignored. A careful clinical correlation is necessary to determine whether further workup is necessary. The patterns of growth on cornmeal agar are helpful in making a presumptive identification and in serving as a quality-control check on species identifications provided by the biocodes indicated by automated and kit systems. Because none of the cornmeal agar patterns are diagnostic (with the exception of the chlamydospore production by *C. albicans*), carbohydrate assimilation studies or the results derived from one of the commercial yeast identification systems are necessary before a definitive identification can be reported.

If a presumptive identification cannot be made from the observation of the cornmeal agar preparation, tests for nitrate reduction, urease activity, inositol assimilation, nitrate reduction, and the production of caffeic acid (using a niger seed agar plate or performing a filter paper strip test), in addition to carbohydrate assimilation studies, are helpful in separating these various species. Although these studies can be accomplished by conventional techniques, packaged yeast identification kits or automated systems are currently used in most laboratories. In those rare

instances in which referral to carbohydrate assimilation profiles are required, the reader is referred to Tables 19-8, 19-9, and 19-10 on pages 1045, 1050, and 1054 in the 5th edition of this textbook.

Chromagar. CHROMagar, a chromogenic differential culture medium, is being used in many laboratories to facilitate the isolation and presumptive identification of certain clinically important yeast species, particularly

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C. albicans. Observation of colony morphology and distinctive patterns of color are used to separate yeast species, particularly when observed in mixed cultures. *C. albicans* forms distinctive yellow-green to blue-green colonies.

Ainscough and Kibbler[3] evaluated the cost-effectiveness and time advantage of CHROMagar in comparison with SDA. An overall sensitivity of 95.2% was observed in the study of 21 yeast isolates recovered from 298 clinical samples from patients with neutropenia and those with AIDS. CHROMagar was found in this study to be 100% sensitive and 100% specific for *C. albicans*. It provided the most economical and least time-consuming approach to the initial culture and presumptive identification of yeast isolates. The authors also recommend the direct inoculation for blood cultures when yeast cells are seen on microscopy.

Candida albicans. Two patterns of growth on cornmeal agar are helpful in identifying *C. albicans*: (1) the production of chlamydospores and/or (2) blastoconidia that arrange in dense clusters evenly distributed along the pseudohyphae (Fig. 21-84). A review for these cornmeal agar patterns is particularly helpful in making an identification for strains that are germ tube–negative.

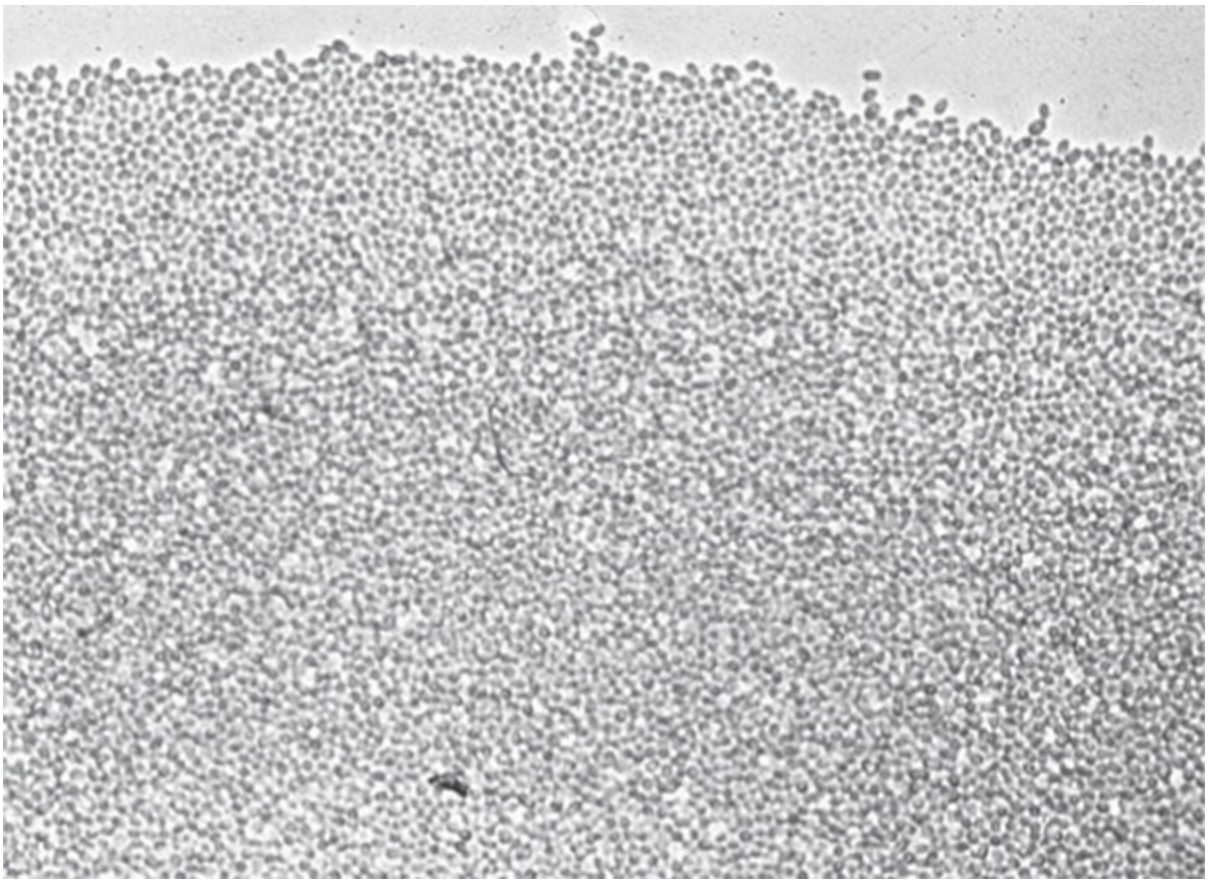
The colony growth of *C. albicans* may also lead to a presumptive identification. The edges of the colonies often present radiating starburst spicules in older cultures (Image Plate 21-6B).

If the germ tube test is negative and chlamydospores are not observed in the cornmeal agar mount, a yeast cell other than *C. albicans* has probably been isolated. Although approximately 5% of *C. albicans* may not produce germ tubes, it would be quite rare to have a strain that also fails to produce chlamydospores. In keeping with Display 21-7, the next step is to observe the patterns of growth on cornmeal agar to presumptively identify other *Candida* spp. To reiterate, it is important to review the patterns of growth on cornmeal agar as a quality-control check on the species identifications provided by automated and kit systems, as they are not always correct. To this end, the classic patterns for these more commonly encountered non- *albicans* *Candida* species are discussed below.

Candida glabrata. *Candida glabrata* is the currently accepted name for this organism. It is an agent of urinary tract infections, comprising about 20% of all yeast isolates from urine specimens.[94] Cases of endocarditis[36] and disseminated infection[125] caused by *C. glabrata* have been reported. *C. glabrata* assimilates both glucose and trehalose, an assimilation pattern that is helpful in making the laboratory identification.

Colonies of *C. glabrata* grow more slowly than other yeasts, often requiring 48 to 72 hours to evolve. Thus, if a slower-growing yeast is recovered on blood agar from a clinical specimen (particularly from the urine), that grows as small, entire, convex, glossy, smooth colonies (Image Plate 21-6 *E*) and that microscopically appears as 2 to 3 mm in diameter, single-budding, regular-sized yeast cells, without the formation of pseudohyphae, *Candida (Torulopsis) glabrata* should be considered. On cornmeal agar preparations, the cells of *C. glabrata* arrange in relatively tight clusters, without the separation or size variation, as seen with *Cryptococcus* spp. (Fig. 21-85).

Candida tropicalis. *Candida tropicalis* produces pseudohyphae with blastoconidia either borne singly or in small irregular clusters along the pseudohyphae at points of constriction (Fig. 21-86). Because this pattern is not specific, carbohydrate assimilation studies or identification through the use of one of the yeast identification systems is necessary.

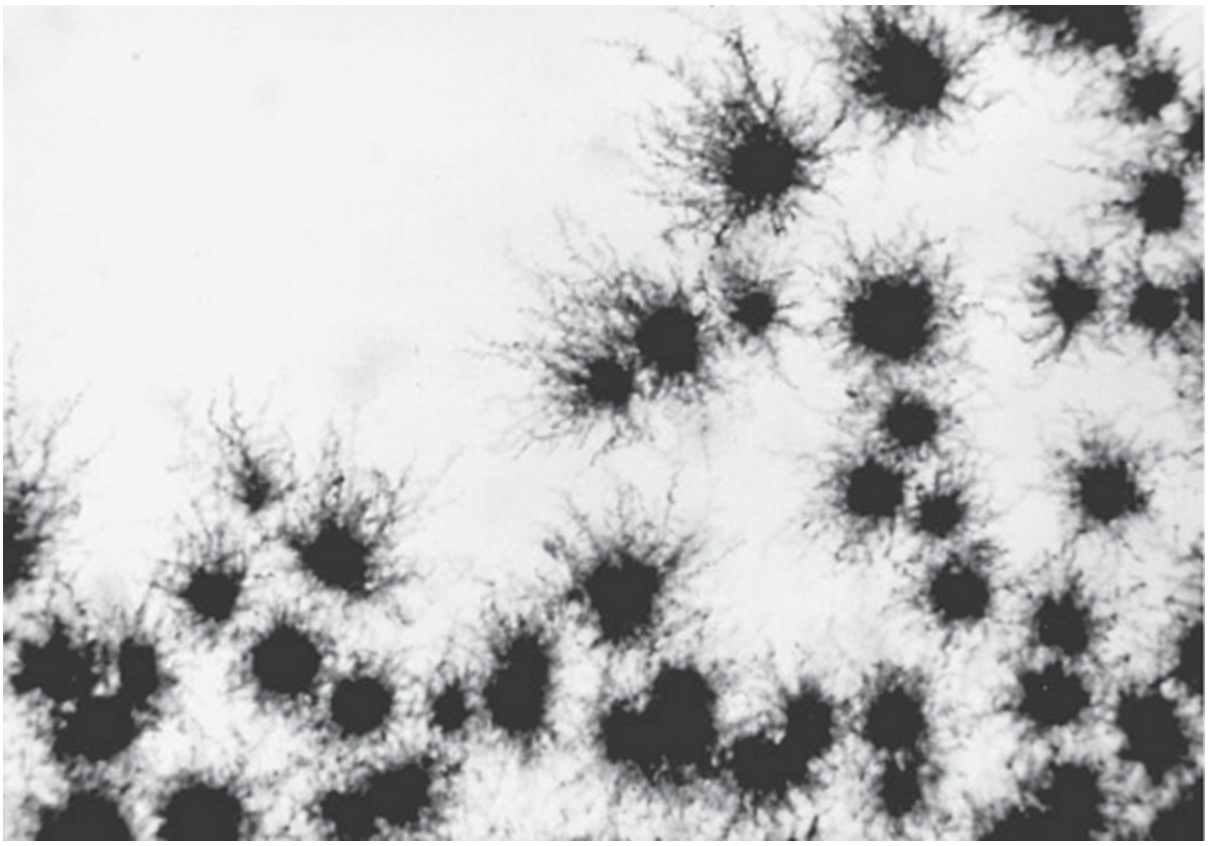


■ **FIGURE 21-85** Photomicrograph of a cornmeal agar preparation of *Candida glabrata,* illustrating tight clusters of tiny, uniform yeast cells not separated by capsular material.

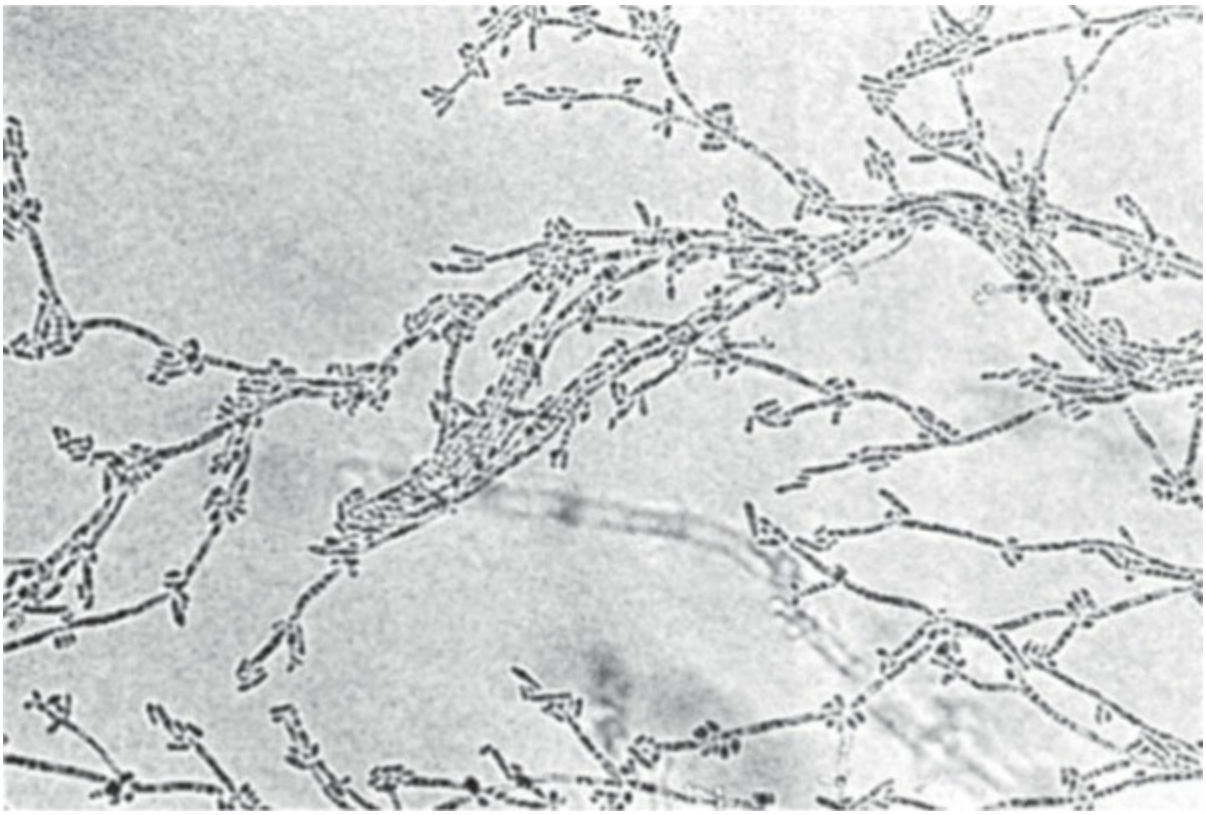
■ **FIGURE 21-86** Photomicrograph of a cornmeal agar preparation of *Candida tropicalis,* illustrating light production of conidia irregularly along the hyphae.

Candida parapsilosis. Key to the presumptive identification of *C. parapsilosis* in cornmeal agar preparations is the observation of multiple focal areas of satellite growth adjacent to the streak lines, forming what has been colloquially referred to as a "sagebrush" pattern (Fig. 21-87). Once observed, this pattern will consistently match the identification codes obtained from commercial systems.

Candida kefyr. Key here is the observation of the abundant production of elongated to rectangular blastoconidia that form distinct loose, criss-cross clusters, likened to the arrangement of "logs in a stream" (Fig. 21-88). *C. krusei* also produces a similar pattern, except that the points of derivation are sequentially clustered more as branches from a tree. Carbohydrate assimilation studies may be required to differentiate these two species.



■ **FIGURE 21-87** Photomicrograph of a cornmeal agar preparation of *Candida parapsilosis,* illustrating the characteristic formation of satelliting "spider or sagebrush colonies" along the streak line.



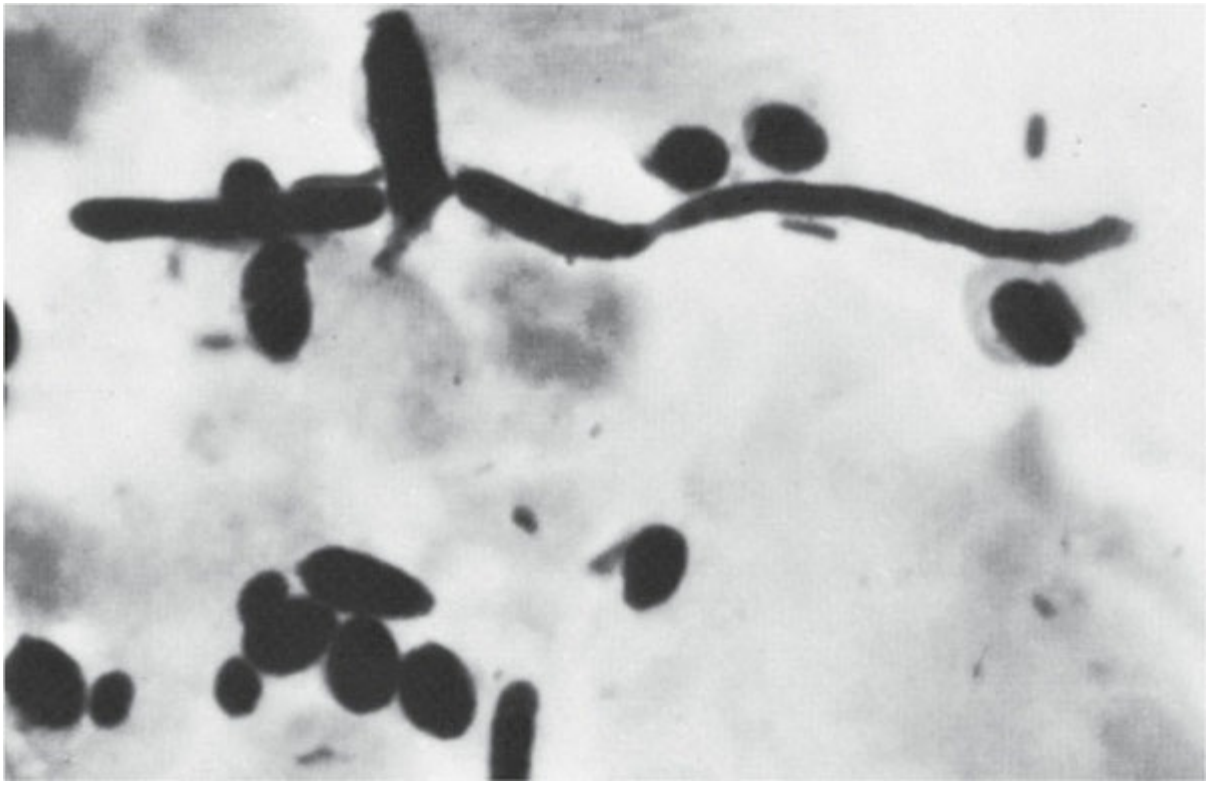
■ **FIGURE 21-88** Photomicrograph of a cornmeal agar preparation illustrating the "log-in-stream" arrangement of elongated blastoconidia characteristic of *Candida kefyr*.

Other Emerging Pathogenic *Candida* Species. The definitive identification of non- *albicans Candida* species is important to detect emerging pathogenic strains, particularly those that have acquired resistance to antifungal drugs.[202] *Candida krusei* has known resistance to fluconazole.[301] *Candida dubliniensis*, phenotypically closely related to *C. albicans*, particularly those strains recovered from patients with oropharyngeal thrush, may have acquired induced resistance to fluconazole.[180] It is unclear if these mechanisms of resistance are acquired because selective pressures or innate, because when large numbers of *C. dubliniensis* isolates have been tested, the resistance profile is similar to that of *C. albicans*. The following characteristics aid in distinguishing *C. dubliniensis* from *C. albicans*.

- The inability to grow at 45°C
- The more abundant production of chlamydospores (see Fig. 21-89).
- Growth characteristics on specialized media.
- Carbohydrate assimilation profile
- DNA sequence or MALDI-TOF pattern
- Germ tube positive, but negative result using the *Candida albicans* PNA FISH

tests.

Sporadic amphotericin B resistance has evolved in certain lineages of *Candida lusitaniae* through a mechanism of switching from previously susceptible colonies.[306] Again, it is unclear if this resistance is acquired or inducible, since most *C. lusitaniae* isolates test susceptible to amphotericin B.



■ **FIGURE 21-89** Photomicrograph of Gram-stained sputum, showing pseudohyphae and budding blastoconidia characteristic of *Candida* species (Gram's stain, 1,000×).

Species That Fail to Produce True Hyphae

Isolates that fail to form hyphae on cornmeal agar include *Cryptococcus* spp., *Candida glabrata, Rhodotorula* spp., and *Saccharomyces* spp. The yeast cells of *Cryptococcus* spp. are spherical, vary in size, and are widely separated by capsular material. The yeast cells of *Candida glabrata* are smaller, regular in size, and tend to arrange in compact clusters. The genus characteristics will be discussed in more detail below, along with the differentiating features of other less commonly encountered genera, such as *Rhodotorula*, *Saccharomyces*, *"Wickerhamomyces* (*Hansenula/Pichia*) *anomalus*, and *Malassezia*. Occasional strains of *Candida* spp. may produce only rudimentary pseudohyphae or none at all; therefore, cornmeal agar

preparations should be set up for any colony that is suspicious for *Candida* spp.

***Cryptococcus neoformans* and Cryptococcosis.** The recovery of *Cryptococcus neoformans* from clinical specimens must always be considered significant until proven otherwise. Until the onset of the AIDS epidemic early in the 1980s, the incidence of cryptococcosis in the United States was low (about 300 cases per year). Most of these early cases occurred in patients with cellular immunodeficiencies, particularly in individuals with Hodgkin's disease.[156] Currently, *C. neoformans* is one of a select group of microorganisms causing infection in patients with AIDS, occurring in 6% to 13% of cases, depending on the place of residence.[190] The incidence of cryptococcosis in patients with AIDS is as high as 15% or greater in some locales in Sub-Saharan Africa.[188]

Of the cryptococci, *Cryptococcus neoformans* is responsible for most human infections. This species is an encapsulated yeast with its natural habitat in the soil. Soil contaminated with pigeon, chicken, or turkey droppings, in which the pH is alkaline and the nitrogen concentration is increased, in particular promotes organism replication. Yeast

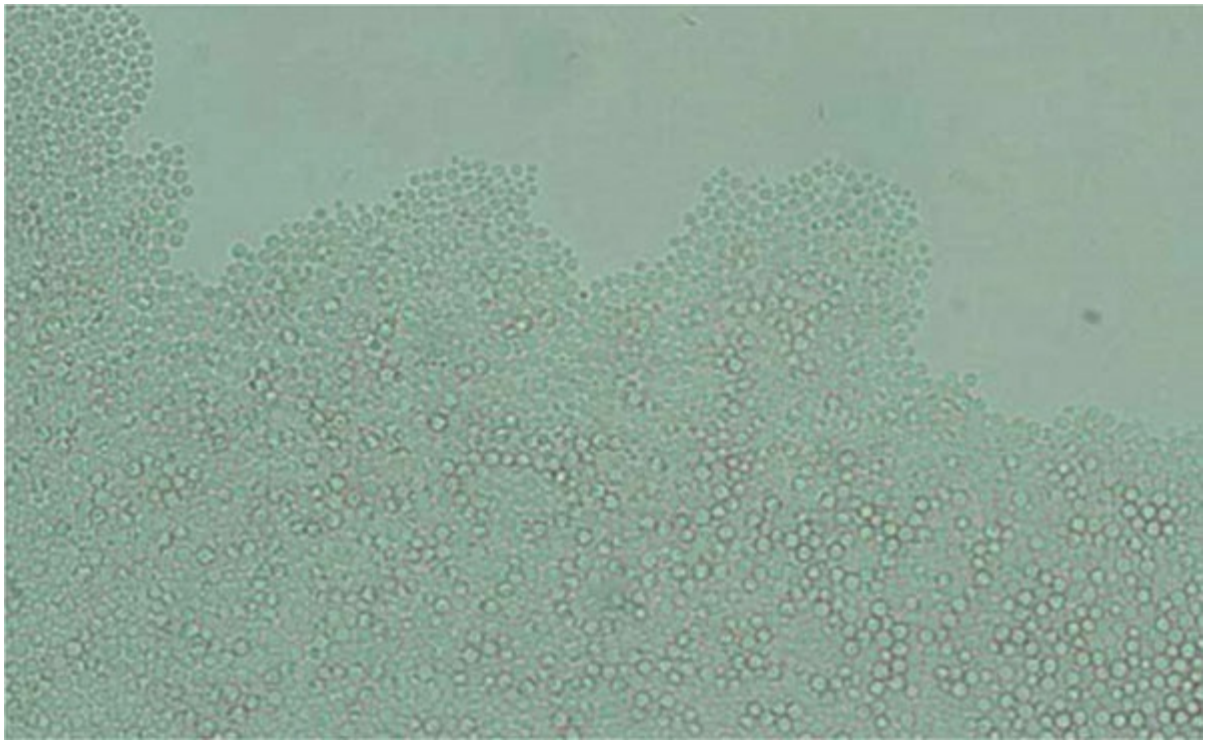
cells may become airborne in clouds of dust created during sweeping, cleaning, and excavating projects. Therefore, poultry farm workers, groundskeepers of city parks frequented by starlings and other birds, and spelunkers, who may explore bat-infested caves, are particularly at risk for acquiring infection. Persons who are immunosuppressed, particularly those with AIDS, are advised not to participate in these activities. In cases in which an immediate exposure is not evident, the onset of acute cryptococcosis may represent unmasking of latent infections.

Capsule synthesis is a major virulence determinant, providing a mechanism for adherence to mucosal linings and protection from phagocytosis, both during transit through the bloodstream and at the sites of infection.[119] *Cryptococcus neoformans* has a particular tropism for the central nervous system, and many cases initially manifest clinically as meningitis. The production of melanin pigment also is a virulence determinant, protecting the yeast against oxidant-induced damage. The production of proteinases, mannoproteins, and release of polyol metabolites account for the invasion and cell destruction observed at the sites of infection.

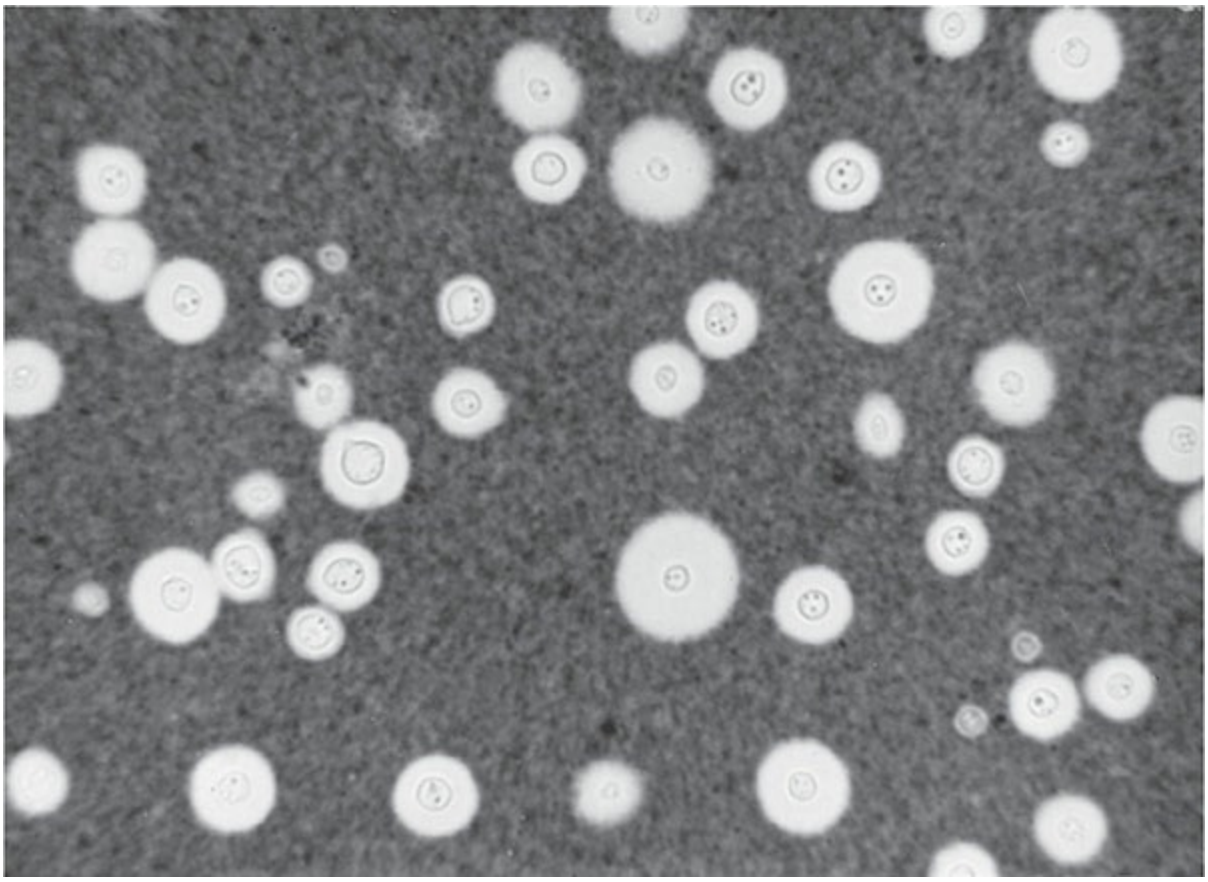
Cryptococcus neoformans can be suspected if a yeast produces colonies that are distinctly mucoid. It should be mentioned, however, that not all strains may produce obvious capsules. Strains with obvious capsules often elicit a more pronounced granulomatous reaction, as observed in tissue sections, in contrast to the liquefaction necrosis that is more commonly associated with invasion by poorly encapsulated

strains. Growth is usually observed within 36 to 72 hours on 5% sheep blood agar and most other primary isolation media (Image Plate 21-6).

Microscopically, the identification of *Cryptococcus* species can be made by demonstrating irregularly sized (4 to 10 µm), spherical yeast cells surrounded by a thick polysaccharide capsule (cells may be as much as 20 µm or more in diameter if the capsule is included in the measurement). In cornmeal agar preparations, pseudohyphae are not produced; irregular-sized, spherical yeast cells are separated one from the other by spaces produced by the capsular material (Fig. 21-90). Encapsulated yeast cells, often with a single bud attached by a hairlike strand, may also be seen in India ink preparations (Fig. 21-91). Because of its insensitivity, the India ink procedure is being performed less commonly on cerebrospinal fluid specimens submitted to clinical laboratories, giving way to the more sensitive cryptococcal antigen-detection tests.



■ **FIGURE 21-90** Photomicrograph of a cornmeal agar preparation of *Cryptococcus neoformans,* illustrating the irregular-sized, spherical yeast cells separated by polysaccharide material. Some mycologists feel that these resemble "glass beads."



■ **FIGURE 21-91** Photomicrograph of an India ink preparation, illustrating the irregular-sized, encapsulated, spherical yeast cells of *Cryptococcus neoformans*.

An isolate suspicious for *C. neoformans* can be confirmed by demonstrating the production of caffeic acid, either through the production of a maroon-red pigment on "bird seed" agar (Image Plate 21-6 *D*) or directly by inoculating a reagent-impregnated filter paper strip with a portion of the unknown colony and observing for the development of a black pigment.

Although other *Cryptococcus* spp. may be recovered from humans, their role as causative agents for infection is uncommon. The clinical presentations of such cases range from skin lesions to fungemia. Most cases of non- *neoformans* fungemia are nosocomially acquired, usually in patients with neutropenia, associated with indwelling intravascular catheters.[138] These authors also report two cases of fungemia caused by *Cryptococcus laurentii*, and warn that most non- *neoformans* cryptococci are more resistant to fluconazole and flucytosine than most *C. neoformans* isolates, thereby requiring a species identification in clinically relevant cases.

Diagnosis by Nonculture Methods. Antigen-detection assays on cerebrospinal fluid specimens are most commonly used to establish the diagnosis of cryptococcal meningitis, largely replacing the time-honored but less sensitive India ink technique.

The capsular material serves as the reactant, resulting in the agglutination reaction for the reagents used in these assays.[91] The latex agglutination assays currently available from several manufacturers are reliable, easy to perform, and sufficiently sensitive and specific to be implemented widely in most clinical mycology laboratories. Special equipment is not required, and the test procedure is easy to master. False-positive reactions can occur secondary to the use of inappropriate specimen transport containers and contaminating disinfectants or soaps.[23] Syneresis fluid from agar plates may contaminate inoculating needles during colony sampling, leading to false-positive results, so the use of these assays for the identification of isolates is not recommended. Additional clinical information on cryptococcosis is presented in Clinical Correlation Box 21-13.

***Rhodotorula* Species.** *Rhodotorula* spp. are environmental fungi that may become part of the human microbiota and have been recovered from the urine and feces. Recovery from respiratory secretions is usually of little significance.

CLINICAL CORRELATION BOX 21-13 Cryptococcosis

Cryptococcosis occurs in both immunocompetent and immunocompromised patients. Most immunocompetent patients may never know they have been infected, unless the nodular cryptococcoma is discovered on chest X-ray. Immunocompromised patients cannot mount the granulomatous response to contain the infection and dissemination via the blood to the central nervous system occurs causing meningitis or meningoencephalitis.[138] Following are the several clinical manifestations of cryptococcosis. Cryptococcosis is an AIDS-defining illness, and HIV testing should be considered whenever *C. neoformans* has been recovered from clinical specimens or when antigen-detection tests are positive.

Although rare, non- *neoformans Cryptococcus* species may also serve as agents of fungemia and disseminated disease.[138] More recently, *Cryptococcus gattii* has emerged as a cause of cryptococcal meningitis in immunocompetent hosts.

Central Nervous System

Central nervous system disease may occur with or without evidence of disease elsewhere. The onset of symptoms may be insidious, with mild headaches, memory lapses, or personality changes being the only clues. Low-grade fever may also be present. As the disease progresses, signs and symptoms suggestive of meningitis slowly develop—namely, nuchal rigidity, tenderness of the neck, and positive knee

and leg flexion tests (Brudzinki's and Kernig's signs). In cases of localized cryptococcal granulomas, signs and symptoms such as paralysis, hemiparesis, and Jacksonian seizures may be evident. Blurring of vision, diplopia, ophthalmoplegia, slurred speech, double vision, and unsteadiness of gait are usually manifestations of an expanding lesion (cryptococcoma). Papilledema is generally a sign of increased intracranial pressure. Weight loss, malaise, persistence of fever, nausea, vomiting, and dizziness may be experienced as the disease progresses. In progressive fulminant or fatal cases, mental changes may be marked (agitation, irritability, confusion, hallucinations, psychosis), developing into delirium, coma, and finally death. Cerebrospinal fluid examination may reveal an increase in lymphocytes, low glucose levels, and elevated protein. Spontaneous onset of CNS cryptococcosis is an AIDS-defining illness. Paradoxically, because of the immunosuppression induced by the HIV in patients with AIDS, there may be a very large number of yeasts in the CSF, but relatively minimal signs and symptoms. This is because the inflammatory response is largely responsible for the signs and symptoms.

Pulmonary Cryptococcosis

Cryptococcus is thought to gain entry into the body via the lungs, although cryptococcal pneumonia is quite rare compared with cryptococcal meningitis. Nevertheless, localized or generalized pneumonia may be seen. Sweeney et al.[268] report the findings of a 10-year-old boy with a malignant fibrous histiocytoma of bone and pulmonary nodule that was found to be secondary to *C. neoformans* infection. This diagnosis was suggested by the appearance of a pulmonary lesion on a computerized tomographic study. Cryptococcal pulmonary disease is often recognized as a result of imaging done for other reasons. Zhu et al.[308] also report four cases of pulmonary cryptococcosis in non-HIV-infected patients, associated with cryptococcal meningitis. Again, all four patients had no apparent symptoms of pulmonary disease.

The natural history, clinical presentation, diagnosis, and therapy of primary pulmonary cryptococcosis have been elucidated in a review of 41 patients by Kerking et al.[143] This group was able to establish the diagnosis when an abnormal chest roentgenogram was associated with the isolation of *C. neoformans* from respiratory secretions and/or the observation of mucicarmine-positive, typical-appearing organisms in sections of lung tissue. The majority of the patients in their series had underlying conditions predisposing to cryptococcal infection: immunosuppressive therapy (28/41), diabetes mellitus (20/41), hematologic or lymphoreticular malignancies (12/41), recent renal transplantation (10/41), and connective tissue diseases or other underlying illness (5/41). Seven of the patients had no detectable underlying abnormalities. Constitutional symptoms, such as fever and malaise, occurred in over half the patients. Chest pain, dyspnea, weight loss, and night sweats in varying combinations were observed in from one fourth to one third of the patients. Only seven of the patients reported cough. Seven patients were asymptomatic and

studied only because of abnormal roentgenograms. The abnormalities observed on chest X-ray examination included circumscribed mass lesions, alveolar or interstitial infiltrates, abscesses with air–fluid levels or cavitary lesions (7/41), solitary coin lesions, and multiple small rounded opacities.

The majority of patients with pulmonary exposure to *Cryptococcus* will develop a subclinical infection that results in a cryptococcoma. This is evidenced by the fact that the incidence of cryptococcomas is far greater than the incidence of cryptococcal pneumonia that comes to medical attention. The cryptococcoma may be discovered years after the primary infection in radiologic studies that are done for other reasons. Aspiration or excision of the "coin lesion" is necessary to exclude malignancy. Other infectious causes in this clinical and radiological differential diagnosis include a histoplasmoma, coccidioma, and tuberculosis.

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Cutaneous Cryptococcosis

Primary cryptococcal infection of the skin is rare; usually cutaneous disease is a manifestation of systemic infection.[111] Murakawa et al.[190] found that in their institution 5.9% of patients treated with cryptococcal infection and AIDS also had skin lesions. The authors describe a variety of appearances of these lesions—umbilicated papules, nodules, and violaceous plaques, with the former resembling molluscum contagiosum and the latter more reminiscent of Kaposi's sarcoma. In this review, lesions were most commonly seen on the head and neck. A comparison of the clinical manifestations of cutaneous cryptococcosis in the pre-AIDS and AIDS era has been presented by Pema et al.[216] As mentioned, although rare primary skin disease may occur. Hamann et al.[117] reported a 75-year-old man from Australia with cryptococcal cellulitis of the right arm. In retrospect, one wonders if this may have been *C. gattii*, which is a *Cryptococcus* species that is morphologically and biochemically similar to *C. neoformans* and causes disease in immunocompetent hosts.

Disseminated Cryptococcosis

C. neoformans produces capsular material that aids the fungus in surviving phagocytosis by segmented neutrophils and mononuclear phagocytes. These fungi may be distributed widely in the human host. In patients with disseminated disease, virtually any viscera or organ system may be involved. The neurotropic nature of this fungus dictates that there is usually a meningeal component, and as mentioned before, dissemination to the skin is common in such patients. Minamoto and Rosenberg[188] report that cryptococcal prostatitis persists after apparently successful therapy in 29% of men with disseminated disease and serves as a reservoir for relapse. Liu[168] culled from the literature 40 cases of cryptococcal osteomyelitis, which 75% of the time was limited to a single bone. The vertebrae were the most common site.

Sarcoidosis, tuberculosis, and previous steroid therapy were the main predisposing causes of cryptococcosis in these patients. Cryptococcal myositis is only rarely reported, most commonly occurring in patients with HIV infection, but may occur in individuals with immunosuppression from other causes. For example, O'Neill et al.[206] reported myositis in a heart-transplant recipient undergoing immunosuppressive therapy. Primary liver disease may predispose to cryptococcal peritonitis, although infection of the peritoneum is a rare event.[263] Cryptococcal infections of the urinary tract are also uncommon.[261] When *C. neoformans* is recovered from the urinary tract, a search for disseminated disease should be made.

Rhodotorula spp. have been recovered from shower curtains, bathtub grout, and toothbrushes. In the past, infections have been rare; however, currently, *Rhodotorula mucilaginosa* (formerly *R. rubra*) and *R. glutinis* are noted as opportunistic pathogens. Fungemia is most often associated with colonized catheters or contaminated intravenous solutions.[44,146] Septicemia related to indwelling catheters, meningitis, and peritonitis in patients receiving continuous peritoneal dialysis are among the uncommon infections caused by this organism.

The observation of a yeast-like colony with a red or orange-red pigmentation (Image Plate 21-6F) is most likely *Rhodotorula* spp. The microscopic morphology is nondiagnostic. Pseudohyphae are not formed; rather, oval budding yeast cells in irregular, loose clusters are observed. Most strains are rapidly urease-positive. Assimilation studies are most commonly used for species-level differentiation.

***Saccharomyces* Species.** The colonies, appearing within 36 to 48 hours, are gray-white and pasty in appearance, with irregular rugae. The appearance is nonspecific, and carbohydrate assimilation study is the most common method used to confirm the identification. Failure to assimilate cellobiose and xylose is helpful in separating *Saccharomyces* spp. from other biochemically related species.

The microscopic morphology is also not specific. Loose clusters of large, oval, budding yeast forms are observed in cornmeal preparations. When colonies are grown on nutritionally poor ascospore agar, which contains potassium acetate, yeast extract, glucose, water, and distilled water, the identification can be confirmed by observing ascospores. After incubation of an inoculated plate for 7 to 10 days, a smear can be prepared from one of the isolated colonies and stained with an acid-fast stain. Observe for ascospores, which appear as large, thick-walled, spherical cells with a distinct red pigment in an acid-fast preparation (Image Plate 21-6H).

Saccharomyces cerevisiae, the common "baker's" or "brewer's" yeast, usually colonizes the mucous membranes of humans but is not commonly considered pathogenic. Occasional instances of fungemia in immunosuppressed patients have

been reported in the older literature.[45,83,198,272] Oliver et al.[205] describe three bone marrow transplant recipients on a hematology unit in whom invasive *S. cerevisiae* infection developed. Two of the patients died. Genotyping of the invasive and carriage strains demonstrated an indistinguishable strain from patients who had been on the unit at the same time, suggesting cross-infection.

***Wickerhamomyces anomalus* (Formerly *Hansenula anomala*).** A rare isolate in clinical laboratories, *Wickerhamomyces anomalus*, is included here because, in addition to *Saccharomyces* spp., it also produces acid-fast ascospores when observed in an acid-fast smear. The ascospores of *Wickerhamomyces* differ from those of *Saccharomyces* by being flattened on one side rather than spherical, with a distinct outer rim or lip at the flat base, simulating the derby hat of an English bobby (Fig. 21-92). Some of the automated identification instruments will identify this fungus by the anamorphic name *Candida pelliculosa.* Isolated infections have been reported in immunosuppressed patients, commonly associated with

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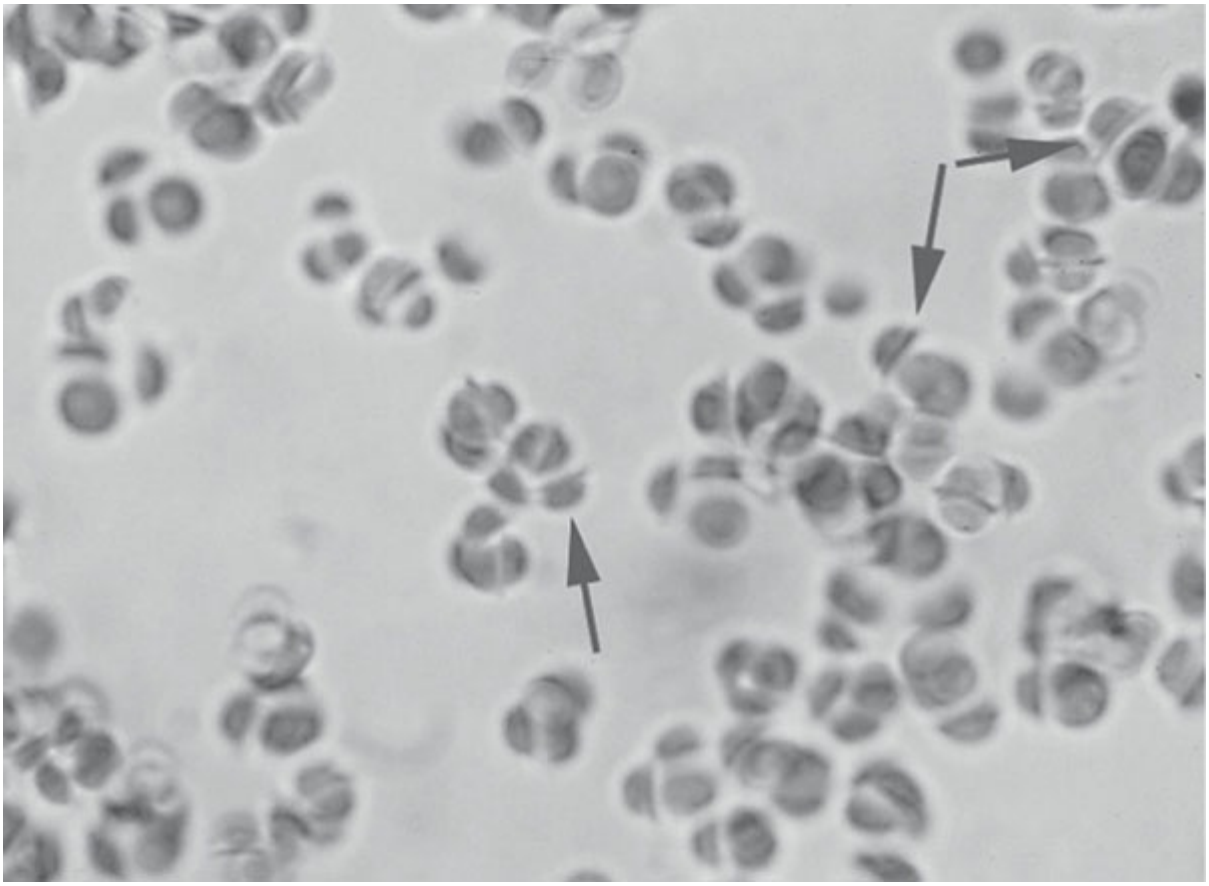


■ **FIGURE 21-92** Photomicrograph of an acid-fast stained preparation, revealing acid-fast yeast cells on *Wickerhamomyces anomalus anomala* (i.e., *Candida pelliculosa*), characteristically shaped like an English bobby hat, complete with brim (*arrows*).

indwelling catheters. Murphy and Buchanan[192] report 52 neonates who were colonized with then called *Hansenula anomala*, some of whom had evidence of systemic infection. Ma et al.[175] report a premature infant with staphylococcal osteomyelitis who was found to have *Wickerhamomyces anomalus* fungemia just before the initiation of the antimicrobial therapy with teicoplanin. The infant was cured after 10 days of antifungal therapy. Kalenic et al.[140]report a recent outbreak of *Wickerhamomyces anomalus* infection in eight adult patients treated at a surgical intensive care unit. The source of the infections and route of transmission were not identified. When searching the medical literature for this or any other fungus for which there have been nomenclature changes, it is important to remember to search under the former and current name, as well as any anamorphic name, such as with this fungus.

***Malassezia* Species.** Recovery in culture of some of these organisms may be difficult because there is a subset of *Malassezia* species that require long-chain fatty acids for growth. When recovery of the organism is anticipated based on the clinical history, a few drops of virgin olive oil should be added to the surface of a sheep blood agar plate immediately after inoculation. In some mycology laboratories, it has been the practice to overlay the agar plates prepared from all blood cultures received from the neonatal intensive care ward because newborns receiving total parenteral nutrition are at high risk for *Malassezia* septicemia.[59] The organisms grow well as small yeast colonies underneath the oil droplets (Image Plate 21-6G). Microscopically, the organism from the plate appears as small budding yeast cells lying singly and in loose clusters. The budding yeast often has a "bowling pin" morphology, and in many instances a "collarette" is discernible at the base of the bud.

In the past, if the *Malassezia* isolate was lipophilic, it was called *M. furfur,* whereas it was called *M. pachydermatis* if it was not lipophilic. However, with the advent of DNA sequencing, it was discovered that there were actually a number of species within each of these groups. For simplicity and for clinical identification purposes, since there are no differences in therapy based on the precise species, the lipid-dependent species are referred to as the *M. furfur "* complex," whereas the lipid-independent species are referred to as the *M. pachydermatis* "complex."

Members of the *Malassezia furfur* complex are lipophilic yeasts that are a common commensal of the skin. This organism causes a superficial cutaneous infection, tinea versicolor. Tinea versicolor presents as scaling lesions of the skin, primarily of the thorax and back, and at times extending to the neck, face, and arms. The lesions are

flat, irregular in outline, and may have a tan or brown pigmentation, but often appear as white blotches of discoloration, particularly in skin exposed to sunlight. The fungal elements in these lesions produce a brick-red fluorescence when examined with long-wavelength ultraviolet light (Wood's lamp). Direct examination of skin scrapings reveal hyphal elements, and 3 to 5 μm diameter, spherical budding yeast cells (colloquially referred to as "spaghetti and meatballs"), with the formation of a distinct collarette at the margin between the mother and daughter cell. Note, the morphology from the lesion is different from that seen from the plate (see above), wherein there is no hyphal element production.

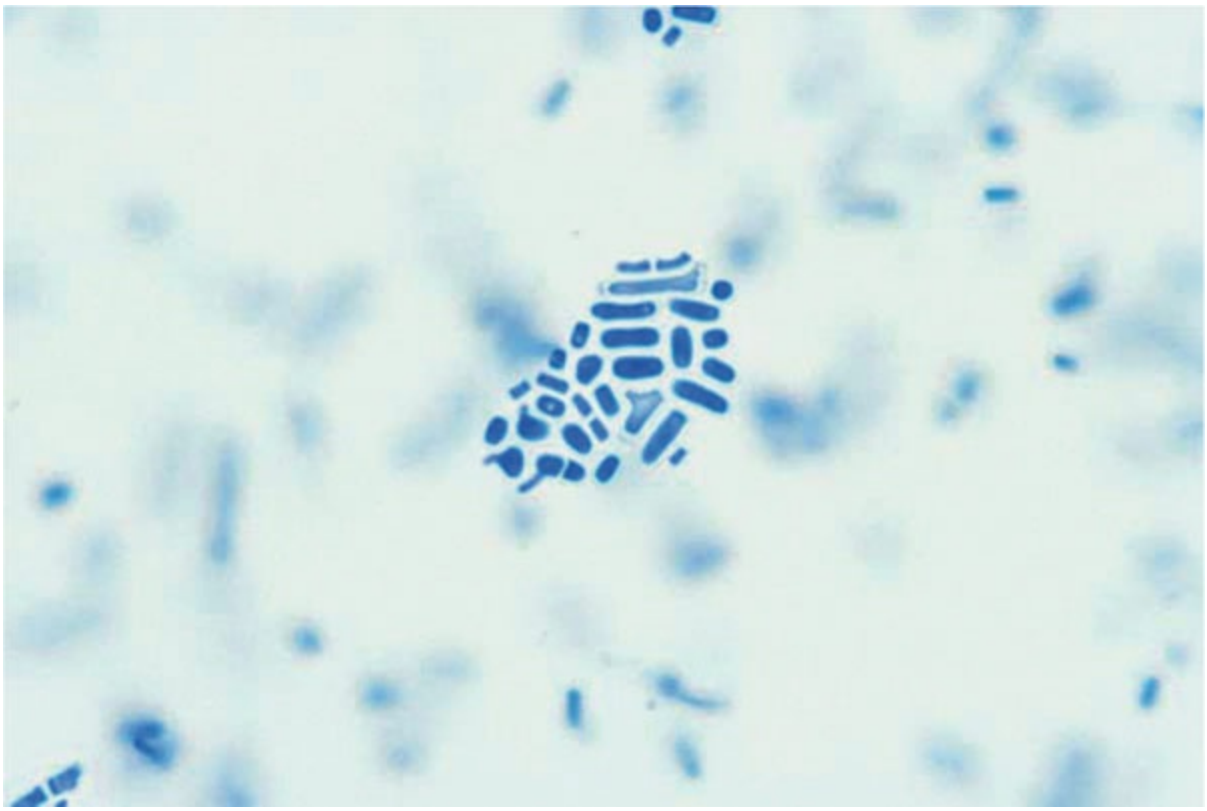
Systemic infections and septicemia have also been reported, most commonly associated with deep-line vascular catheters, particularly in patients receiving long-term hyperalimentation therapy that contains a lipid supplementation. Rosales et al.[238]reported a very-low-birth-weight infant receiving total parenteral nutrition and intralipid fluid infusion into the subarachnoid space, which developed *Malassezia furfur* meningitis. Many of the hyperalimentation emulsions are rich in long-chain fatty acids. This is an ideal microenvironment to support the growth of the endogenous lipophilic organisms that are present on the skin surface. The catheter provides a barrier break in the skin through which the proliferating organisms can enter the bloodstream.

Species That Produce True Hyphae

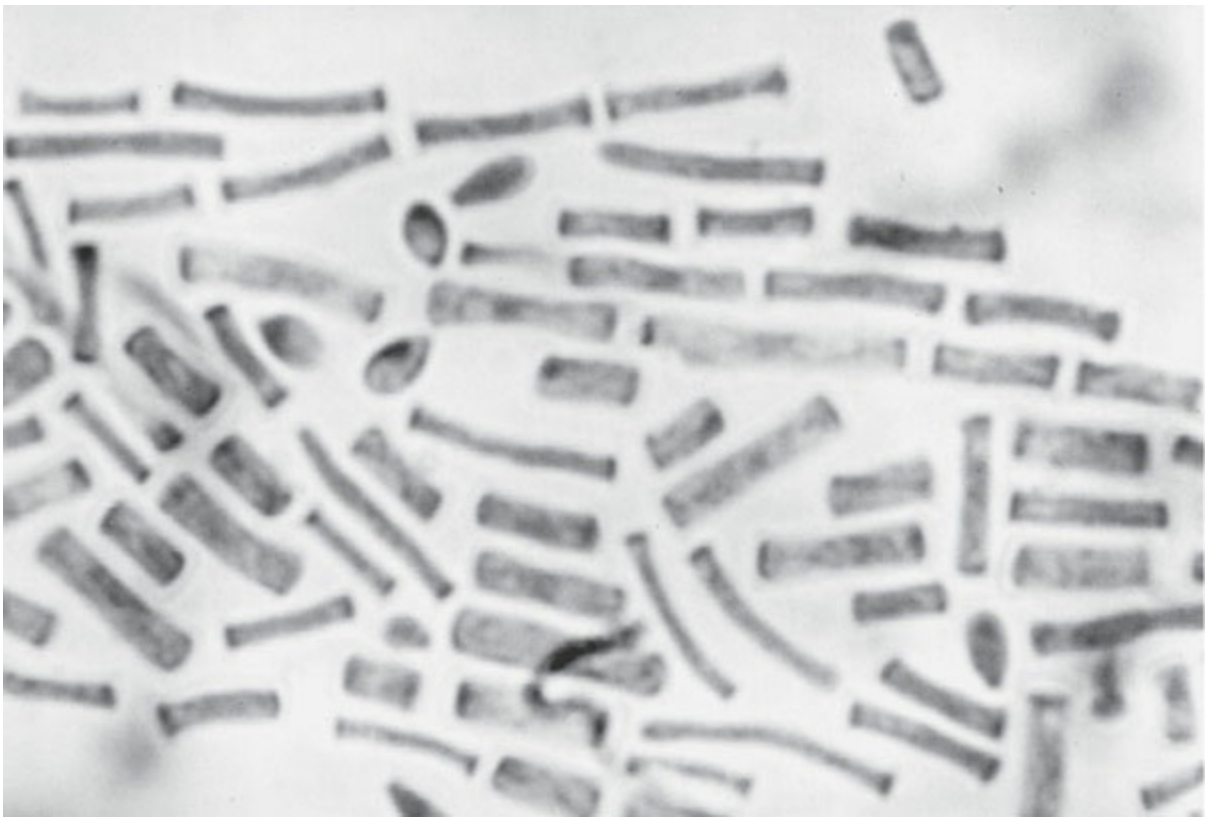
There are three organisms that are often considered together because of morphologic similarities and difficulties in differentiating. They are *Geotrichum candidum, Trichosporon* spp., and *Blastoschizomyces capitatus.* The colonies of these fungi are white-to-cream and initially yeast-like. Soon after isolation, however, they develop a delicate, silk-like surface as true hyphae and arthroconidia on maturity. Morphologic and biochemical features are used for differentiation.

In cornmeal agar mounts, yeasts, pseudohyphae, and true hyphae are produced. The true hyphae become transformed into arthroconidia in some areas. Morphologically, *Geotrichum candidum* often produces a single germ tube from one corner of the arthroconidium, simulating a hockey stick (Fig. 21-93). In contrast, *Trichosporon* spp. typically form blastoconidia from both corners of an arthroconidium, simulating "rabbit ears" (Fig. 21-94). *Blastoschizomyces capitatus* produces clusters of annelloconidia, which are flattened at the point of connection.

Biochemically, *Trichosporon* spp. are urease-positive and often is cycloheximide-resistant, but there is strain variability for the latter test. *Geotrichum candidum* is urease-negative and susceptible to cycloheximide. *Blastoschizomyces capitatus* is resistant to cycloheximide and does not produce urease.



■ **FIGURE 21-93** Photomicrograph of *Geotrichum candidum,* illustrating arthroconidia, a few of which present the characteristic germ-tube-like extension from one corner, simulating a "hockey stick."



■ **FIGURE 21-94** Photomicrograph of a *Trichosporon* species, illustrating arthroconidia, one of which presents the characteristic budding blastoconidia from two corners, simulating "rabbit ears."

The clinical manifestations of infections by these organisms are presented in Clinical Correlation Box 21-14.

Laboratory Identification of Fungi with Both Hyaline and Dematiaceous Components

***Aureobasidium pullulans* and *Hormonema dematioides*.** Both *Aureobasidium* and *Hormonema* grow as smooth, moist colonies tan colonies upon early isolation, which become darker and more mold-like with extended incubation. Microscopically, these produce large, dark, dematiaceous hyphae that often appear as arthroconidia; hyaline septate hyphae are also produced. Myriad small, elliptical, single-celled, hyaline conidia are produced from buds from the centers of individual hyphal segments (Fig. 21-95). Occasional cases of cutaneous phaeohyphomycoses secondary to *Aureobasidium pullulans* have been reported in humans.[240] Rare cases of disseminated nosocomial fungal infection due to *Aureobasidium pullulans* var. *melanigenum* in a severely traumatized patient has been reported by Bolignano and

Criseo.[27] A 50-year-old patient with an infected corneal ulcer by this organism was reported by Gupta and Elewski.[108] In this patient, scleritis developed 5 days after surgery, and the patient became asymptomatic after antifungal therapy, surgical debridement, and cryotherapy. It is acceptable to finalize an isolate as *Aureobasidium/Hormonema* complex, if it becomes difficult to determine a definitive identification, as these are usually contaminants. Differentiation is achieved by denoting synchronous versus asynchronous conidiation, and, more easily determined, if the conidia are produced by the hyaline cells only or by both the hyaline and dematiaceous cells. *Aureobasidium* undergoes synchronous conidiation and the conidia arise only from hyaline cells. In contrast,

CLINICAL CORRELATION BOX 21-14 Arthroconidia-Producing Yeasts and Yeast-Like Fungi

Trichosporonosis

The *Trichosporon* species have undergone significant reclassification based on DNA sequencing. *Trichosporon beigelii* is no longer recognized as a species, but it is important to remember this previous nomenclature when searching the medical literature. There are now many recognized *Trichosporon* species, with *T. asahii, T. mucoides, T. cutaneum, T. inkin,* and *T. ovoides* being the most clinically relevant. The latter three species are important causes of white piedra. This fungus may be carried on the skin, particularly around the anus. White piedra is an infection involving the hair of the scalp, body, or pubic hairs, presenting as small, circumscribed nodules or concretions on the hair shafts. Each nodule contains twisted fungal hyphae, which can be readily observed in direct KOH/calcofluor white preparations, and from which the organism can be readily recovered. The infection can often be eradicated simply by shaving; only rarely is the use of topical or systemic azoles necessary to eradicate the infection.

Disseminated disease, most likely endogenous in origin, representing extensions from colonized cutaneous or gastrointestinal sites, may develop in persons who are immunosuppressed, and become life-threatening in some cases.[82] Hoy et al.[130]reviewed 19 patients with disseminated *Trichosporon* (called *T. beigelii,* but likely represented *T. asahii*) infections in patients with a variety of neoplastic diseases being treated at M.D. Anderson Hospital in Houston over a 10-year period. A nonspecific febrile illness or pneumonia was the most common clinical

manifestation among this group of patients. Three fourths of these patients died; in relation to the discussion elsewhere, the diagnosis was not suspected prior to death in 25% of the autopsied cases. Other *Trichosporon* species, may cause invasive infection, particularly in patients with hematologic malignancy.[142]

The report by Sweet and Reed[269] indicates that neonates are not immune to disseminated disease. Yoss et al.[307] found that low-birth-weight infants in particular were susceptible, a situation of great concern because most infections in this group have been fatal.

Unfortunately, in many instances the diagnosis is often delayed either because the condition may not be recognized or because the organism is not quickly identified in the laboratory. It is important that microbiologists recognize the characteristic arthroconidia in cornmeal mounts as the initial clue to the identification and perform appropriate confirmatory tests.

Trichosporon asahii is rapidly urease-positive and quite saccharolytic, producing acid from most carbohydrates. As a precautionary note, *Trichosporon* spp. share antigenic determinants with *Cryptococcus neoformans*; therefore, serum and cerebrospinal fluid from patients with disseminated disease may give a false-positive reaction with the cryptococcal latex agglutination test.

Geotrichosis

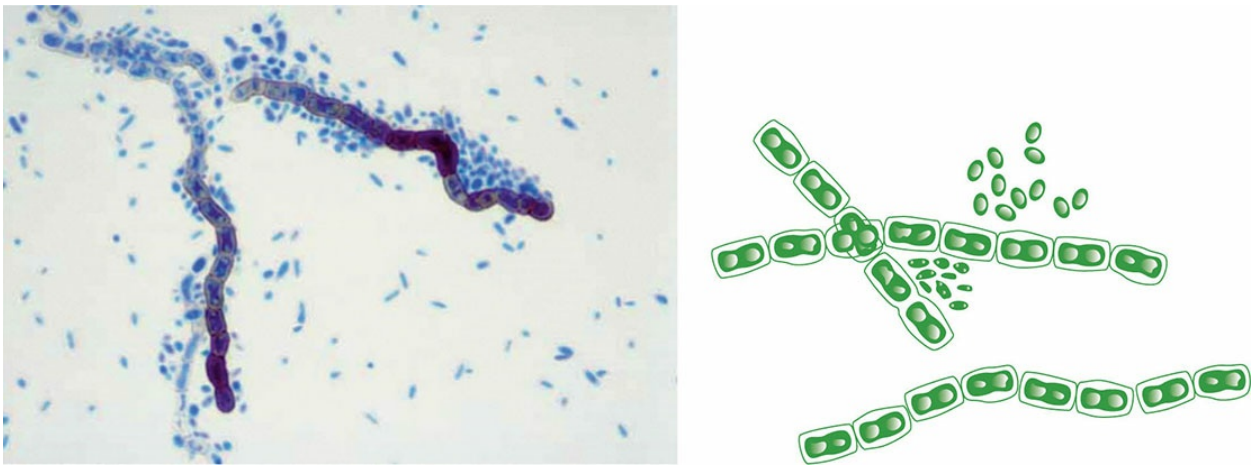
Disseminated infections with *Geotrichum candidum* are less commonly encountered. Isolated patients with disseminated *Geotrichum candidum* infection have been reported, and two in patients with acute leukemia.[248] In one of these patients, renal failure developed 3 days before death. The glomeruli were blocked with hyphal segments of *Geotrichum candidum*. The other patient, reported by Andre et al.,[14] was a young girl being treated for hepatoblastoma. After complications with secondary bacterial infections, this patient survived following a 5-week course of intravenous amphotericin B followed by 6 months of oral itraconazole. Although treatment regimens in patients with disseminated *Geotrichum* infection have not been established, the use of high doses of itraconazole and liposomal amphotericin B is recommended.[248]

Although patients with pneumonitis secondary to Geotrichum species have been reported, the recovery of this organism from respiratory secretions usually represents either contamination or commensal habitation, and other causes of disease should be ruled out. *G. candidum* assimilates only glucose, galactose, and xylose, which, except for the xylose utilization, is a pattern similar to that of *T. capitatus*. Most strains of *Geotrichum* are urease-negative, which is useful in differentiating this organism from *Trichosporon* species.

Blastoschizomycosis

Infections by *Blastoschizomyces capitatus* are rare. These usually occur in patients with significant underlying disease and are more commonly seen in the

immunocompromised host. Birrenbach et al.[22] report their experience with five patients infected by *B. capitatus*. Three were immunosuppressed because of chemotherapy for leukemia, whereas two were immunosuppressed secondary to renal transplantation. Three of the patients had fungemia, whereas the yeast was isolated from peritoneal fluid in one and the urine in another. All three of the patients with fungemia had pulmonary involvement, one had additional involvement of the brain, whereas the third also had involvement of the liver, spleen, skin, and brain. The three patients with fungemia died, which is not uncommon, as there is a high mortality associated with *B. capitatus* infections.



■ **FIGURE 21-95** Photomicrograph and sketch of the microscopic appearance of *Aureobasidium pullulans*, illustrating chains of large, dark-staining arthroconidia-like cells with small, hyaline conidia in the background.

1401

Hormonema undergoes asynchronous conidiation and the conidia arise from both the hyaline and the dematiaceous cells.

Laboratory Identification of "Black Yeasts"

Hortaea (Phaeoannellomyces) werneckii. The genus "*Phaeoannellomyces*" was created in 1985, by McGinnis et al.[183] "to accommodate those black yeasts that are characterized by the development of yeast cells that function as annellides" (*phaeo* = dark; *annello* = annellides; *myces* = fungus). Unfortunately, this beautifully named fungus has been taxonomically reclassified into the genus *Hortaea.*

Hortaea werneckii microscopically produces two-celled yeast cells. The daughter

cell is produced from an extension from the mother cell, which then contracts to form a scar (annellide), appearing as a deep-staining septum that separates the two cells (Fig. 21-96). Engleberg et al.[81] have reported a patient with a phaeohyphomycotic cyst caused by this or a related organism. More commonly, this fungus is known of as the cause of tinea nigra, a fungal infection of the palms of the hands. The yeast-like cells with this species are unicellular and may develop thick dark walls and pseudohyphae on maturity.

Controversy still exists with this group of organisms,[160] which are morphologically similar to the black yeast-like forms seen in early cultures of *Exophiala* species (covered above).

■ **FIGURE 21-96** Photomicrograph and sketch of the yeast forms of *Hortaea werneckii*, revealing elongated double yeast cells separated by a distinct deeply staining transverse bar

(annellide).

Commercially Available Yeast Identification Systems

Several packaged kits for identifying yeasts have been introduced by commercial companies.[281] These change over time, with the development of newer and improved products. Most recently has been the introduction of MALDI-TOF mass spectrometry for which there are two, excellent FDA-approved applications for yeasts. Although the initial capital costs are somewhat higher for mass spectrometry than for other identification systems, the cost is recovered quite quickly due to savings in reagent costs. Another advantage of the MALDI-TOF is the more rapid time to identification, which potentially could have a clinical impact.

The use of any of these systems requires technical skills and sufficient familiarity with each test performed to enable accurate interpretations. In each instance, the manufacturer's instructions must be closely followed. Each laboratory director or supervisor must weigh factors such as cost, availability and supply, shelf life and stability of reagents, adaptability to workflow, and the specific needs of the clinical services before a decision can be made to implement these systems in the home laboratory.

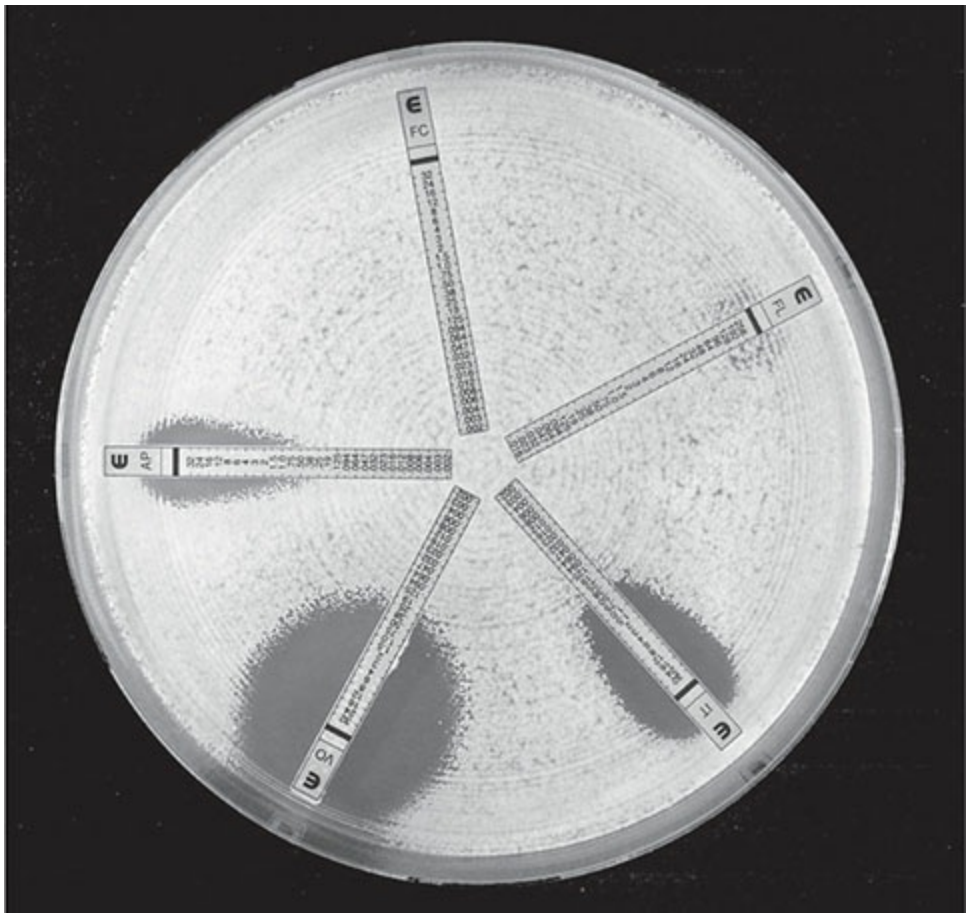
Antifungal Susceptibility Testing

The CLSI Subcommittee on Antifungal Susceptibility Testing has established standardized and reproducible methods for the antifungal susceptibility testing of yeasts.[196,223,220,231] Establishing these laboratory standards, particularly for the yeasts, has provided valuable guidelines for antifungal therapy with improved correlation with clinical outcomes.[99] There are established standards for broth dilution susceptibility testing for yeasts (M27-A2), disk diffusion susceptibility testing for yeasts (M44-A), and the broth dilution standards for filamentous fungi (M38-A).[48–51,55]

Sewell and coworkers,[253] in a comparative study of susceptibility tests for fluconazole, have shown that the Epsilometer "Etest" (bioMerieux) compares favorably with the reference methods, with an overall 84% agreement, reaching as high as 90% for most *Candida* species, except for *C. tropicalis* (56%). *Candida* (*Torulopsis*) *glabrata* also had low comparison values in this study (34%).

The Etest uses a plastic strip containing a defined continuous gradient of antimicrobial drug, which is placed on the surface of an agar plate after it has been

evenly plated with an inoculum containing a standardized concentration of the yeast organisms to be tested (Fig. 21-97). The plastic strip releases the antifungal agent into the surrounding agar, producing a logarithmic increase in the concentration of antibiotic from as low as 0.002 µg/mL to as high as 256 µg/mL, depending on the antifungal agent being tested. The details for performing the Etest, including the recommended test medium, the preparation of the standard inoculum, the steps of the procedure, and the readout can be found in the publication by Colombo et al.[53] This report also included the results for the Etest against 200 yeast isolates, compared with the standard methods for several individual drugs. The essential agreements between the two methods within a ±1 dilution were 71% for ketoconazole, 80% for fluconazole, and 84% for itraconazole. Since the publication of this paper, the use of



■ **FIGURE 21-97** Photograph of the surface of an agar plate demonstrating an Etest. Several gradient diffusion strips have been placed on a lawn of growth of *Candida kruzei*. The MIC interpretations of the strips reading clockwise beginning with fluconazole in the 11-o'clock position are: FL (fluconazole), >256 µg/mL; FL (flucytosine) >32 µg/mL; IT

(itraconazole), 0.38μg/mL; VO (voriconazole) 0.38 μg/mL, AP (amphotericin B), 2 μg/mL.

a standardized medium (RMPI 1640), a final reading at 48 hours, and other adjustments have raised the essential agreement percentage sufficient to establish the Etest as an alternative method for the fungal susceptibility testing of most yeast species. As mentioned before, because the Etest is less labor intensive and easy to perform, in-house antifungal susceptibility testing is now within the reach of many clinical microbiology laboratories. Some have employed the Etest for molds.

Pfaller et al.[221] in a multi-institutional study demonstrated a high level of reproducibility of the Etest minimal inhibitory concentration (MIC) method between four participating institutions. Each participating laboratory received subcultures of two American Type Culture Collection (ATCC) QC *Candida* strains (*C. parapsilosis*, ATCC 22019 and *C. krusei*, ATCC 6258), Etest strips, and sufficient RPMI agar plates to perform 20 replicate tests with each of five drugs—amphotericin B, fluconazole, flucytosine, itraconazole, and ketoconazole. In almost all of the drug/bug combinations tested, a very tight distribution was observed, ranging between 98% and 100% of the MICs, falling within a three-dilution range. The selective discrepancies of results between the Etest and the standard method for flucytosine and fluconazole against *C. krusei* was related to uneven growth between the glucose-enriched Etest agar and the unsupplemented broth used in the reference macrobroth test.

The Etest detected a resistant subpopulation of strains not detected by the reference method. After analyzing all the data, the authors concluded that the study established excellent interlaboratory and intralaboratory levels of agreement between the two methods, providing further documentation of the utility of the Etest in determining antifungal susceptibility profiles. This standardization of the test methods, the establishment of interpretive breakpoints for certain drug–yeast combinations, and reproducibility of results both within and between clinical laboratories will provide the basis on which clinical outcomes can be measured.[84]

The motivation to implement in vitro antifungal susceptibility tests in clinical laboratories goes beyond establishing the resistance profiles of a given isolate. The emergence of antifungal resistance is becoming an issue of increasing concern. For example, with the more widespread use of fluconazole, reports of fluconazole resistance in *Candida* species and *Cryptococcus neoformans* have increased.[279] Peyron et al.[218] have found that the Etest provides the optimal means for discrimination between *C. lusitaniae* isolates that are amphotericin B–resistant and amphotericin B–susceptible. Decreased susceptibility to itraconazole has been observed against select *Aspergillus fumigatus* isolates. Azole resistance has also been noted in *C. albicans* isolates from patients with AIDS, presumably resulting from the extended use of this drug in the treatment of oropharyngeal candidiasis (thrush). The

intrinsic resistance of *Candida krusei* to the imidazoles and emerging resistance of *Candida glabrata* to this group of antifungal agents are also problematic. White et al.[295] have published a review of the clinical, cellular, and molecular factors that underlie the development of resistant strains, particularly of *C. albicans*. This work serves as a primer for those who would like more information. More recently, the CLSI has provided species-specific breakpoints for the commonly encountered yeasts for which there were adequate data, as well as epidemiologic cutoff values, the latter of which reflect the wild-type susceptibility distribution to a particular drug. Both of these represent advances in our ability to provide more tailored therapy for patients. In summary, it is becoming imperative that clinical microbiology laboratories provide in vitro antifungal susceptibility data in order to guide the most appropriate use of the newer antifungal agents, as well as to detect these emerging patterns of resistance.

Unusual Fungi

Pneumocystis jirovecii

Pneumocystis jirovecii, as the genus name implies was originally thought to be a parasite. However, based on DNA sequence analysis, *Pneumocystis jirovecii* has been shown to be more genetically more closely aligned with the fungi, and thus has been reclassified as an ascosporogenous fungus. Additionally, it has been demonstrated that the species that infects humans, *P. jirovecii,* is distinct from that which infects rats, *P. carinii,* which is the reason for the change in nomenclature. Criteria note by Hadley and Ng[121] regarding the reclassification of this organism include the following:

- There are ultrastructural similarities between the cell walls of the cyst of *P. jirovecii* and those of other fungi.
- The cristae in the mitochondria of *P. jirovecii* are lamellar, whereas protozoans have tubular cristae.
- The cyst forms containing intracystic bodies resemble those of ascospores formed by the Ascomycetes.
- A high homology of the more conserved domains of the rDNA gene compared with that of Ascomycetes.
- The β-tubulin gene is 89% to 91% homologous with that of some of the filamentous fungi.
- *P. jirovecii* possesses separate proteins for thymidylate synthase and dihydrofolate reductase activities, whereas protozoa produce a single bifunctional protein.

- NADH dehydrogenase subunits 1, 2, 3, and 6; cytochrome oxidase subunit II; and a small subunit of rRNA have an average similarity of 60% with fungi, but only 20% with protozoa.

Pneumocystosis was recognized early in the HIV epidemic as a rapidly progressive, bilateral pneumonia that often contributed to the death of the patient. Although infections with this organism had been recognized prior to the HIV epidemic, infections were rare. There unusual infections were reported as comorbid conditions in patients with cancer and severe malnutrition. This disease is now recognized in patients with other immunocompromising conditions secondary to receiving chemotherapy or immunosuppression to control transplantation rejection or autoimmunity. In patients with more profound immunosuppression, frothy material fills the terminal alveoli of the lungs, which appears as "ground glass infiltrates" on chest roentgenogram. In patients with a lesser degree of immunosuppression or reconstitution of their immune system, the organisms appear within granulomas; in this presentation, the pathologist must be careful not to mistake the cyst forms of *Pneumocystis* with the morphologically similar yeasts of *H. capsulatum.*

Until recently, the morphologic assessment of respiratory specimen has been the mainstay of diagnosis and is still commonly used in most laboratories. Nonspecific staining options include the Diff-Quik stain, calcofluor white, toluidine blue, and the GMS stain. All of these, except for the Diff-Quik, rely on the detection of the cyst form of the organism, some of which will disclose the characteristic parentheses-shaped, intracystic bodies. Immunofluorescent stains are also an option. Although these stains are useful, studies at the Cleveland Clinic have demonstrated that they are significantly less sensitive than PCR, which may emerge as the new diagnostic standard.

Microsporidia

The tiny organisms of phylum *Microsporidia* do not stain well with most stains. The Weber stain[284a] and the Ryan stain[238a] have been used with success in clinical laboratories. The chromotrope R stain has been developed at the Centers for Disease Control and Prevention (CDC) to stain this group of organisms. In this stain, the concentration of the chromotrope 2R component has been increased by 10 times to facilitate the penetration of dye into the minute fungal forms. It is important to prepare a thin smear using approximately 10 μL of 10% formalin-fixed unconcentrated stool suspension on a glass slide (formalin concentrates may also be used, but the number of organisms, which do not concentrate well, per field of view is not substantially increased). The quick hot-Gram-chromotrope procedure is an alternative stain that is fast, reliable, and simple to perform.[194] Using this technique, a routine Gram stain is first performed on the thin smear, but instead of safranin, the slide is placed in a

warmed (50°C to 55°C) chromotrope stain for at least 1 minute. The microsporidia spores appear as dark-staining violet ovoid structures against a pale green background. A control slide that contains microsporidia spores from a 10% formalin-preserved specimen should be included for each staining run. This control provides a point of reference to distinguish true spores from similarly appearing objects. These stains are described in more detail below.

The microsporidia are obligate intracellular parasites that are sufficiently unique to be classified into a separate phylum, *Microsporidia*.[169] The unique feature of the *Microsporidia* is the production of spores that contain a complex tubular extrusion mechanism (i.e., the polar tubule or polar filament) by which the infective material, "sporoplasm," is injected into host cells. This occurs when an appropriate host cell stimulates the microsporidian spore (e.g., changes in pH and in ionic concentration of the intestinal contents for intestinal microsporidia). Upon appropriate stimulus, the coiled tubule is everted and penetrates the host cell. Within the host cell, asexual replication occurs either in a parasitophorous vacuole or within the cytoplasm, depending on the species. Eventually, the microsporidia are released from the cell fully functional and ready to infect the next host cell.

Over 140 genera and 1,200 species are included in this collective group called *Microsporidia*. This single-celled, minute parasites cause disease in a variety of nonhuman hosts, including insects, fish, laboratory rodents, rabbits, fur-bearing animals, and primates.[24] They are true eukaryotes (perhaps arising as a very early branch of the eukaryotes), having a nucleus, a nuclear envelope, and an array of intracytoplasmic membranes. Within the phylum, 15 genera have been shown to cause human diseases. Among these, the most important are species within *Encephalitozoon, Nosema, Pleistophora*, and *Enterocytozoon*. A former genus of medical importance, *Septata,* has been reclassified within the *Encephalitozoon*. Species differentiation is virtually impossible by light microscopy. Differentiation has classically been achieved using ultrastructural morphology achieved by transmission electron microscopy. Molecular studies are also important for definitive characterization.

Laboratory Identification. The diagnosis of microsporidiosis can be made by identifying microsporidia spores in the stool, as noted above, or in formalin-fixed, paraffin-embedded tissues. Although clusters of intracellular microsporidia may be seen in H&E-stained sections, they are better seen in tissue Gram stain preparations. They may also be seen in PAS, silver (GMS), acid-fast, or Giemsa-stained sections. The spores appear within intestinal enterocytes, characteristically located between the cell nucleus and the lumen of the intestine. In PAS-stained preparations, a PAS-positive granule may be observed at the anterior end of each spore. These appear as dark-staining dots when stained with Giemsa. Several photomicrographs demonstrating these spores can be found in Garcia.[102]

Electron microscopy continues to serve as the morphologic gold standard. The identification of spores with a polar tube is characteristic of all genera. Members of the genus *Encephalitozoon* are enclosed in a host-produced, phagosome-like, limiting vesicle (i.e., the parasitophorous vacuole). Both *Enterocytozoon* species and *Nosema* species develop in direct contact with host-cell cytoplasm without a limiting vesicle; *Nosema* species are distinguished by having paired, abutted nuclei.[24]

The laboratory diagnosis is most commonly made in the clinical parasitology section by demonstrating spores in stained preparations of fecal specimens.

Microsporidia have the following characteristics:

- The spores are tiny, ranging from 1.5 to 2.5 × 2.5 to 4.0 μm.
- They are oval to cylindrical in shape and possess thick walls that render them environmentally resistant and difficult to stain.

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- A transverse pink-staining band midway in the cells is a key identifying feature (Image Plate 22-4H).
- The spores stain poorly with H&E, but can be better visualized with Gram, acid-fast, PAS, Giemsa and a modified trichrome stain.
- They are gram-positive and some are acid-fast; in PAS stains an anterior PAS-positive granule is observed.
- The stain devised by Weber et al.[280] can be highly recommended for the detection of microsporidia spores in duodenal aspirates and in fecal material (Box 21-8).

DeGirolami et al.[65a] found that both Weber's modified trichrome stain and the fluorochrome Uvitex 2B stains were equally and highly sensitive compared with duodenal biopsies in detecting microsporidia spores in smears of duodenal aspirates biopsy material from 43 patients. Ryan et al.[238a] describe a modified stain in which the phosphotungstic acid is decreased in concentration to 0.25 g/dL and aniline blue instead of fast green was used as the counterstain. Each staining procedure requires 90 minutes, and selection between the Weber and the Ryan stain is one of personal preference. Kokoskin et al.[152a] found that performing the stain at a temperature of 50°C and decreasing the time of staining to 10 minutes produced a deeper, easier-to-interpret stain. Because the microsporidia spores are very small and may resemble bacteria or very small yeast cells, staining of positive control material is always necessary when performing either of these stains.

Although all species of microsporidia may cause disseminated infections, and organisms may be found in several organs, including liver, kidney, and brain,

Enterocytozoon bieneusi is the species that tends to preferentially infect enterocytes in the small bowel mucosa, particularly in patients with AIDS.[91] However, several case reports, as reviewed by Pol et al.,[225a] indicate that *E. bieneusi* may colonize the bile-duct epithelium leading to cholangitis. The estimated prevalence of microsporidium intestinal infection in AIDS patients may be as high as 12%,[163] or even higher if special techniques including cytocentrifugation, fluorescent antibody detection, and serology are used. *Encephalitozoon cuniculi* is most commonly associated with disseminated infection, with a predilection for infecting the brain and kidneys. *Nosema corneum* has been found in several cases of keratoconjunctivitis[32]; however, *Encephalitozoon cuniculi* and *Encephalitozoon hellem* have also been recovered from the cornea and conjunctiva in patients with AIDS with keratoconjunctivitis.[73] Conjunctivitis, scleritis, sensation of foreign body, and blurred vision are common presenting symptoms. Interestingly, infections of the cornea are often devoid of significant inflammation, even though abundant organisms are present, even in immunocompetent hosts.

Those with experience may be able to identify the characteristic, tiny (2 μm in diameter) intracytoplasmic spores in stained tissue sections of intestinal biopsies. Spores are best demonstrated using Brown-Brenn or Brown-Hopps tissue Gram stains or Giemsa-stained touch preparations. Organisms may also be seen in semithin plastic sections stained with methylene blue-azure II with basic fuchsin or toluidine blue counterstains.[24] Positive control slides should always be prepared in parallel when using any of these stains. Spores have also been demonstrated in CSF and urine by the use of immunofluorescence light microscopy. The infrequency of recognized infection caused by these organisms, particularly in resource-rich countries with effective HIV therapy, makes diagnosis a challenge.

Aldras et al.[6a] demonstrated the superiority of detecting microsporidia spores in the stools of patients with AIDS with indirect immunofluorescence antibody stains, using polyclonal antisera to *E. cuniculi* and *E. hellem* and monoclonal antibodies raised against *E. hellem*. Similarly, Zierdt et al.[309] used a variety of polyclonal mouse and rabbit antisera raised against *E. cuniculi* and *Encephalitozoon hellem* spores in an indirect fluorescent antibody assay to successfully identify microsporidial spores in 11 of 12 fecal samples, in addition to detection of antigen in colon and duodenal fluids and in duodenal biopsy touch preparations. Franzen et al.[91a] successfully used microsporidian DNA amplification by PCR on six known positive duodenal biopsy specimens to

Weber Stain for the Detection of Microsporidia Spores in Stool Specimens[284a]

1. Prepare slides for light-microscopic examination of stool taking a 10-µL aliquot of unconcentrated liquid stool concentrate in 10% formalin (12:3 ratio), spread thinly over an area 45× 25 mm^2.
2. Fix smears in methanol for 5 minutes.
3. Stain for 90 minutes in the Weber chromotrope-based stain. To prepare the stain, mix:
   - Chromotrope 2R (Harleco, Gibbstown, NJ), 6.00 g
   - Fast green (Allied Chemical & Dye, New York), 0.15 g
   - Phosphotungstic acid, 0.70 g
   - Allow these ingredients to stand for 30 minutes in 3 mL of glacial acetic acid. Then mix with 100 mL of distilled water.
4. After staining, rinse slides in acid alcohol (4.5 mL of acetic acid and 995.5 mL of 90% ethyl alcohol) for 10 seconds and then rinse briefly in 95% alcohol.
5. Successively dehydrate smears in
   - 95% alcohol for 5 minutes
   - 100% alcohol for 10 minutes
   - Hemo-De (xylene substitute, Fisher Scientific) for 10 minutes
6. Examine 100 oil immersion fields per slide, a reading time of approximately 10 minutes. Look for the small, 1–4 µm, cylindrical spores that stain bright pink-red.

detect a 353-bp DNA fragment specific for *Enterocytozoon bieneusi*. They suggest that PCR may be a useful approach to diagnosing microsporidiosis in HIV-infected patients. In fact, molecular diagnosis with species-specific PCR primers is likely the gold standard for the detection and identification of microsporidian species.[59] A challenge for the molecular diagnostic approach for microsporidia is that PCR-based assays are species-specific (i.e., a "broad-range" assay that detects all microsporidia species has not been developed). An approach is to test for the most common species, with the recognition that negative results due not exclude microsporidiosis caused by a species not covered by the assay.

BOX 21-8

Effective therapy for microsporidiosis is limited. Treatment of intestinal infections due to *Enterocytozoon bieneusi* with pyrimethamine, metronidazole, or trimethoprim–sulfamethoxazole (SXT) may be effective. De Groote et al.[65b] report successful treatment of a patient with AIDS using albendazole, a situation wherein PCR was used

to provide an early diagnosis. For a comprehensive review of human microsporidial infections, including over 250 references, see Weber et al.,[284b] who introduce several diagnostic options that will facilitate future studies on the incidence, risk factors, origins of infection, modes of transmission, clinical manifestations, pathogenesis, and treatment of this emerging pathogen.

Serologic Diagnosis of Fungal Diseases

Fungal serology is usually performed in reference laboratories. The time-honored procedures, including tube precipitin, immunodiffusion, latex agglutination, and CF tests are giving way to newer, more sensitive technologies. Among these, CF and immunodiffusion are still commonly used. EIA techniques that use species-specific monoclonal antibodies are being more widely used in clinical mycology laboratories.[118]

As a general rule, single species-specific serologic reactions in titers of 1:32 or greater indicate disease; however, demonstrating a rising titer fourfold or greater in samples drawn 3 weeks apart may be of greater significance.[61] Titers less than 1:32 or that do not show a fourfold increase between the paired samples usually indicate the presence of either early infection or nonspecific cross-reactivity with other antigens. The results from all serologic studies, nonetheless, must be considered presumptive, and correlated with the clinical and microbiologic findings.

Antibodies of the IgM class are commonly detectable about 2 weeks after the disease is acquired, and indicate recent infection. Usually IgM antibodies are no longer detectable after 6 months. IgG antibodies appear shortly after the rise in IgM titer, do not peak until about 6 to 12 weeks, and may remain elevated for many months after infection. Thus, a single elevated IgG antibody titer cannot be used to distinguish between recent and remote infections. The use of IgM and IgG assays is becoming more widespread and can help with distinguishing acute from remote infection.

The nonculture serologic and molecular techniques pertinent to the various types of fungal infections have been reviewed in the respective sections. Much attention is now shifting to sensitive assays being developed for the direct detection of fungal antigens in clinical specimens and in biologic fluids, often leading to same-day results. The continuous advances made in molecular diagnostics make it mandatory for the directors and supervisors of clinical mycology laboratories to peruse the current medical literature to determine which of these techniques may be applicable to specific clinical settings.[151] The following are proposed or confirmed taxonomic name changes of medically-important fungi. The traditional nomenclature has been predominantly retained throughout this chapter, but readers should be aware of these changes (Table 21-8).

TABLE 21-8 Taxonomic Name Changes of Medically-Important Fungi

Former Name	New Name
Bipolaris australiensis	*Curvularia tsudae*
Bipolaris hawaiiensis	*Curvularia hawaiiensis*
Bipolaris spicifera	*Curvularia spicifera*
Blastoschizomyces capitatus	*Saprochaete capitata*
Cunninghamella bertholletiae	*Cunninghamella elegans*
Geotrichum candidum	*Dipodascus geotrichum*
Madurella grisea	*Trematosphaeria grisea*
Ochroconis constricta	*Scolecobasidium constrictum*
Ochroconis gallopava	*Verruconis gallopava*
Paecilomyces lilacinus	*Purpureocillium lilacinum*
Paecilomyces variotii	*Paecilomyces divaricatus*
Penicillium marneffei	*Talaromyces marneffei*
Rhizopus oryzae	*Rhizopus arrhizus*
Scedosporium prolificans	*Lomentospora prolificans*

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Preliminary Observations Suggestive of the Zygomycetes

- Colonies rapidly growing with a poorly defined margin, filling the Petri dish in 48–72 hours
- Colony surface is wooly in consistency, and may become speckled as sporagnia are produced
- The hyphae are irregular in diameter, broad and ribbon-like, and aseptate-to-pauciseptate
- Sporangiospores are borne within spherical or cylindrical, sack-like sporangia, supported at the tips of sporangiophores

Root-Like Rhizoids are Formed

Yes / No

Sporangiophores borne nodally	Sporangiophores borne internodally. Prominent flare-like apophysis	Spherical conidia borne from the surface of a globose sporangium.	Sporangia borne in cylindirical sporangia radiating in daisy-head fashion from the surface of a spherical columella.	Sporangiophores broadly curved back on themselves	Sporangiophores often branched, with no swelling where sporangiophore and sporangium merge.
***Rhizopus* species**	***Lichtheimia* species**	***Cunninghamella* species**	***Syncephalastrum* species**	***Circinella* species**	***Mucor* species**

■ **DISPLAY 21-1** Algorithm showing genus identification of the *Zygomycetes*.

Preliminary Observations Suggestive of a Hyaline Mold

- Colonies rapidly growing, entire, cottony to granular, white or with varying pastel colors
- Hyphae are narrow, septate, hyaline, and have parallel walls

Conidia in Chains / **Conidia in Clusters** / **Conidia Borne Singly**

Colonies green and granular; phialides branching and blunt; conidia 2-3 µm, spherical.	Colonies green to yellow-tan or light purple; phialides tapered; conidia 2-3 µm elliptical, irregular in size and staining.	Colonies buff, granular, and radially rugose; 4-6 µm, circular, rough-walled annelloconidia with truncated bases.	Colonies gray-white to salmon-colored, glabrous; elliptical conidia arranged in clusters at the tip of the phialide.	Colonies fluffy, intense rose or purple pigment surface and reverse; large, multi-celled, sickle-form macroconidia.	Colonies spreading as green lawn without a border; balls of spherical conidia supported by tapered, finger-like phialides.	Colonies spreading as green or yellow lawn without a border; balls of spherical conidia borne laterally at tips of tapered phialides.	Colonies house-mouse gray and cottony; elliptical conidia, turning dark with age, borne from tips of delicate conidiophores.	Colonies gray-white and cottony; elliptical non-pigmented conidia borne from tips of delicate conidiophores, resembling *Blastomyces dermatitidis*.	Colonies gray-white and cottony; large, spherical, rough-walled macroconidia resembling *Histoplasma capsulatum*.	Colonies gray-white and cottony;small elliptical conidia in loose clusters, each attached by a delicate denticle to a geniculate conidiophore.
***Penicillium* species**	***Paecilomyces or Pleurostomophora* species**	***Scopulariopsis brevicaulis***	***Acremonium* species**	***Fusarium* species**	***Gliocladium* species**	***Trichoderma* species**	***Pseudallescheria boydi* complex**	***Chrysosporium* species**	***Sepedonium* species**	***Beauveria* species**

Genus *Aspergillus*

Sporulation from Top Half of Vesicle / **Phialides Circumferentially Placed** / **Cleistothecia Seen**

Phialides uniseriate	Philides biseriate Compact aleuroconidia produced.	Phialides biseriate Loose penicillus-like structures produced.	Conidia smooth and clear or buff. Roughened. Conidiophores may be biseriate or uniseriate.	Conidia rough and black. Condiophores are very long.	Colonies white and smooth. Conidiophores are short and pigmented brown.	Colonies variegated, green, yellow, white.
Colonies blue-green, granular and often rugose	Colonies cinnamon, granular and radially rugose	Colonies gray-green to tan with yellow patches	Colonies yellow/green, cottony or granular	Colony surface peppery black;		
Aspergillus fumigatus	***Aspergillus terreus***	***Aspergillus versicolor***	***Aspergillus flavus***	***Aspergillus niger***	***Aspergillus nidulans.***	***Aspergillus glaucus***

■ **DISPLAY 21-2** Algorithm showing laboratory identification of the rapidly growing hyaline molds.

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Preliminary Observations Suggestive of the Dermatophytes

- Colonies grow in 3-5 days with distinct margins
- Colony surface is cottony to granular in consistency. Surface pigmentation ranging from gray white to yellow-buff
- Hyphae are narrow, septate, and hyaline
- Combinations of microconidia and/or macroconidia are produced, the size, shape, and arrangement of which provide for genus/species identifications

MICROSCOPIC EXAMINATION

Macroaleureospores large, club-shaped, walls thin and smooth, multi-celled, walls thin and smooth.	Macroaleureospores multi-celled, walls thick and rough. Microconida few or absent.		Microconidia abundant; macro-aleureospores few or absent, walls thin and smooth			Aleureospores usuall not present; hyphae only observed.
No microconidia produced.	Macroaleureospores are spindle-shaped, pointed, curved tip.	Macroaleureospores are broad club-shape with rounded tip.	Microconidia spherical, uniform in loose clusters.	Microconidia tear-shaped, borne laterally from hyphae.	Microconidia irregular in size, balloon forms seen.	Antler hyphae and chlamydospores in chains observed.
Colony khaki-colored	Reverse of colony lemon-yellow	Colony cinnamon-colored		Wine-red pigment in agar	No growth on Trichophyton #1 agar	Slow-growing. Colony smooth on surface, heaped and partially submerged.
Epidermophyton flocossum	*Micrsporum canis*	*Microsporum gypseum*	*Trichophyton mentagrophytes*	*Trichophyton rubrum*	*Trichophyhton tonsurans*	*Trichophyton verrucosum*

■ **DISPLAY 21-3** Algorithm showing genus identification of the commonly encountered dermatophytes.

Preliminary Observations Suggestive of a Dimorphic Fungus

- Colonies grow slowly usually 7–14 days required for primary isolation
- Colony surface is gray-white and cob-web in consistency.
- Hyphae are delicate, septate, and hyaline. Arrangement in parallel bundles may be seen
- Species identification are made based on the size, shape, morphology, and arrangement of conidia

MICROSCOPIC EXAMINATION

Conidia, spherical, with thin-smooth walls, borne at the tips of short, straight conidiophores. (lollipops)	Hyphae segment into barrel-shaped arthroconidia, separated by non-staining cells. (alternate staining)	Tiny, microconidia produced early. Later, large, spiked spherical macroconida are important for identification.	Small, club-shaped conidia produced radially at the tip of a straight conidiophore, in a daisy head and/or sleeve arrangement.	Small, thin-walled conidia borne at the tips of long, straight conidiophores, when produced.
Blastomyces dermatitidis	*Coccidioides species*	*Histoplasma capsulatum*	*Sporothrix schenckii complex*	*Paracoccidioides brasiliensis*
CONFIRM SPECIES IDENTIFICATION BY YEAST CONVERSION (OR NUCLEIC ACID PROBE)				
Spherical yeast cells, 10–15 µm in diameter, with a single broad-based bud.	Large spherules, 75–200µm in diameter, containing 3-5µm endospores observed in stained tissue sections.	Tiny, spherical, hyaline, smooth-walled conidia arranged in clusters. Seen intra-cellularly in stained tissue sections.	Elongated, 2–8 µm in length, single-budding yeast, often referred to as cigar bodies.	Large, spherical, smooth, thick-walled mother cell with multiple buds, mariners wheel.

■ **DISPLAY 21-4** Algorithm showing genus identification of the dimorphic fungi.

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Preliminary Observations Suggestive of a Dematiaceous Mold

- Colonies grow in 3-5 days, are cottony or wooly, gray-brown or brown black; reverse jet black
- Hyphae are narrow, septate, brown-pigmented, and have parallel walls

Muriform Macroconidia				Transverse Macroconidia			Pycnidia or Conidia Single			
Multi-celled, muriform, macroconidia, beak or drumstick-shaped, borne in simple or branched chains, with the blunt end of one abutting the tapered end of the next.	Multi-celled muriform macroconidia borne singly from the tips of geniculate conidiophores, in a sympodial derivation.	Multi-celled muriform macroconidia, each borne singly from the tips of short conidiophores, simulating a bale of cotton on a stick.	Multi-celled muriform macroconidia, mature and immature, borne singly, aggregating in loose clusters.	3-4 -celled macroconidia divided by transverse septa only. The subterminal cells grow more rapidly, become enlarged, resulting in a boomerang appearance.	Conidiophores dark, simple or branched, geniculate, bearing cylindrical conidia in a sympodial pattern. Conidia with multiple cells, encased in thickened septal walls. Germ tubes from each end of germinating conidia.	Conidiophores and conidia similar in appearance to those of *Bipolaris* species. A single germ-tube is observed emanating at right angles from the germinating conidia. Any cell may germinate in the germ tube test, not just the terminal cells.	Conidiophores dark, erect, and geniculate. Multiseptate, elongated pencil-shaped conidia borne singly in a sympodial pattern. Each conidium has a dark, prominently protruding knob from the hilar cell.	Large, jet-black-spherical to oblong conidia borne singly from the tips of short, broad, conidiophores, each with a cup-like concavity within which the conidia rests.	Small, hyaline, spherical to elliptical conidia borne within sack-like, globose to subglobose pycnidia, scattered amid brown-staining septate hyphae with parallel walls.	Large perithecia, dark-brown to black, globose to flask-shaped, formed in the sub-stratum amid brown-staining, septate hyphae. Distinctive elongate, hairlike-hyphae protrude from the inside, simulating the legs of a spider
***Alternaria* species**	***Ulocladium* species**	***Stemphilium* species**	***Epicoccum* species**	***Curvularia* species**	***Bipolaris* species**	***Drechslera* species**	***Exserohilum* species**	***Nigrospora* species**	***Phoma* species**	***Chaetomium* species**

■ **DISPLAY 21-5** Algorithm showing laboratory identification of the rapidly growing dematiaceous molds.

1409

Preliminary Observations Suggestive of the Agents of Phaeohyphomycosis/Chromomycosis/Mycetoma

- Colonies grow in 3–5 days with distinct margins
- Colony surface is cottony to granular in consistency. Surface pigmentation gray-white to brown black; reverse jet black
- Hyphae are relatively broad, have parallel walls, are distinctly septate, and are dark yellow to brown in color
- Conidia are borne in chains, clusters, or singly from the tips or sides of phialides distinctive for each genus

MICROSCOPIC EXAMINATION

Conidia Borne in Chains			Conidia Borne in Clusters			Conidia Borne Singly		
Short branching chains of elliptical conidia with dysjunctor scars	Long chains (35+ cells) of elliptical conidia without dysjunctor scars	Colonies rapidly growing; no growth at 42°C. *Cladosporium* type sporulation	Phialides short, urn-shaped		Phialide long and tapered	Sympodial phialides bearing elliptical conidia (acrotheca-type sporulation)		Conidia borne laterally from the hyphae (rhinocladiella-type sporulation)
			Tip of phialide bottle-shaped	Tip of phialide saucer-shaped		Conidia arranged loosely	Conidia arranged tightly	
Cladophialophora carrionii	***Cladophialophora bantiana***	***Cladosporium* species**	***Phialophora verrucosa***	***Pleurostomophora richardsiae***	***Exophiala* species**	***Fonsecaea* pedrosoi***	***Fonsecaea compacta***	***Rhinocladiella* species**

*Different *Fonsecaea* species may show several combinations of cladosporium,-phialphora, acrotheca, and cladosporium-types of sporulation.

■ **DISPLAY 21-6** Algorithm showing genus identification of the agents of chromoblastomycosis, phaeohyphomycosis, and mycetoma.

1410

Preliminary Observations Suggestive of Yeasts

- Colonies grow in 24–72 hours
- Colony surface is smooth to mucoid; may develop feet or low aerial mycelium. Surface pigmentation ranging from white to yellow to rose red to black
- True hyphae may be present or absent.
- Pseudohyphae may or may not be present
- Blastoconidia are produced, the size, shape, and arrangement of which provide for genus/species identifications

MICROSCOPIC EXAMINATION

Pseudohyphae and Blastoconidia Produced		True Hyphae and Arthroconidia Produced		Hyphae Absent			Black Yeasts	
Germ Tube Test		Urease Test		Urease Test			Annelloconidia	
Pos	Neg	Pos	Neg	Pos	Pos	Neg	No	Yes
Chlamydospores on cornmeal Agar	No chlamydospores on cornmeal Agar			Caffeic Acid Test	Orange to red colonies		Dark-staining hyphae producing hyaline blastoconidia	Two-celled yeast cells with dividing cross septa (annellide)
				Pos / Neg				
Candida albicans	***Candida* species**	***Trichosporon* sp.**	***Geotrichum* sp.**	***Cryptococcus neoformans*** / ***Cryptococcus* species**	***Rhodotorula* species**	***Saccharomyces* sp. *Wickerhamomyce* sp. *Malassezia* sp. *Blastoschizomyces* sp.**	***Aureobasidium pullulans***	***Exophiala* spp.**
	Make presumptive ID based on patterns of growth on cornmeal agar.	Look for blastoconidia on corners of arthroconidia--rabbit ears on cornmeal agar	Look for right angle germ tubes from corners of arthroconidia (hockey sticks) on cornmeal agar	Look for irregular-sized yeast cells surrounded by capsular material	Inositol Neg			May produce hyphae and phialides when mature

■ **DISPLAY 21-7** Traditional algorithm showing genus identification of the commonly encountered yeasts.

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CHAPTER 22

Parasitology

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Introduction

Knowledge of common parasites dates back to antiquity. Worms fitting the description of *Enterobius vermicularis*, *Ascaris lumbricoides*, and the tapeworms have been mentioned in the ancient writings from many countries; additionally, certain symptoms known to be caused by these worms have also been described.[124] Ectoparasites, such as lice, fleas, and ticks, could not have been overlooked. Ruffer[217] reported the observation of calcified *Schistosoma haematobium* eggs in the remains of two mummies of the 20th dynasty in Egypt (1200 to 1090 BC), proving the existence of this infection in ancient Egypt. Chinese settlers in ancient times living in infected areas were familiar with the clinical picture of severe hookworm infection, which was called "lazy yellow disease."

Imaginary parasites have played an important role in medicine from ancient times. The pain of toothache was attributed to the work of a special boring "tooth-worm." Similarly, pus exuding from the ear and the eye resulted from the action of earworms and eye worms, respectively. Heartworms were responsible for sudden death; echo worms, possibly related to *Ascaris* balls in the intestine, were described, with the advice to those taking medication to remain silent during treatment for fear that the worm would be listening and not be removed.[124]

For centuries, spontaneous generation was thought to account for the origin of parasites, among other small creatures. It was commonly believed that out of a certain combination of the elements, parasites as well as other living organisms can be created. Tapeworms were known to be associated with the ingestion of raw meat; therefore, they were thought to take their origin in the stomach from macerated food, before being passed out in the stool. Since Roman times, tapeworms were thought to

be transformed strips of intestinal mucosa; according to many, *Ascaris* worms originated in phlegm. According to ancient theorists, something imperceptible or invisible is endowed with all potentialities—then through the action of a metaphysical principle, or of a primordial force, visible living beings can be created.[24]

An understanding of the protozoan parasites developed closely with improvements in the compound microscope. van Leeuwenhoek is credited with making the first observation of protozoa, a "motile animalcule" in the gut of a horsefly, shortly thereafter found to be present in his own feces. The current opinion is that he was looking at *Giardia duodenalis*, but this remains speculative. After these observations, however, over a century passed before progress in the study of the protozoans was made. With the widespread use of the compound microscope, by the turn of the 20th century, the morphology and life cycles of virtually all human parasites afflicting humans were well defined. The chronology of these advances is well presented in Foster's historical account for those who desire further reading on the subject.[76]

Despite the implementation of preventive measures to minimize the prevalence of parasitic diseases in most of the resource-limited countries, human parasites still account for inestimable loss of life, widespread morbidity, and the slowing of economic development. To quote from a report of the distribution of human parasites in China, "prevalence rates (of parasitic diseases) are of such staggering magnitude that the mind has difficulty in conceiving the descriptive statistics."[275] As further stated in this report, "the total number of protozoan and helminthic infections currently existing worldwide far outnumbers the total world population since multiple infections is the rule rather than the exception." Box 22-1 lists the estimated worldwide prevalence of human parasitic diseases, based on statistics published by the World Health Organization and from other sources.

In the United States, certain parasite species are encountered more frequently than others, and expertise in their identification must remain foremost. From a Centers for Disease Control and Prevention (CDC) intestinal parasite surveillance program, parasitic forms were found in 64,901 (15.6%) of the over 400,000 stool samples examined.[26] *Giardia intestinalis* (previously *G. duodenalis*) was found in 3.8% of all stool specimens, *Trichuris trichiura* ova in 2.7%, *Ascaris lumbricoides* ova in 2.3%,*Enterobius vermicularis* ova in 1.6% (not a true reflection of the incidence of this disease, since stool specimen examination is not the most sensitive method for establishing a diagnosis), and *Entamoeba histolytica* in 0.6% of all stool specimens. Diphyllobothriasis, sparganosis, and anisakiasis are also seen with increased frequency as ingestion of fresh fish becomes more in vogue. Private practitioners and public health officials must be on the alert for a possible increase in *Taenia* infections, including cysticercosis and other parasitic diseases, with the advent of more frequent travel and a more free exchange of foodstuff between North America and other countries.[223]

Bruckner and associates[21] reported a summary of the parasites found in a 6-month survey of stool specimen examinations on outpatients treated at Olive View Medical Center and Harbor General Hospital in Los Angeles (Box 22-2). In this study, other protozoa were identified in about 3% of all stool specimens; other nematodes were identified in 3% and cestodes in 0.5% of specimens. Similarly, Amin,[6] in a study conducted through the Parasitology Center in Tempe, Arizona, reported that one-third of 5,792 fecal specimens that were surveyed from 2,896 patients in 48 states and the District of Columbia during the year 2000 tested positive for intestinal parasites. Multiple infections with

BOX 22-1

Estimated Worldwide Prevalence of Parasitic Diseases[a]

Parasitic Disease	Global Prevalence (Millions of People)
Amebiases	500
Giardiasis	200
Ascariasis	800
Trichuriasis (whipworm)	800
Hookworm	900
Strongyloidiasis	50–100
Enterobius (pinworm)	42 (resource-rich countries)
Taenia (including cysticercosis)	1% of population in endemic countries
Diphyllobothriasis	5–10
Schistosomiasis	200
Fascioliasis	1–2
Clonorchiasis	20 (Asia)
Paragonimiasis	4–5
Malaria	100–270

Leishmaniasis	12
Trypanosomiasis (African)	20,000 new cases/year
Trypanosomiasis	15 (Chagas' disease)
Onchocerciasis	20
Filariasis	90
Echinococcus disease	1/100,000–150/100,000

[a]The incidence figures recorded here are rough estimates, intended only to illustrate the huge magnitude of infections. There are variations in numbers published in different sources, which probably reflect incomplete access to information or to differences in extrapolation of known data to the population at large.

BOX 22-2

Incidence of Parasites in Stool Specimens[21]

Organism	Olive View (1,350 Samples)	Harbor General (493 Samples)
	%	%
Giardia intestinalis	14.5	8.7
Endolimax nana	13.0	8.5
Entamoeba coli	10.5	7.7
Entamoeba histolytica	4.5	5.3
Ascaris lumbricoides	3.9	2.0
Hymenolepis nana	3.3	1.4
Dientamoeba fragilis	2.1	2.8

two to four parasitic species constituted 10% of 916 infected cases. *Blastocystis hominis* was found in 662 patients (72% of the 916 cases). Eighteen other species of intestinal parasites were identified—*Cryptosporidium parvum* and *Entamoeba histolytica/E. dispar* ranked second and third in prevalence, respectively. Prevalence

of infection was lowest (22% to 27%) in winter, gradually increased during the spring, and reached peak at 36% to 43% between July and October; then, prevalence gradually decreased to 32% in December. These studies provide examples of the types of parasites most likely to be encountered. The types of parasites present in a community are dependent on a number of variables, such as immigration and travel among the local populous.

Malaria is uncommon in the United States, usually found in individuals who have traveled into endemic areas. The CDC received reports of 1,687 cases of malaria with onset of symptoms during 2012 among persons in the United States or one of its territories.[268] Although this was a 12% decrease from the 1,925 cases reported in 2011, there has been an overall steady increase in the trend of the total number of cases. On average, there has been an increase of 28.6 cases per year since this surveillance began in 1973. *P. falciparum, P. vivax, P. malariae*, and *P. ovale* were identified in 54.4%, 16.6%, 3.2%, and 3.5% of cases, respectively. The remainders were either mixed or the species was not determined. The infections were acquired in Africa (n = 1,200), Asia (n = 200), Central America and the Caribbean (n = 68), South America (n = 48), or elsewhere or the location of the acquisition was unknown.

Because of expanded international travel and expanded military presence abroad, students and technologists working in diagnostic laboratories must maintain competence in the recognition of the agents of all parasitic diseases. Trade agreements between countries, some known to be endemic for parasitic diseases, also open up borders to the influx of goods, particularly foodstuffs that potentially are contaminated with animal parasites. Travelers from the United States to foreign lands must also remember that a variety of parasitic diseases are endemic in most resource-limited countries and that appropriate precautions must be taken to avoid infection. Unfortunately, accurate numbers of parasitic diseases and the prevalence of specific parasites are not readily available in many countries because of the lack of mandatory system of reporting for certain diseases. Parasitic diseases are often overlooked or misdiagnosed since symptoms are frequently nonspecific and have similarities with other diseases.

The frequent occurrence of certain parasitic infections in immunocompromised patients has spurred a new interest in laboratory parasitology among laboratory workers in nonendemic areas. Although this began with the introduction of HIV, the increasing number of transplant recipients and individuals receiving immunomodulating therapeutics for a variety of diseases contributes significantly to the cohort of immunocompromised patients in our communities.[253] For example, *Cryptosporidium* species were rarely recognized in humans before 1983, but in 1984, comprised 13.8% of all pathogenic protozoa. In a survey conducted at the New York Columbia-Presbyterian Medical Center of 41,958 stool specimens submitted for examination during the period 1971 to 1984, *Strongyloides stercoralis* was approaching the

incidence of *Trichuris trichiura* as the most frequently identified

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nematode. Harms and Feldmeier[111] postulate that HIV and parasitic infections may interact and mutually affect one another. For example, it is known that individuals with other sexually transmitted diseases, such as trichomoniasis, are at increased risk for contracting HIV, likely through alterations in mucous membranes.[170]

Risk and Prevention of Parasitic Infections

The risk factors for acquiring parasitic infections during travel to infested areas of the world and prophylactic measures have been reviewed by Warren and Mahmoud.[257] At lowest risk is the businessman who stays in first-class hotels in large cities for short periods. At the opposite end of the spectrum are volunteers and missionaries who live in tents or native dwellings in rural settings.

Most parasitic diseases are contracted either through ingestion of contaminated food or water or through the bite of an arthropod vector.[264] Drinking untreated water or brushing teeth with contaminated water can be particularly hazardous. Because most intestinal parasites withstand freezing, contaminated ice water is equally unsafe. This is a factor often forgotten by the traveler. They consider themselves safe by drinking soda, but forget the ice made with local, possibly contaminated water. Hot tap water is relatively safe because the infective forms of most intestinal parasites are heat-sensitive; however, tap water may not consistently exceed the critical temperature of 43°C (110°F); thus, its safety cannot be guaranteed. The ingestion of fresh, unpasteurized milk should also be avoided in endemic areas for a variety of reasons. Bottled, pasteurized milk and carbonated beverages are usually safe.

The ingestion of undercooked meats or raw/smoked fish can lead to infection with flukes, tapeworms, nematodes such as *Trichinella spiralis,* and protozoa such as *Toxoplasma gondii*. Raw vegetables are relatively safe if peeled before eating; however, lettuce and other leafy vegetables are particularly difficult to rid of infective parasitic eggs and cysts.

Precautions should be taken to avoid insect bites in tropical regions. The use of screens, bug bombs, insect repellents, and long-sleeved protective clothing is highly recommended. It is recommended by the CDC and other experts that insect repellents should contain ≥20% DEET (i.e., *N, N*-diethyl-*m*-toluamide or *N, N*-diethyl-3-methyl-benzamide), which is present in many commercially available products. Travelers to foreign countries, particularly to resource-limited countries in the tropical or subtropical climates, should consult the CDC Yellow Book, which is available online

(e.g., http://wwwnc.cdc.gov/travel/page/yellowbook-home-2014), concerning appropriate immunization programs and other notices to travelers.

Travelers to tropical regions should also be warned against swimming in natural freshwater estuaries. The infective cercariae of *Schistosoma* species abound in many freshwater rivers, lakes, and canals and can easily penetrate the unbroken skin of an unsuspecting wader. Chlorine in the concentration used in swimming pools may not make the water safe. Schistosomal cercariae that infest humans are not found in seawater; however, swimmer's itch may occur after wading in brackish water following penetration of the skin by cercariae of species that infect animals.

Examining physicians should make an effort to obtain a history of recent travel into regions where parasitic diseases are endemic and question patients carefully about the conditions under which they lived. The laboratory should be informed if a physician suspects a parasitic disease so that relevant specimens can be collected and the proper procedures performed for optimal parasite recovery. Serologic studies to screen for subclinical infections with *S. stercoralis* should be performed prior to initiating immunosuppressive drugs; a stool examination, albeit less sensitive, may also be considered.

Clinical Manifestations of Parasitic Disease

The most common symptom of intestinal parasitic infection is diarrhea that may be watery, bloody, and/or purulent. Cramping abdominal pain may be a prominent feature in diseases in which the bowel mucosa or wall is invaded by the parasite, such as in infections with hookworms, schistosomes, or intestinal flukes. Heavy infections with *Ascaris lumbricoides* can result in small bowel obstruction. Patients with tapeworms may be asymptomatic, except for weight loss despite an increased appetite and food intake. Bloating, belching, and steatorrhea may be seen in patients with giardiasis.[238,272]

Peripheral blood eosinophilia (15% to 50% or higher) is one of the more important markers for certain parasitic infestation. Eosinophilia may also be seen in various body secretions, such as sputum, diarrheal stools, suppurative exudates, or fluids from pseudocysts or various body cavities. However, the lack of eosinophils in either the blood or body fluids does not preclude the possibility of a parasitic disease, since eosinophilia is not a common manifestation for some diseases or the load of parasites may be minimal. Infections by helminthic parasites commonly generate an eosinophilic inflammatory response, whereas most protozoal infections do not. *Cystoisospora*, however, may be an exception to this rule. A generalized urticarial skin rash, thought to be a hypersensitivity reaction secondary to metabolic or lytic products of dead organisms that are absorbed into the circulation, may also suggest parasitic infection.

Although nonspecific, elevation in serum immunoglobulin levels, particularly IgE, may add support to the presence of a parasitic disease, particularly if coupled with eosinophilia.

Hepatosplenomegaly is a common manifestation of leishmaniasis (kala-azar) and liver fluke infection.[179] Portal hypertension, in particular, can be caused by *Schistosoma mansoni* and *S. japonicum*, with jaundice being a common presenting sign. Space-occupying cystic lesions of the liver, brain, and other organs can be found in amebiasis, echinococcosis, and cysticercus (i.e., infection by the larval stage of *Taenia solium*) infections.

Suprapubic pain, frequent urination, and hematuria are highly suggestive of *Schistosoma haematobium* infection. The presence of *Salmonella* serogroup Typhi in the urine is

evidence of an infection with *S. haematobium,* as it is thought that this bacterium colonizes the eggs of the parasite that are imbedded in the bladder wall and act as a nidus. Transient pneumonitis (i.e., Loeffler's pneumonia) may be experienced during the larval migratory phases of *Ascaris,* hookworm, or *Strongyloides* infections. This condition can be suspected if large numbers of eosinophils are observed in the sputum. When more severe cough, chest pain, and hemoptysis occur with the formation of parabronchial cysts, then the lung fluke infection should be suspected (i.e., paragonimiasis). Low-grade fever, weight loss, facial edema, and skeletal muscle pain indicate possible infection with *Trichinella spiralis*. Focal itching of the skin may occur at the sites of penetration of hookworm larvae or the cercariae of *Schistosoma* species.

Generalized constitutional symptoms are more commonly experienced after infections with blood parasites. Fever, chills, night sweats, lassitude, myalgias, and weight loss are common manifestations of malaria, leishmaniasis, and trypanosomiasis. Varying degrees of hepatosplenomegaly and lymphadenopathy are also seen with these diseases. Neurologic signs and symptoms secondary to encephalitis, meningitis, or localizing neuropathies may be seen in a variety of parasitic diseases. Central nervous system (CNS) involvement is commonly diffuse in African trypanosomiasis (i.e., sleeping sickness) and falciparum malaria, while space-occupying abscesses or cysts are more commonly seen with *Entamoeba histolytica*, *T. solium* (cysticercosis), and *Echinococcus granulosus* infections. Primary infections with *Toxoplasma gondii* present with lymphadenopathy and constitutional symptoms resembling acute mononucleosis, whereas reactivation disease in the CNS of the immunocompromised patient presents radiologically as a "ring-enhancing lesion" and often clinically as encephalitis/meningoencephalitis.[144] Cardiac myopathy is one of the most serious

complications of South American trypanosomiasis (*Trypanosoma cruzi*), of toxoplasmosis, and of several larval migratory infections.[165] Huge swellings of the legs, arms, and scrotum (elephantiasis) are common symptoms of filariases, because the adult worms block the lymphatic vessels, resulting in extensive chronic inflammation and fibrosis. Localized subcutaneous nodules or serpiginous inflammatory areas in the skin may be seen in diseases such as onchocerciasis, dracunculiasis, or cutaneous larva migrans, representing the larval migratory forms of hookworms of dogs and other animals.

A succinct overview of the clinical and laboratory aspects of several common and uncommon parasitic diseases has been published by Tan.[243]

Collection, Transport, and Specimen Processing

Appropriate specimens must be collected from the patient and transported to the laboratory sufficiently preserved to allow for the detection and identification of any parasites or parasite eggs. The diagnosis of parasitic infections relies in large part on macroscopic or microscopic examination of feces, urine, blood, sputum, and tissues. The implementation of reliable laboratory processing techniques is an integral step. It is not possible to review more than a few of the commonly used laboratory procedures that can aid in the recovery and identification of parasitic forms in clinical specimens. For a succinct, current, and practical overview of these procedures, the reader is referred to the section "Diagnostic Procedures" found in the current edition of the classic text by Garcia.[87]

Fecal Specimens

Stool specimens should be collected in a clean, widemouthed container with a tightly fit lid. Specimens that are mixed with water (i.e., contamination from the toilet bowel or bed pan) or urine are unsuitable because trophozoites may lose their motility or undergo lysis. Medications containing mineral oil, bismuth, antibiotics, antimalarials, or other chemical substances may compromise the detection of intestinal protozoa. Thus, examination of specimens must be delayed for one or more weeks after diagnostic procedures (barium enema) or therapy is stopped. Patients who have received a barium enema may not excrete organisms in their stools for at least one week following the enema. The container lid should be tightly affixed immediately after collection of the sample to maintain adequate moisture. Every specimen container must be properly labeled, as outlined in Chapter 1 of this text.

In obtaining fecal specimens for the examination of parasites, cathartics with an oil base should be avoided, since oils retard the motility of trophozoites and distort the

morphology of other forms. Specimens should also be collected before the administration of certain drugs and compounds that may compromise examination, or collection should be delayed until after the effects of such agents have passed. Such substances include antacids, kaolin, mineral oil and other oily materials, nonabsorbable antidiarrheal preparations, barium or bismuth (7 to 10 days needed for clearance of effects), antimicrobial agents (2 to 3 weeks needed for clearance), and dyes used for the visualization of the gallbladder (3 weeks needed for clearance).

The collection of three fecal specimens usually suffices to establish the diagnosis of intestinal parasitic diseases—two obtained on successive days during normal bowel movement and a third after a Fleet's phosphosoda or magnesium sulfate purge is recommended. No more than one fecal sample per day should be accepted. Some advocate that only a single specimen is required to diagnose most intestinal parasitic infections, unless there is a very high clinical suspicion of a parasitic infection. This approach depends on the experience of the microscopist and the parasitic load in the specimen, or it may be implemented if the patient becomes asymptomatic between sample collections.[87] Hiatt et al.[121] found that, with additional examinations, the yield of *Entamoeba histolytica* increased by 22.7%, of *Giardia duodenalis* by 11.3%, and of *Dientamoeba fragilis* by 31.1%. In contrast, Morris et al. evaluated results of the yield of 2,015 stool examinations to determine the yield of specimens obtained from patients who had been hospitalized >3 days.[178] They conclude that parasitic infections are unlikely in this patient population. Others have corroborated this finding, but exceptions exist for the elderly and patients with an immunocompromising condition. Posttherapy specimens should be examined 3 to 4 weeks after treatment of patients with protozoan infections and 5 to 6 weeks after therapy for *Taenia* infections.

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If a polymerase chain reaction (PCR)-based assay is requested on a stool specimen, implementation of the specimen transport protocol recommended by the manufacturer should be followed. The following suggestions are made for laboratory-developed tests:

1. Collect the specimen in the absence of typical preservatives used for the microscopic examination of stool (e.g., formalin and polyvinyl alcohol [PVA]).
2. Store and ship the specimen either refrigerated (4°C) or frozen (shipped with dry ice).
3. Alternatively, mix stool specimens in potassium dichromate (1:1 dilution with 5% w/v) or in absolute ethanol (1:1 dilution) and ship the specimen refrigerated.

Details of the extraction procedure and steps to follow in the DNA amplification have

been published.[113]

Preservation of Clinical Stool Specimens. Many stool specimens for examination of ova and parasites are collected at home, in a physician's office, or in a clinic some distance from the laboratory that performs the examination. Because trophozoites disintegrate rapidly after defecation and do not encyst, liquid stool specimens should be examined within 30 minutes after collection (not 30 minutes after receipt in the laboratory), and semiformed stools within 60 minutes, in order to detect motile trophozoites, particularly in suspected infections with *E. histolytica*. Formed stools, in which trophozoites are not expected, may be examined up to 24 hours after passage for the presence of helminth eggs. Stool specimens should never be frozen and thawed or placed in an incubator because parasitic forms may deteriorate rapidly.

Several preservatives are available for permanent fixation of stool specimens that are held for future teaching purposes or that must be sent to reference laboratories for analysis Chart 22-6. Box 22-3 presents the formulation and preparation of several currently used preservatives. The advantages and disadvantages are summarized below.

Formalin and Polyvinyl Alcohol. Stool specimens submitted in two vials, one containing formalin and the other PVA, had been the standard method used for the preservation of stool specimens for the morphologic examination for eggs, larvae, and protozoal parasites. These remain excellent preservatives, but as noted below acceptable alternatives exist. As noted in previous editions of this text, the eggs of *Trichuris trichiura* and the cysts of *Giardia* do not concentrate as well from PVA as from formalin-fixed material; similarly, the morphology of larval forms of *Strongyloides stercoralis* is poor in PVA-fixed stools and the oocysts of *Cystoisospora* (*Isospora*) *belli* may be undetectable.

Alternative Fixatives. Concerns over the toxicity of formalin and the difficulty of disposal of preservatives containing mercuric chloride have led several commercial companies to market alternatives to the gold standard, low-viscosity PVA (LV-PVA) reagent. In a study of 68 fresh stool specimens, collectively containing 31 parasitic forms, Jensen and colleagues[135] concluded that Proto-Fix (Alpha-Tec Systems, Vancouver, WA) and EcoFix (Meridian Diagnostics, Cincinnati, OH) were environmentally safe substitutes for PVA, resulting in the least parasite distortion. Pietrzak-Johnston et al.[203] also concluded that EcoFix is comparable to traditional LV-PVA for the visualization of protozoa in permanent-stained smears in a study of 20 positive stool specimens containing one or more parasites with stages consisting of eggs, larvae, and cysts; these findings confirmed the earlier findings of Garcia and Shimizu.[85] In each of these studies, the parasitic forms had well-defined nuclear detail, with certain parasites even easier to identify in the EcoFix-preserved specimens than in the traditional PVA-fixed samples.

When properly stained by standard techniques, the organisms have a blue-green to purple cytoplasm and red to purple-red chromatin against a green-staining background. Helminth eggs and larvae have a red or purple appearance.

More recently, several one-vial fixatives have been marketed. These have a number of advantages and relatively few limitations. Both the permanent smear and the concentrate can be made from a single vial. These fixatives are free of mercuric chloride, which makes disposal simpler and less expensive. Fortunately, most stool immunoassays are compatible with these fixatives. In some instances, these may be slightly more expensive than traditional fixatives. There are some color differences compared the staining of stool specimens with traditional stains, but this can be learned. Overall, these are an acceptable, environmental-friendly option to the traditional formalin/PVA fixatives. Several commercially available fecal concentration devices exist, including the FPC JUMBO tube and the FPC HYBRID connector systems (Evergreen Scientific, Los Angeles, CA), the PARA-SED concentration system (Medical Chemical, Torrance, CA), and the MACRO-CON concentration system (Meridian Diagnostics, Cincinnati, OH), in addition to an automated countertop workstation for the microscopic analysis of fecal concentrates (DiaSys, Waterbury, CT), as presented by Garcia.[84] Each of these products, in current use in clinical laboratories, can be recommended.

Visual Examination. Freshly passed stool specimens submitted for the detection of parasites should be visually examined for the presence of barium, oils, or other materials that may render them unacceptable for further processing. Patches of blood or mucin should be specifically selected for microscopic study because they may be derived directly from ulcers or purulent abscesses, where the concentration of amoebae or other parasites (e.g., *Balantidium*) may be highest. Visual examination can also be used to determine the appropriate procedures to perform. Formed stools are unlikely to contain trophozoites; thus, wet mounts are usually unnecessary and only concentrates need to be prepared. Helminth eggs, larvae, and protozoan cysts can be seen in the sediment of concentrates. The preparation of direct mounts should be limited to liquid to semiformed stools, and stained smears should be prepared to better demonstrate the internal structures of any parasitic forms observed. These approaches are described in more detail in the sections to follow.

Processing Fresh Stool Specimens for Ova and Parasite Examination. If fresh stool specimens are to be examined, which is uncommon, then liquid stool specimens should be examined within 30 minutes of passage (not within 30 minutes of arrival in the laboratory) in order to maximize the chance for observing motile trophozoites. Soft specimens, which may contain either trophozoites and/or cysts, should be examined within 1 hour of passage. In instances wherein there will be delays in examination (e.g., due to prolonged specimen transport), then the specimen should be preserved as

previously discussed to avoid possible disintegration of trophozoites. Formed stool specimens should be similarly preserved or can be kept for up to 1 day with refrigeration; it will not contain protozoal parasites, but may contain the eggs of helminths. Three types of preparation should be made for fresh liquid and soft stool specimens that have been submitted for parasitic examinations: (1) direct wet mounts, (2) concentrates, and (3) permanent-stained smears. Only concentrates and permanent-stained smears need to be prepared on stool specimens received in preservative.

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BOX 22-3

Common Fecal Specimen Fixatives: Formulations and Preparation

10% Formalin-Saline

In an appropriate storage container with a tightly fitting lid, add 100 mL of formaldehyde in 900 mL of 0.85% sodium chloride.

Advantages

- It is readily available, is easy to prepare, and serves as an all-purpose fixative.
- The prepared reagent has a long shelf life.
- The morphology of helminth eggs, larvae, protozoan cysts, and coccidial forms is well preserved.
- Formalin-preserved specimens are suitable for concentration procedures, epifluorescence microscopy, and performance of acid-fast, safranin, and chromotrope stains, and compatible with immunoassay kits.

Disadvantages

- Formalin-preserved specimens are not suitable for the preparation of trichrome-stained smears.
- Preservation is inadequate to maintain the morphology of protozoan trophozoites.
- Formalin levels must be monitored per current regulations, although these are usually very low and within established safe levels.

  Some parasitologists prefer a formula containing 5% formalin, which is purported to be less damaging to protozoa and emits less formalin vapor into the laboratory environment.

LV-PVA

Formulation

PVA, 10.0 g
95% ethyl alcohol, 62.5 mL
Mercuric chloride, saturated aqueous, 125.0 mL
Glycerin, 3.0 mL

Preparation

The liquid ingredients are mixed in a 500-mL beaker and the PVA powder added without mixing. Cover the beaker with paper or foil and allow the PVA to soak overnight. Slowly heat the mixture to 75°C and gently swirl for about 30 seconds until a light milky suspension is achieved.

Advantages

- The morphology of protozoan trophozoites and cysts is well preserved.
- Permanent smears stained with trichrome are easily prepared.
- Parasite forms are both preserved and adhere to the slide.
- Preserved samples remain stable for several months.

Disadvantages

- The morphology of helminth eggs and larvae, coccidian, and microsporidia is inadequately preserved.
- Mercuric chloride is the main ingredient, making it difficult and expensive to dispose of the fixative.
- The reagent is difficult to prepare in the laboratory.
- Cannot be used for concentration procedures or immunoassay kits.
- Unsuitable for acid-fast, safranin, and chromotrope stains.

MIF

Formulation

The two solutions must be prepared and stored separately and mixed together only immediately before use.

Solution I

Tincture of merthiolate, 1:1,000, 40 mL
Formaldehyde, 10% aqueous (USP), 5 mL
Glycerol, 1 mL
Distilled water, 50 mL

Solution II

Potassium iodide crystals (KI), 10 g
Iodine crystals (add after KI dissolves), 5 g of distilled water, 100 mL

Preparation

The shelf life of each solution is many months if stored at room temperature in a brown bottle. In a small vial, combine 9.4 mL of solution I with 0.6 mL of solution II just before use. Add the equivalent of about 1/4 teaspoon of fresh feces, and thoroughly mix with an applicator stick. Allow the suspension to settle

for 24 hours; then, with a pipette, remove a small portion of the pale orange middle layer and the deeper-staining lower layer for preparation of smears.

Advantages

- The reagent components both fix and stain the parasitic forms.
- Preparation is easy and shelf life is long.
- Suitable for concentration procedures.

Disadvantages

- As with formalin preservative, it is not suitable for preparation of permanent trichrome smears.
- The morphology of protozoan trophozoites is poorly preserved.
- The iodine component may interfere with other stains and fluorescence and may cause distortion of protozoa.

Sodium (SAF)

Formulation

Formaldehyde (37%–40% solution), 0.6 mL
Glacial acetic acid, 0.3 mL
Sodium acetate, 225.0 mg
Distilled water, 13.88 mL

Preparation

Dissolve the sodium acetate in the distilled water, slowly add the glacial acetic acid and formalin solutions, mix, and store. The shelf life is several months.

Advantages

- Easy to prepare and reagent has long shelf life.
- Suitable for acid-fast, safranin, and chromotrope stains.
- No interference with immunoassay kit assays.
- Free of the toxic chemical mercuric chloride.

Disadvantages

- Albumin-glycerin or similar additive must be used for adhesion of specimen to slides.
- Permanent stains are inferior to those obtained with PVA or Schaudinn's fixative.

Schaudinn's Fixative

Formulation

Mercuric chloride, saturated aqueous solution ($HgCl_2$), 110 g
Distilled water, 1,000 mL

Preparation

Into a beaker in a water bath placed in a hood, boil the mercuric chloride until dissolved, and let stand for several hours until crystals form.

Schaudinn's Fixative (Stock)

Mercuric chloride, saturated aqueous solution, 600 mL

Ethyl alcohol, 95%, 300 mL

Add 5 mL of glacial acetic acid per 100 mL of stock solution just before use.

Advantages

- Protozoan trophozoites and cysts are well preserved.
- Permanent-stained smears are easily prepared.

Disadvantages

- Mercuric chloride is the main ingredient, making it difficult and expensive to dispose the fixative.
- The morphology of helminth eggs and larvae, coccidian, and microsporidia is poorly preserved.
- Albumin-glycerin or a similar additive must be used for adhesion of the specimen to slides.
- Less suitable than other preservatives for concentration procedures.

Modified PVA (Copper or Zinc)

Advantages

- Suitable for making permanent trichrome-stained smears.
- No mercuric chloride.

Disadvantages

- Inconsistent staining.
- Organism morphology poor, particularly with the Copper modifications.

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Single Vial Fixatives

(Includes EcoFix, Unifix, Proto-fix, STF, and Parasafe)

Advantages

- Both the concentrate and the permanent smear can be made from a single vial.
- No mercuric chloride.
- Many immunoassays can be performed with this fixative.

Disadvantages

- There are some limitations regarding which stains can be used with which fixatives.
- May be more expensive.
- Some staining differences and possible staining inconsistencies.

Direct saline mounts are valuable for the detection of motile trophozoites. Helminth eggs and larvae and protozoan cysts may also be observed in direct saline mounts; the addition of a drop of iodine may aid visualization of these forms. For fecal specimens that are watery or liquid, centrifugation of the specimen alone may suffice because trophozoites do not concentrate well from liquid stools, and cysts that may be present will be seen in the sediment. The direct examination may be omitted for the processing of semiformed stools since any parasitic forms present will still be detected in the concentrated preparation.

Final identifications of parasites should not be made from examination of direct mounts alone; rather, permanent-stained smears should be prepared and examined to confirm the characteristic morphologic features. Permanent stains should be prepared on any specimens for which a delay in transport is anticipated (see below).

Preparation of Direct Wet Mounts. The wet mount procedure is useful for the identification of protozoan trophozoites, cysts, oocysts, and both helminth eggs and larvae. The saline mount is prepared by emulsifying a small portion of fecal material in a drop of physiologic saline on a microscope slide, then overlaying the mixture with a coverslip. Ideally, two mounts can be prepared on the same slide, the second one using iodine. Mounts should be just thick enough that newspaper print can be read through the slide. If the mounts are too thick, particularly iodine preparations, parasitic forms often stain poorly and may be difficult to differentiate from background debris. If the smear is too thin, parasitic forms in low numbers may be diluted out and missed during routine microscopic examination. Saline mounts are also prepared to observe the motility of trophozoites. Protozoan cysts also appear more refractile in saline than in iodine preparations. The internal structures of trophozoites or protozoan cysts are often poorly delineated in saline preparations, making definitive identification difficult. Permanent-stained smears should always be prepared, particularly if *Giardia* is suspected.

The oocysts of *Cyclospora* species may be observed microscopically in wet preparations using UV fluorescence microscopy, on which they appear intensely blue (UV excitation filter set at 330 to 365 nm). The use of both brightfield and fluorescence microscopy provides an efficient and reliable approach to secure this diagnosis. Additional smears should also be prepared in the event that an identification is not made from the original preparations.

Iodine is used as a stain to highlight the internal structures of intestinal parasites. One percent solutions of iodine (e.g., D'Antoni's iodine, prepared by adding 1.0 g of potassium iodide and 1.5 g of powdered iodine crystals to 100 mL of distilled water) should be used. Lugol's iodine, used for Gram's stains, in full strength is too strong to stain protozoan forms but can be used if a freshly prepared solution is diluted 1:5 with

distilled water. Examination of iodine mounts alone, however, may not be satisfactory because trophozoites are no longer motile. Both saline and iodine mounts can be prepared on a single microscope slide, making it easy to compare any suspect forms.

If desired, the coverslip(s) may be sealed. A preparation of molten petroleum jelly and paraffin in a 1:1 ratio can be applied to the margins of the coverslip using the tip of a cotton-tipped swab. The paraffin and petroleum jelly must be heated to approximately 70°C to mix both just before use. The seal is made by first securing the four corners by placing a drop of hot sealant to anchor the coverslip. A thin layer of the mixture is then spread around the edges. Sealing the coverslip keeps organisms from moving when using oil immersion objectives and prevents the preparation from drying out. Other suitable sealing preparations can be used, if desired (e.g., clear nail polish).

The entire coverslip area should be systematically scanned with an overlapping back and forth motion, using the 10× objective. If a suspect form is observed, a higher magnification may be necessary to study internal details.

Fecal Concentration Methods. Eggs, cysts, and trophozoites are often in such low numbers in fecal material that they are difficult to detect in direct smears or mounts;

therefore, concentration procedures should always be performed. The two most commonly used are (1) flotation and (2) sedimentation. Both are designed to separate intestinal protozoa and helminth eggs from excess fecal debris. The details for these procedures are found in Chart 22-1.

Flotation techniques. Flotation techniques are less commonly used in clinical laboratories than the sedimentation procedure. The flotation method uses solutions that have higher specific gravity than the organisms to be floated so that certain parasitic forms rise to the top, while fecal debris sinks to the bottom. Most commonly, zinc sulfate with a specific gravity of 1.18 is used. In comparison, the specific gravity of protozoa and many of the helminth eggs is lower. For example, the specific gravity of a hookworm egg is 1.055, an *Ascaris* egg 1.110, a *Trichiura* egg 1.150, and *Giardia* cysts 1.060.

The chief advantage of the flotation technique is that it produces a cleaner material in which parasitic forms are easy to distinguish. The major disadvantages are that the walls of eggs and cysts will often collapse, thus hindering identification; also, some parasite eggs do not float. Also, the operculated trematode and cestode eggs may not be detected because the high concentration of the zinc sulfate suspension causes the opercula to open and the egg to fill with fluid and sink to the bottom of the tube. For this reason, both the top filtrate and the bottom sediment should be examined microscopically.

Bartlett and associates[13] have described a modified zinc sulfate flotation technique that may be adapted for use with specimens that have been formalin-fixed. The formalin fixation not only prevents operculated eggs from opening so that they can be detected in flotation eluates, but it also prevents the distortion of morphology caused by salt solutions of high specific gravity.

Sedimentation techniques. Solutions of a specific gravity lower than the parasitic organisms are used, thus concentrating the eggs, cysts, and other forms in the sediment. Sedimentation techniques are recommended for general diagnostic laboratories because they are easier to perform and less prone to technical errors.

The concentration of intestinal parasites by either gravity or centrifugation leads to the efficient recovery of protozoa, eggs, and larvae, although they may be more difficult to detect in microscopic mounts and in stained smears because of the comparatively large amount of background debris. Ethyl acetate has been substituted for diethyl ether in the formalin concentration procedure used in most laboratories. Young and associates[284] have demonstrated that ethyl acetate is less flammable and less hazardous to use than diethyl ether and that the capability to concentrate cysts and eggs is not compromised. Care must be taken during the washing steps in the procedure to decant the supernatant carefully; otherwise, a significant number of parasitic forms can be lost. Neimeister et al.[185] have demonstrated that Hemo-De, available from commercial distributors, is an effective replacement for ethyl acetate. Hemo-De is a solvent with a specific gravity and solubility similar to that of ethyl acetate; it is nonflammable, nontoxic, and biodegradable (classified as GRAS [generally regarded as safe]) by the US Food and Drug Administration).

Permanent-Stained Smears. Although temporary wet mounts of fecal material for direct microscopic examination facilitate the rapid detection of intestinal parasites in fecal specimens, smaller protozoan organisms may be missed. Therefore, Garcia[87]and others recommend that permanent stains be prepared as part of the examination of every stool specimen submitted for parasitologic examination. The detection and identification of *Entamoeba histolytica, Giardia duodenalis,* also known of as *G. lamblia* and *G. intestinalis* and other protozoan infections can be greatly enhanced through the examination of permanent smears.[231] The morphology of cysts and trophozoites is better visualized in stained smears. Permanent mounts may also be prepared from stained smears, for future use in teaching collections, and for future consultation with experts when unusual forms are observed.

Two types of permanent stains are commonly used to visualize intestinal protozoa in fecal smears: (1) the iron hematoxylin stain and (2) the modified (Wheatley's) Gomori's trichrome stains. The iron hematoxylin stain is the time-honored technique used for the most exacting definition of the morphology of intestinal parasites. The staining procedure is somewhat difficult to control and is best performed by an experienced person to achieve optimum results. Therefore, the trichrome stain is

widely used in diagnostic laboratories. It is easy to perform, and good results are obtained with both fresh and PVA-preserved fecal material. The trichrome staining procedure is reviewed in detail in Chart 22-2.

There are parasitic diseases caused by protozoa that are not detectable or not readily detected in the iron hematoxylin or modified trichrome stain. These include cryptosporidiosis, which most commonly occurs in immunocompromised patients and cyclosporidiosis, which is usually food-borne.[267] Infections by the microsporidia, which were initially thought to be parasites but are now known to be fungi, are covered in the chapter dedicated to mycology.

New causes of diarrhea were discovered early in the HIV epidemic, which are now known to infect a wider range of individuals. Foremost among these were *Cryptosporidium* species. *Cystoisospora belli*, formerly known of as *Isospora belli*, is also known to infect similarly immunocompromised populations. Finally, *Cyclospora*, a parasite usually associated with food-borne disease, was described, and may infect immunocompetent and immunocompromised hosts alike. These organisms all stain well with the modified acid-fast stain, in which 1% sulfuric acid is used as the decolorizer. Additionally, a fluorescent antibody method is also available for the visual detection of *Cryptosporidium* species. In many products, this is available in combination with a monoclonal antibody directed against *Giardia duodenalis* cysts (discussed in detail later in this chapter). Either fresh or formalin-fixed material must be used to perform the acid-fast and/or the monoclonal antibody techniques; PVA-preserved specimens cannot be used.[84]

Examination of Intestinal Specimens Other Than Stool

Parasites such as *Giardia duodenalis* and *Strongyloides stercoralis* commonly inhabit the duodenum and jejunum. Specimens of duodenal contents may be required to demonstrate these organisms. A saline wet mount can be prepared from the aspirated material and examined microscopically. If motile organisms are seen, examination of a second iodine preparation may be helpful to highlight the internal structures so

that a definitive identification can be made. Again, the examination of fresh duodenal or jejunal aspirates is infrequently performed. More commonly, a small intestinal biopsy will be performed and evaluated by the surgical pathologist.

Duodenal contents were more commonly examined in the past by the string test.[137] The implement used is a weighted gelatin capsule containing a coiled length of nylon string (commercially available as Enterotest). One end of the string protrudes from the capsule, which is taped to the face of the patient. The capsule is swallowed, and peristalsis carries the weighted string into the duodenum. After 4 to 6 hours, the string

is removed and any bile-stained mucous adhering to the distal end is used to prepare direct mounts and stained smears for microscopic study. The examination of duodenal contents should be performed only on patients with signs and symptoms suggestive of giardiasis. McHenry et al.[172] obtained duodenal aspirates from 144 patients who were undergoing endoscopy for other reasons. *Giardia duodenalis* was recovered in only one patient (0.7%).

Enterobius vermicularis infects the large intestine, but the female migrates and deposits eggs in a perianal location. This infection is best detected using cellulose tape or similar, now commercially available products. In brief, the adhesive surface of a 3- or 4-inch strip of clear cellulose tape is applied to the perianal folds of a patient in whom pinworm infection is suspected Chart 22-5. Specimens collected in the early morning, soon after the patient arises and before bathing, are optimal for detecting ova. A tongue blade can be used to provide a firm backing for the tape. The tape is then placed adhesive side down on a glass microscope slide and examined for the characteristic ova of *E. vermicularis*. A commercially available "paddle" with a flat, sticky surface (BD Falcon SWUBE Pinworm Paddle) can be used to obtain the specimen. The microscopic appearance of a positive preparation is illustrated in Figure 22-1.

Examination of Extraintestinal Specimens

Sputum and Other Respiratory Tract Specimens. On rare occasions, the larval stages of hookworm, *Ascaris lumbricoides* or *Strongyloides stercoralis*, or the eggs of *P. westermani* may be seen in sputum or other respiratory tract specimens. The preparation of a direct saline mount is usually sufficient. If the sputum is unusually thick or mucoid, an equal quantity of 3% *N*-acetyl-L-cysteine can be added to liquefy the specimen, which is then mixed for 2 or 3 minutes and centrifuged. After centrifugation, a wet mount of the sediment is prepared for microscopic examination. If examination of the sputum specimen is delayed for any reason, 10% formalin should be added to preserve helminth eggs or larvae. The organisms present in the respiratory tract may also be identified by the cytopathologist.

■ **FIGURE 22-1** Photomicrograph of a positive cellulose tape preparation from a patient with suspected pinworm infection, illustrating several oval, thin-walled eggs of *Enterobius vermicularis*.

Urine and Body Fluids. Samples of large volume should be allowed to settle for 1 or 2 hours. About 50 mL can then be taken from the bottom sediment for centrifugation. The highly concentrated sediment can then be examined by preparing a direct wet mount. If objects suggestive of parasites are seen, examination of an iodine mount or a stained preparation may be helpful in highlighting the diagnostic internal structures.

Tissue Biopsies and Aspirates. Cutaneous ulcers (as seen in leishmaniasis), skin nodules (seen in onchocerciasis and in *Mansonella streptocerca* infections), and lymph nodes may be either aspirated with a fine needle or biopsied. In suspected cases of cutaneous leishmaniasis, material should be aspirated with a needle and syringe from beneath the ulcer bed. A "skin snip" can be obtained to diagnose subcutaneous leishmanial disease by grasping a small portion of skin with a forceps or elevating it with the tip of a needle. The tip of the small "cone" of skin is then sliced with a scalpel. The snip should be sufficiently deep to include the dermal papillae; however, it should not be so deep as to produce extensive bleeding.

All biopsy tissue must be submitted to the laboratory without the addition of formalin fixative. If a delay in processing cannot be prevented, the specimens should

be placed in PVA fixative. If the specimen is soft, a small portion should be scraped free and placed in a drop of saline for wet mount examination. Impression smears should also be prepared, performed by pressing a freshly cut surface of the tissue against the surface of a glass slide and placing the slide immediately into a fixative, such as Schaudinn's solution. Trichrome and other stains can be applied.

The remaining portion of the biopsy material should be submitted in 10% buffered formalin for histologic examination. As denoted above, the histologic examination of intestinal biopsies may reveal a wide variety of infections. The eggs of *Schistosoma, Cryptosporidium, Giardia, Entamoeba histolytica*, and in some instances cross sections of *E. vermicularis* may be seen in biopsied materials, among others. Other examples include the evaluation of excised or biopsied pulmonary specimens which may disclose the larvae of *Strongyloides,* the eggs and/or worms of *Paragonimus,* or the degenerate nematode *Dirofilaria immitis.* The infectious disease pathologist is a partner with the microbiologist in securing the cause of infectious diseases. Collaborative communication translates into the best patient care possible.

Corneal Scrapings or Biopsy. Corneal scrapings are helpful in making the diagnosis in suspected cases of *Acanthamoeba* keratitis. The corneal scrapings, obtained by

a physician, are placed on a slide and fixed in methyl alcohol for 3 to 5 minutes. Garcia[87] suggests that staining should be done using calcofluor white, a commercially available textile whitener. A solution of 0.1% calcofluor white and 0.1% Evans blue are dissolved in distilled water. A few drops of this solution are placed on the methanol-fixed smear for 5 minutes. The slide is then tipped and the fluid drained into an absorbent paper towel; a coverslip is added and examined for characteristic amebic cysts (trophozoites do not stain), which will have an apple-green or blue-white fluorescence depending on the exciter light/filter combination used.

Muscle Biopsy. The characteristic spiral larval forms of *Trichinella spiralis* are best demonstrated in a tease mount made from a skeletal muscle biopsy (Image Plate 22-11G). Garcia[87] suggests that the biopsy material be treated with a digestion fluid before examination. The digestion fluid is prepared by adding 5 g of pepsin to a mixture of 1,000 mL of distilled water and 7 mL of concentrated HCl. The tissue is placed in a widemouthed Erlenmeyer flask, and digestion fluid is added in a ratio of 1 part tissue to 20 parts fluid. The digestion mixture is held at 37°C for 12 to 24 hours. After digestion, examine a few drops of the eluate under the microscope for the presence of larvae. If none are seen, centrifuge a 15-mL aliquot of the mixture and examine the sediment.

Blood. Although the examination of fresh blood is rarely performed, it can be done for the evaluation of motile parasites. A drop of anticoagulated blood can be placed on a microscope slide, a coverslip put in place, and the specimen microscopically examined for large, often motile, extra-erythrocytic forms, such as trypanosomes and microfilariae. The presence of microfilariae in the circulating blood exhibits a marked periodicity depending on the species involved; therefore, the time of specimen collection is critical. If a filarial infection is suspected, the optimal collection times for demonstrating microfilariae are *Loa loa*—midday (10 AM to 2 PM); *Brugia or Wuchereria*—at night, after 8 PM; *Mansonella*—any time; *Onchocerca*—any time. There is no advantage for the direct evaluation of blood over the standard evaluation of thick and thin blood films. Additionally, such an evaluation exposes one unnecessarily to blood-borne pathogens. Therefore it is not recommended.

When first examining a stained blood film, screen the smear under low-power magnification (×250 or less) in search for these larger parasitic forms that may be missed if only oil immersion is used. The morphology of intraerythrocytic parasites (i.e.,*Plasmodium* and *Babesia*) is best observed in Wright- or Giemsa-stained peripheral blood films.

Blood smears prepared for the detection of blood parasites should be collected specifically for that purpose. Smears should be prepared on anticoagulated blood specimens as soon after collection as possible since the long exposure to the anticoagulant may distort the morphology of mature schizonts and gametocytes. Also, exflagellation may occur and sexual stages may develop during storage of the blood sample in a laboratory environment, which is cooler than body temperature. Exflagellated gametocytes may be confused microscopically with *Borrelia* species. Merozoites, particularly those of *Plasmodium vivax,* may be released from mature schizonts and reinvade erythrocytes, where they may appear similar to the small "accolade" ring forms of *Plasmodium falciparum*. These are the reasons that the collection of capillary blood samples with the immediate preparation of thick and thin blood films is preferable. This is commonly performed in field studies, but rarely in the hospital environment.

Both thick and thin blood films should be prepared. The thick film is the sensitivity portion of this assay and is usually simply used to detect the presence of a blood parasite. In contrast, the thin film is the specificity arm of the assay and is used to identify the etiologic agent, often to the species level.

Thick smears consist of a thick layer of dehemoglobinized (lysed) erythrocytes. A thick smear of proper density is one that allows one to barely read the words of newsprint through the film. Thick blood smears contain a 30× concentration of parasitic elements compared with an equal area of a thin smear. Thus, the preparation of thick smears is particularly useful in detecting malarial parasites in light infections; however, the morphology of trophozoites is often compromised, making it difficult to make a species identification. If elements suspicious for malarial parasites are

observed, a thin smear should be prepared to make a species identification.

The thick smear is prepared by placing two or three drops of blood on the surface of a glass microscope slide to cover an area the size of a dime. If capillary blood is obtained from a fingerstick, it should flow freely; "milking" of the finger should be avoided. Stir the drop, if uncoagulated blood is used, for about 30 seconds to prevent formation of fibrin clots. If anticoagulated blood is used, which currently is almost the universal practice, stirring is not necessary because fibrin strands do not form. Potassium ethylenediaminetetraacetic acid (EDTA) is the anticoagulant of choice.

Lay the slides flat on the tabletop and allow the smears to dry thoroughly, as any residual moisture may cause the film to detach. The time to drying will take at least 30 minutes, but may take much longer in a humid climate. The drying time can be shortened by ventilating the smear with a fan or hair dryer set on "cool." The chance for detachment is increased with smears made from anticoagulated blood. Once the film is dry, the blood should be laked by placing the slide in water or a buffer solution immediately before staining.

The thin blood smear is used primarily for the definitive genus- and species-level identification of the blood parasite present. It is prepared exactly as a peripheral smear is prepared for a differential blood count. At least two smears per patient should be prepared. Thin smears consist of blood spread in a layer such that the thickness decreases progressively toward the feathered edge, where the erythrocytes do not overlap. The thin-feathered edge should be 1.5 to 2.0 cm long. Care must be taken in preparing the thin film to see that the feathered edge is evenly spread and free of holes, streaks, or other artifacts. The preparation of thin and thick blood smears is described in Chart 22-3. At least three specimens should be taken on successive days if initial samples are negative for parasites.

Garcia (personal communication) has described a technique by which both a thick and thin smear can be accomplished on the same slide. Two or three drops of anticoagulated blood are placed at one end of a glass slide, then with an applicator stick, smear the drop over the surface of the remainder of the slide using a continuous rolling motion.

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Both thick and thin areas will develop where the concentric circles intersect. Both thin and thick smears should be stained as soon as possible after preparation, always within 48 hours, with Giemsa or Wright's stain. The pH of both the stain and the buffer must be carefully controlled and should be between 7.0 and 7.2. The stains used in the routine hematology laboratory are closer to pH 6.8, eliminating any possibility of observing Schuffner's dots or Jame's stippling. The use of automated staining instruments is to be avoided. Thick smears may require a slightly longer exposure to the stain than the time used for the thin smear preparations.

It is recommended that at least 300 oil fields be microscopically examined at 1,000× magnification on the thin film and approximately 100 fields on the thick film. The number of organisms may be very few in patients in relapse, in those who have an early infection, or in those who have received inadequate treatment or partial prophylaxis. In these instances, the number of fields examined should be doubled. Proper interpretation of the thick smear requires considerable experience, and positive controls for comparison should be made available.

Identification and Differentiation of Parasites

Although certain clinical signs and symptoms may suggest the possibility of a parasitic disease, the final diagnosis is made by demonstrating the causative organism in properly collected specimens. Because many artifacts resemble parasitic forms, the final identification must always be made based on well-established morphologic criteria. Microscopic interpretations, in particular, cannot be left to guesswork, and a laboratory diagnosis of a parasitic disease should not be rendered until adequate identifying features can be clearly and objectively demonstrated. Select artifacts that have features suggesting true parasites are illustrated in Image Plate 22-1.

Traditionally parasites are separated into various morphologic and/or taxonomic groups (e.g., protozoa, nematodes, cestodes, trematodes, etc.). One should recognize that even closely related or taxonomically grouped parasites may have very different modes of infection and mechanisms of transmission. For example, even though hookworms and pinworms are taxonomically included with the nematodes, there are considerable differences in their life cycles, modes of infection, and the seriousness of the diseases they cause. In fact, each species of parasite should be considered unique unto itself, as attempts to group them by whatever criteria will meet with some degree of failure.

The microscope will be the tool most commonly used to identify parasites. Therefore, it should have an eyepiece fit with an accurately calibrated ocular micrometer. The procedure for calibrating an ocular micrometer is presented in Chart 22-4. The ability to measure exactly the size of parasitic forms encountered in clinical specimens is often critical for the correct identification of the parasites. In the discussions that follow, emphasis is placed on the size ranges of various diagnostic parasite forms that are reviewed.

Life Cycles of Human Parasites

Many of the life cycles of human parasites are included in the sections on the laboratory identification that follow. It is not intended that students memorize every phase of every life cycle; rather, these descriptions are helpful in pinpointing the

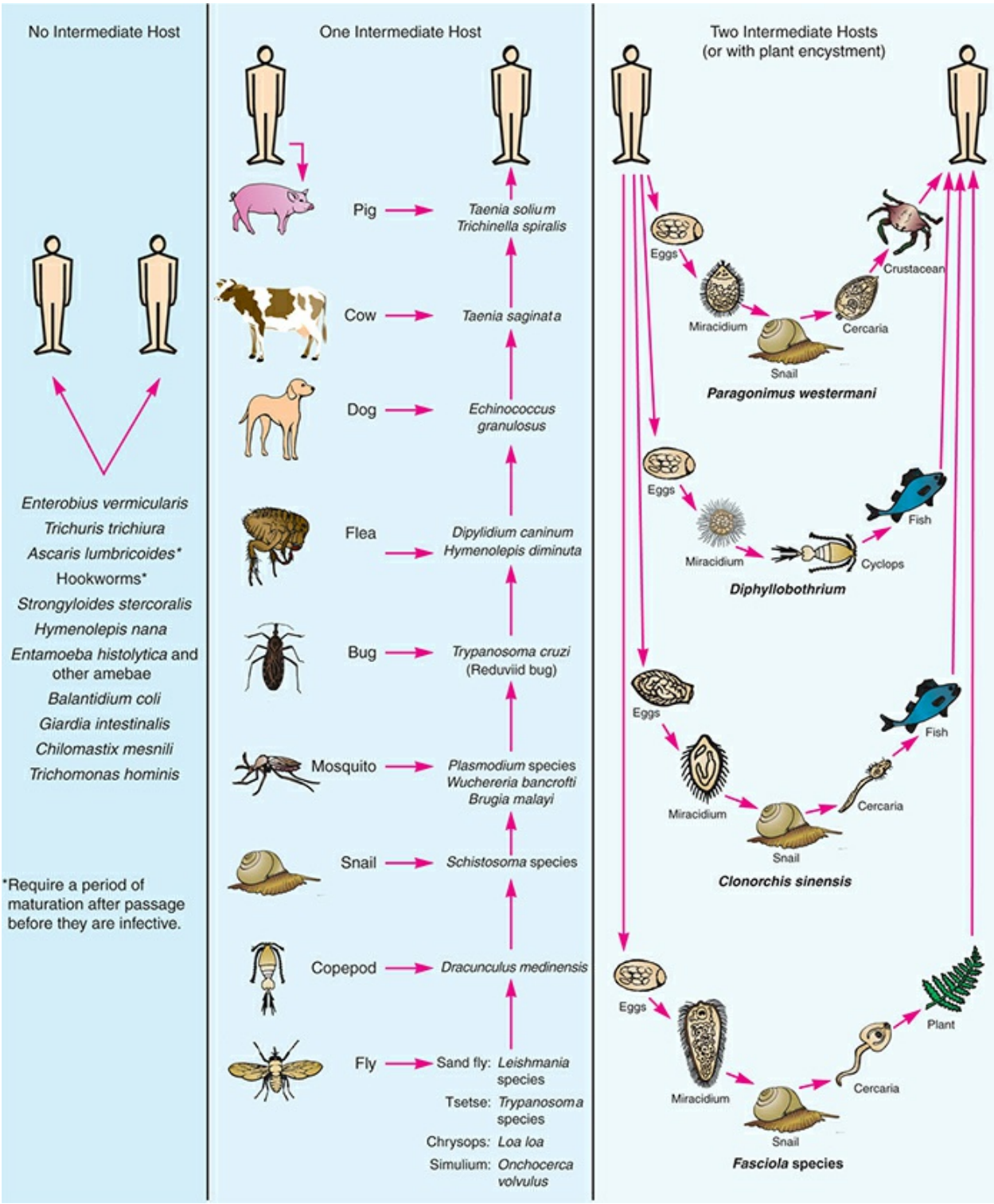
infective, invasive, and diagnostic forms of each parasite, an awareness of which is necessary to make a diagnosis. DPDx is an excellent resource for students and practitioners alike. This site (http://www.cdc.gov/dpdx/) is maintained by the Division of Parasitic Diseases and Malaria of the CDC. It contains up-to-date information, color images, and illustrated life cycles of the most common, and some less common, parasites.

Figure 22-2 has been designed to provide an overall orientation to the life cycles of parasites of importance to humans. Parasites can be divided into three major groups on the basis of their life cycles: (1) those having no intermediate hosts, (2) those using one intermediate host, and (3) those for which two intermediate hosts are necessary.

Parasites that have no intermediate host are transmitted directly between humans, or animal to animal, through fecally contaminated food or water. This is true for most of the intestinal protozoal parasites and for some nematodes such as *Enterobius vermicularis* and *Trichuris trichiura*. Human-to-human transmission occurs through the transfer of cysts or ova that can survive external environmental conditions and contaminate food and water supplies. The eggs of *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworms require a period of maturation after the stool is passed into the environment before they become infective.

Direct Versus Indirect Lifecycles of Nematodes. *Enterobius vermicularis* and *Trichuris trichiura* are the two common human parasites with a direct lifecycle. This means that once the egg is ingested and the internal larva has emerged, it develops within the intestinal tract into the adult form. In contrast, in indirect life cycles, the immature larvae migrate to and through the lungs after which it is swallowed and then develops into an adult form in the intestinal tract. This may occur with parasites transmitted through the fecal–oral route, such as the eggs of *Ascaris lumbricoides*, or with larval parasites that are able to penetrate through intact human skin, such as the hookworms and *Strongyloides stercoralis.*

Parasites requiring one intermediate host commonly involve a large mammal, rodent, crustacean, or insect vector within which they complete their life cycle. This process may be either simple or complex. For example, humans serve as the definitive host for *Taenia* spp., which reside in the intestinal tract. The definitive host is the host in which sexual reproduction occurs. The pig and cow, for *T. solium* and *T. saginata,* respectively, are intermediate hosts for these tapeworms. They are present as encysted larvae and reside in skeletal muscle of the animal. The parasitic cycle is completed when humans ingest the encysted parasitic form in raw or undercooked meat. The released larvae ultimately find their way to the intestine to mature into adult worms. A caveat to *T. solium* infections is that humans may also act as the intermediate host for the parasite, if they ingest a viable egg. When the larval form of this tapeworm is present



■ **FIGURE 22-2** An overview of the life cycles of some parasites of human importance.

The parasite species are divided by their need for zero, one, or two intermediate hosts. Sketches of the common intermediate hosts are also included.

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in humans, the disease is called cysticercosis.[259] Echinococcosis is another example of cystic larval disease in humans. In echinococcosis, canines are the definitive or primary host, harboring the adult echinococcal tapeworm in its intestine. In the normal life cycle of echinococcosis, sheep normally serve as the secondary or intermediate host, in which, following ingestion of infective eggs, the cystic lesions develop primarily in the liver. The life cycle of the parasite is completed when dogs and other carnivores ingest the larval-infected offal of dead sheep. Humans become accidentally infected when they ingest the eggs found in the feces of the dog.

Parasites involving insects usually go through a complex series of developmental stages before the infective form is released. In malaria, for example, the plasmodia undergo sexual recombination with ookinete production within the mosquito before infective sporozoites are formed in the salivary glands of the mosquito. These sporozoites are then injected into a human host during a blood meal. There are similarities in the life cycles in diseases such as trypanosomiasis, leishmaniasis, and filariasis.

Parasites that require two intermediate hosts (*Diphyllobothrium latum* and most trematodes) follow similar life cycles. Eggs that are passed from the adult parasite within the definitive host hatch in a suitable aqueous environment and release ciliated, free-swimming forms called miracidia. The miracidia are highly motile and move very rapidly (2 mm/sec). Through the action of photoreceptors, touch receptors, and chemoreceptors, miracidia are attracted to their complementary snail host. Attachment must take place within a few hours because their life span is very short. They possess anteriorly placed glands that secrete proteolytic enzymes to penetrate into the snail's integument. Penetration into the snail takes about 30 minutes, during which time the miracidium loses its cilia. Once in the snail, each transforms into the sporocyst stage, in which asexual replication occurs to produce the next form, the rediae. Thereafter, numerous free-swimming, infective cercariae are formed.

The cercariae occur in a variety of shapes depending on the species. Upon release from the snail, the cercariae seek out and invade the flesh of crustaceans or fish (i.e., the second intermediate host), through the action of enzymes produced by several paraoral penetration glands. The definitive host becomes infected by ingesting the metacercaria contained in raw or inadequately cooked crustacean or fish meat. There are some exceptions to this general life cycle among the trematodes. For example, in the life cycle of *Fasciola* and *Fasciolopsis* species, the cercariae become attached to water plants as inactive, encapsulated metacercaria, rather than infecting a second

intermediate host. Humans are infected by these parasites by ingesting raw or poorly cooked water plants on which these metacercariae are attached.

Intestinal Protozoa

Four broad groups of intestinal protozoa are currently recognized, as listed below: (1) the amoebae, (2) the flagellates, (3) the ciliates, and (4) the coccidia. The task of learning the differential features of these protozoa is somewhat lessened when one realizes that only a few species are of medical importance within each of these major groups. Following the classification proposed by Levine et al.,[152] the following are the intestinal protozoa that will be presented here:

Ameba
- *Entamoeba histolytica/Entamoeba dispar/Entamoeba hartmanni*
- *Entamoeba dispar*
- *Entamoeba coli*
- *Entamoeba polecki*
- *Iodamoeba bütschlii*
- *Endolimax nana*

Flagellates
- *Giardia intestinalis* (formerly *G. duodenalis*)
- *Chilomastix mesnili*
- *Dientamoeba fragilis*
- *Pentatrichomonas hominis*

Ciliates
- *Balantidium coli*

Coccidia
- *Cryptosporidium* species
- *Cystoisospora (Isospora) belli*
- *Sarcocystis* species
- *Cyclospora* species

The Intestinal Amoebae

Three genera of amoebae may inhabit the intestinal tract of humans: *Entamoeba*, *Iodamoeba,* and *Endolimax*. Some of these, such as *Entamoeba coli,* are considered nonpathogenic. It is important that these are objectively identified in stool specimens and not be confused with other protozoa that may cause disease. Additionally, it is usually evident that the patient has been exposed to unclean food or water. Of the amoebae, *Entamoeba histolytica* is the principal pathogen for humans. At the onset of

training, students must develop an approach by which they can consistently identify this parasite when seen in stool specimens. The *Entamoeba histolytica* "look-alikes," *E. hartmanni*, and the newly designated species *Entamoeba dispar*, to be described in more detail later, have been found to be nonpathogenic and of little clinical significance when observed in stool specimens.[59]

Amebiasis and *Entamoeba histolytica*. Amebic dysentery is caused by pathogenic strains of *E. histolytica*.[210] The organism is transmitted via the direct fecal–oral route through contaminated water or food supplies, either directly from the excreta of infected persons or indirectly by cockroaches or flies that can act as mechanical vectors. Improvements in sanitation, socioeconomic conditions, and alterations in specific practices such as hand washing, fly control, improved water quality, and sanitary facilities are necessary to control parasite transmission. An estimated, 5% to 50% of individuals in endemic areas are intestinal carriers of *Entamoeba histolytica*. Ten percent of these individuals will develop invasive, dysenteric bowel disease, whereas 0.5% will develop extraintestinal disease, most commonly liver abscesses. Of those with liver abscesses, 2% to 10% die. The death rate can reach as high as 70% in patients with fulminant colitis. The clinical syndromes associated with *E. histolytica* infections are presented in Clinical Correlation Box 22-1.

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CLINICAL CORRELATION BOX 22-1 Amebiasis

The disease syndromes resulting from infection with *E. histolytica* have been outlined by Guerrant.[105] The majority of humans infected with *E. histolytica* (80%–99%) are asymptomatic, as determined by observing for excreted cysts in fecal screening examinations or from the data of serologic surveys. A majority of those with symptoms have disease limited to the gastrointestinal tract, following an average incubation period of 1–4 weeks after infection. The typical large bowel lesions are called "buttonhole" ulcers because the trophozoites that penetrate through the mucosa are incapable of digesting the musculature of the bowel wall, and therefore extend laterally along the submucosa. (Image Plate 22-2D illustrates several trophozoites in the submucosa.) Lower abdominal pain, low-grade fever, and bloody diarrhea with or without tenesmus are the usual presenting symptoms. Pregnancy, malnutrition, underlying metabolic diseases, and corticosteroid therapy predispose to more serious disease. Complications are relatively uncommon, developing in 1%–4% of cases, and include bowel perforation and peritonitis. Paracecal amebomas, at times forming an annular inflammatory mass simulating colonic carcinoma, have been reported.

Extraintestinal amebiasis occurs with varying frequency in different geographical areas. In Mexico, invasive disease develops in about one in every five infected individuals, compared with only one in 100–1,000 in the United States. The onset of symptoms may be within days after the acute bout of dysentery, or it may be delayed by months or even years. The liver is the most common organ of extraintestinal involvement. Adams and McLeod[3] report that in up to 50% of cases of amebic liver abscesses, a history of intestinal amebiasis is lacking and trophozoites or cysts cannot be detected in stool specimens. Symptoms include weight loss, low-grade fever, weakness, and vague right upper quadrant discomfort and elicitation of pain between the ribs on palpation. Anemia, leukocytosis, and an elevated alkaline phosphatase are other supporting findings. The diagnosis is usually made by observing the characteristic single, large defect in the right lobe with a liver scan. Direct extension into the pleura or pericardium or metastatic foci in the brain, lung, and kidney is an uncommon event.

The life cycle of *E. histolytica*, as a prototype for all intestinal ameba, is shown in Figure 22-3.

Laboratory Identification. The cysts and trophozoites of the intestinal amoebae must be differentiated from a variety of confusing background artifacts that may be present in microscopic mounts and smears of fecal material.[96] Welsh[265] denotes that the correct identification of amoebae is dependent on the accurate observations of skilled laboratory workers, which laments the high frequency of both overidentification and underidentification of amoebae in stool specimens. This is evidenced often by the poor performance of national proficiency testing surveys. Several studies were cited, indicating that in up to one-third of laboratories in the United States and other countries, *E. histolytica* was not correctly identified; furthermore, several outbreaks of presumed amebiasis investigated by the CDC were false alarms because of laboratory misidentification errors. Pollen grains, incompletely digested vegetable elements, and host-derived somatic cells are among artifacts that can be confusing. Several commonly encountered artifacts are illustrated in Image Plate 22-1.

In particular, amebic cysts must be differentiated from active polymorphonuclear leukocytes that may have two or more lobes to their nuclei. The cytoplasm of leukocytes, however, is coarse, the nucleus is without evidence of a distinct karyosome, and the nuclear membrane is thick with no evidence of attached chromatin (Image Plate 22-2A). Microscopists can minimize the chances of error by making sure that parasitic structures are objectively identified before making a positive identification. If there is any doubt, one should search for more convincing forms in the same preparation, examine additional mounts from the same specimen, consult with a more experienced person, or request that additional stool specimens be obtained.

Once it is determined that a form observed in a stool specimen is indeed an ameba, either a trophozoite or a cyst, a detailed examination of the nucleus should be undertaken to determine whether chromatin has been deposited on the nuclear membrane, giving the appearance of a densely stained ring (Image Plate 22-2B). This ring of chromatin is characteristic of an "*Entamoeba*"-type nucleus. The nuclei of amoebae belonging to the other genera, *Iodamoeba* and *Endolimax*, are devoid of this peripheral placement of chromatin and appear more as a "ball in socket," with a large karyosome in a nucleus devoid of the thickened peripheral chromatin (Image Plate 22-2I, J, and K).

Entamoeba histolytica Versus *Entamoeba coli*

Definitive Microscopic Criteria for Differentiation

- The trophozoites of *E. histolytica* have a unidirectional motility; that is, they extend pseudopods along only one plane when observed in a microscopic mount. Conversely, the trophozoites of *Entamoeba coli* extend pseudopods in multiple planes. These features can only be observed in fresh stool preparations, which are not commonly available.
- The trophozoites of *E. histolytica* also have the unique capability of ingesting erythrocytes; it is extremely rare that ingested red cells will be seen in *Entamoeba coli* trophozoites. Gonzalez-Ruiz et al.[95] found that erythrophagocytic strains of *E. histolytica* always demonstrate a pathogenic zymodeme pattern. They concluded that erythrocyte phagocytosis is virtually 100% specific for *Entamoeba histolytica* and when observed in fecal mounts is predictive of infection with an invasive strain.

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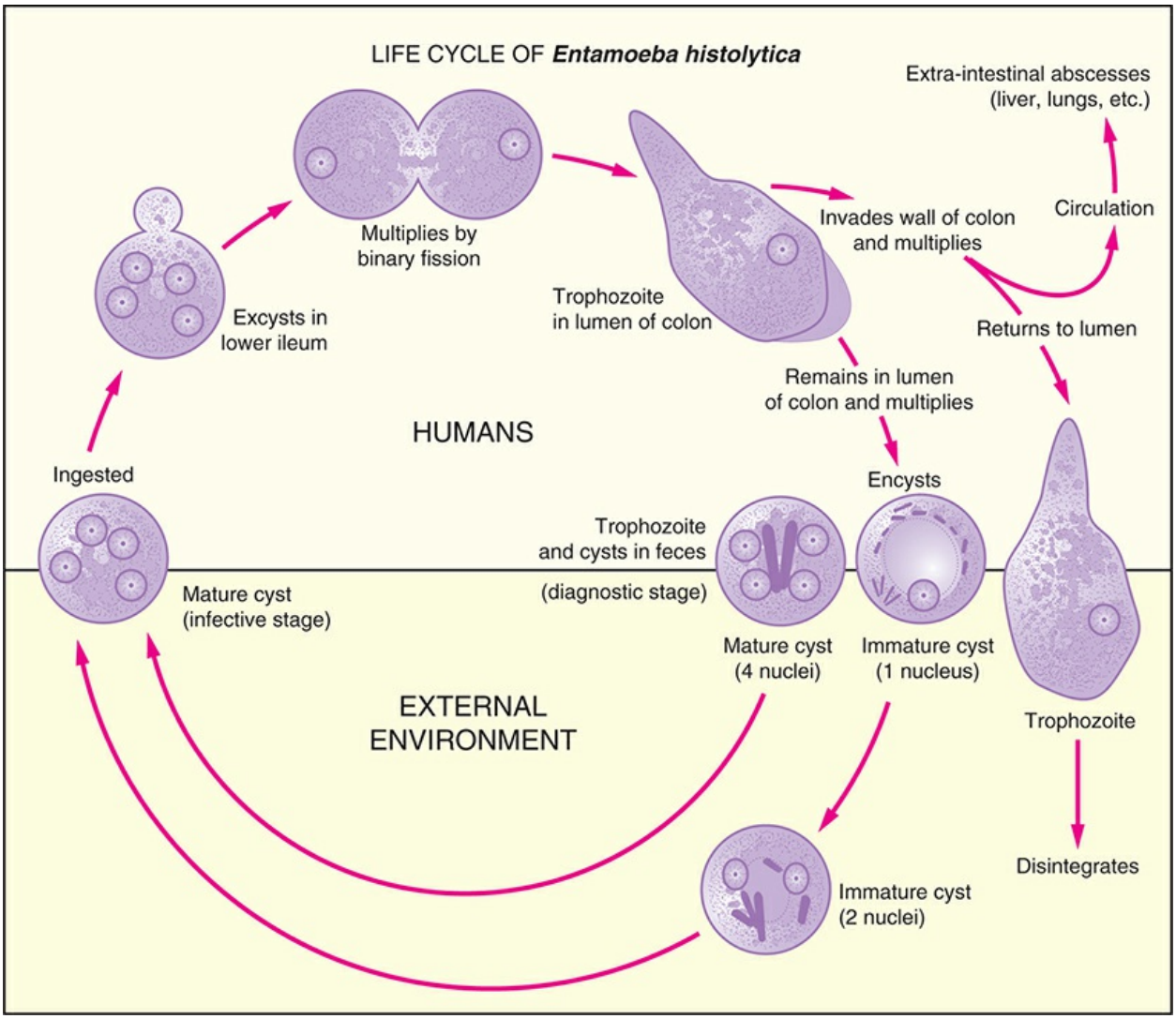

■ **FIGURE 22-3** The life cycle of *Entamoeba histolytica* is simple, with no intermediate hosts required. Resistant cyst forms are found in the external environment, which, when ingested by a human in contaminated food or water, excyst in the intestine and develop into free-living, motile, feeding trophozoite forms. These trophozoites may invade the intestinal mucosa, and potentially can reach the circulation and cause extraintestinal abscesses in various organs, particularly the liver.

- An *Entamoeba*-type cyst that possesses more than four nuclei rules out *E. histolytica* (Image Plate 22-2F).

Secondary Microscopic Criteria for Differentiating *E. histolytica* From *E. coli.* All other identifying characteristics between these two amoebae are secondary, may be inconsistent, and can lead only to a presumptive identification when observed.

- The trophozoites and the cysts of *Entamoeba* coli (15 to 50 µm [usual range: 20 to 30 µm] and 10 to 35 µm [usual range: 15 to 20 µm], respectively) on the average are larger than those of *Entamoeba histolytica* (12 to 40 µm [usual: 10 to 20 µm] and 10 to 20 µm [usual: 12 to 14 µm], respectively); however, there is sufficient overlap in size to reduce the significance of this observation.
- The intranuclear karyosomes of *E. histolytica*, whether observed in trophozoites or in cysts, tend to be tiny and more centrally placed (Image Plate 22-2B), in contrast to the relatively larger and more eccentrically positioned karyosomes of *Entamoeba coli* (Image Plate 22-2E).
- The ring of nuclear chromatin of *E. histolytica* is evenly dispersed and beadlike (Image Plate 22-2B), whereas it is more blotchy (i.e., chunky) in distribution in *Entamoeba coli* (Image Plate 22-2E).
- The cytoplasm of *E. histolytica* trophozoites tends to be smooth or finely granular (Image Plate 22-2B), whereas cytoplasm of *Entamoeba coli* trophozoites is more filled with debris and phagocytosed bacteria or yeasts (Image Plate 22-2E).
- Although chromatoid bars are seen in only about 10% to 15% of cysts, when present, those of *E. histolytica*

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(and *E. hartmanni*) have smooth, rounded ends (Image Plate 22-2H), whereas those seen in the cysts of *Entamoeba coli* are fragmented and frayed in appearance, possibly with pointed ends (Image Plate 22-2F).

By combining two or more of these features when observing several amebic forms in a given microscopic mount, a highly presumptive if not definitive identification can usually be rendered.

The critical observation of an endameba-type nucleus of an amebic form in a microscopic mount may also be useful in two other situations. The precysts of both *E. histolytica* and *Entamoeba coli* can form prominent intracytoplasmic vacuoles, closely simulating the glycogen mass characteristic of *Iodamoeba bütschlii* (Image Plate 22-2I). *Entamoeba hartmanni* develops both trophozoites and cysts that are on the average smaller, within the 5 to 15 µm, range that is the same size range as *Endolimax nana*. Again, differentiating the endameba-type nuclei characteristic of the "ball-in-socket" nucleus present in both *Endolimax and Iodamoeba* will minimize confusion between these two species (In Image Plate 22-2, compare Frames B and K).

Entamoeba polecki is another member of the *Entamoeba* genus that may rarely be found in human stool specimens, particularly in individuals who may have close association with pigs. Considerable experience is necessary before the microscopic forms of this organism can be objectively identified in stool specimens. The

trophozoite resembles *Entamoeba coli* in size, sharing the sluggish pattern of motility, the eccentric location of the nuclear karyosome, and the "dirty" appearance of the cytoplasm. The cysts typically have only one nucleus (never more than two) with a small, eccentric karyosome and fine, even distribution of chromatin along the nuclear membrane. Small, multiple, iodine-positive glycogen masses may be seen in the cytoplasm. A human infection caused by this parasite has been reported in a Japanese refugee.

Serologic Diagnosis of Amebiasis. Sensitive and specific serodiagnosis of invasive amebiasis has been demonstrated by Lotter et al.,[160] who used immunoblotting and enzyme-linked immunosorbent assay (ELISA) techniques to detect a specific recombinant surface protein of pathogenic *E. histolytica*. Cummins et al.[48] did pioneering work to use rapid latex agglutination test to detect patients with extraluminal amebiasis. More recently, van Doorn and colleagues assessed three assays, a rapid dipstick test, a latex agglutination test, and an ELISA, on well-characterized patients with extraintestinal amebiasis, intestinal amebiasis, and cyst passage. All assays had a sensitivity of 93.3%, with specificities that varied from 98.1%, 99.5%, and 97.1%, respectively.[249] A number of enzyme immunoassays (EIAs) are commercially available for the serologic detection of patients with *E. histolytica.* These have replaced more cumbersome serologic assays used previously. These assays are most useful for patients with extraintestinal disease, who do not have detectable parasites present in the stool. The CDC reports that these EIAs detect approximately 95% of patients with extraintestinal disease. The sensitivity decreases to 70% for patients with active intestinal disease and 10% for asymptomatic carriers, but performs well (95%) in the patient population wherein the diagnosis may be most challenging because of the absence of parasites in the stool.

There are at least two assays that detect the antigens of *E. histolytica.* These assays unfortunately cannot be performed on preserved stool specimens and require either fresh or frozen stool. Although Haque and associates[108] reported these assays to be useful for the diagnosis of patients with amebic liver abscess, this case is not corroborated by Zeehaida and colleagues, who found it positive in only 8.6% of patients with liver abscess. This latter finding is not surprising, since many of these patients may no longer have detectable parasites in the stool. Far better sensitivities have been demonstrated when antigen detection was compared with stool microscopy. Further guidance regarding parasitic serology and antigen testing is available through the CDC (http://www.cdc.gov/dpdx/diagnosticProcedures/serum/tests.html) and additional publications.[109]

Nonpathogenic *Entamoeba histolytica: Entamoeba dispar*. As early as 1925, Brumpt first recognized that severe dysentery or amebic liver abscess develops in only

about 10% of individuals infected with *E. histolytica*, while 90% of intestinal carriers remain asymptomatic. This prompted the hypothesis that amebic infections are caused by two species with the same morphology but with different pathogenicities.[22] Thus, the presence of *E. histolytica* cysts in stool specimens does not necessarily indicate active infection; it indicates that only certain strains are capable of tissue invasion.[234]

In 1978, Sargeaunt and Williams[219] used starch gel electrophoresis to demonstrate that extracts obtained from amoebae grown in axenic cultures had different isoenzyme patterns. Specifically, the reactivity of four enzymes, glucose phosphate isomerase, phosphoglucomutase, malate dehydrogenase, and hexokinase, resulted in 18 so-called zymodeme patterns. This group of investigators later found that only *E. histolytica* strains demonstrating certain zymodeme patterns were associated with human infections. Furthermore, only nonpathogenic zymodeme patterns were found in the 20% of homosexual men attending sexually transmitted disease clinics who harbored intestinal *E. histolytica*.[5] The further demonstration by Weinke et al.[262] that only *E. histolytica* isolates with pathogenic zymodemes were recovered from five travelers returning from the tropics, in contrast with a group of 320 male homosexuals who had neither pathogenic zymodemes nor symptoms of amebiasis, led to the realization that methods must be developed by which these pathogenic and nonpathogenic strains could be differentiated.[220]

During the late 1980s and early 1990s, many questions remained unanswered concerning the pathogenicity of certain strains of *E. histolytica.* The indigenous bacterial microbiota and the ability of *E. histolytica* to modulate its virulence depending on culture conditions were thought to play a role.[173] Geurrant[105] and Ravdin[209] discuss other virulence mechanisms characteristic of *E. histolytica* to produce disease. These included (1) chemically defined adherence factors (galactose-specific adhesin) that determine whether the organism can attach to the bowel mucosa, (2) the secretion of cytotoxins, including hyaluronidase, trypsin, pepsin, gelatinase, and hydrolytic enzymes for casein, fibrin, and hemoglobin, and (3) the production of proteolytic enzymes.

The host uses several defenses to protect against invasion by parasites.[200] Pancreatic proteases, bile salts, and bacterial

glycosidases may destroy the galactose-specific adhesin on the ameba surface and block adherence. Colonic mucins, produced in response to contact of the epithelial cells by amoebae, can also effectively block adherence by binding the carbohydrate recognition domain of the galactose adhesin. Humans who have debilitating disease, are malnourished, or have defective immune responses are particularly prone to both

intestinal and extraintestinal disease.

The issue of the underlying mechanism for pathogenicity of *E. histolytica* was finally brought to conclusion by Clark and Diamond,[39] who used molecular methods based on analysis of stable DNA polymorphisms to identify the DNA patterns unique to different strains of *E. histolytica*. It was through these studies based on molecular biology, coupled with those of Orozco and colleagues,[191] who cloned, sequenced, and characterized various *E. histolytica* clones and strains, that the differences in pathogenic and nonpathogenic strains of *E. histolytica*, and the expression of their various differences in zymodeme patterns, were found to be based on encoded genetic differences. Clark and Diamond[39] have officially retained the name of the classic, pathogenic strain as *Entamoeba histolytica*, and given the nonpathogenic strain the designation *Entamoeba dispar*.[22]

Efficient techniques have been developed to differentiate between these two species. Antigen-detection and PCR-based methods based on amplification of rRNA genes are most commonly used. Antigen-detection tests are relatively simple to perform, provide for rapid results, and are best suited for use in most diagnostic parasitology laboratories, and for use in the field. Enzyme immunoassay (EIA) kits are commercially available for detection of fecal antigens for the diagnosis of intestinal amebiasis. These assays use monoclonal antibodies that detect the galactose-inhibitable adherence protein in the pathogenic *E. histolytica*. The primary drawback of these assays is the requirement for fresh, unpreserved stool specimens. Several EIA kits for antigen detection of the *E. histolytica*/*E. dispar* group are available in the United States; when assessing these for use, it is important to determine whether the assay is specific for *E. histolytica* or whether it detects both *E. histolytica* and *E. dispar*.

Yau et al.[281] developed monoclonal antibodies against a recombinant 170-kDa subunit of the Gal or GalNAc lectin of *Entamoeba histolytica* that specifically recognizes *E. histolytica* but not *Entamoeba dispar* in preserved stool samples. These antibodies were found not to cross-react with other bowel protozoa, including *Entamoeba coli, Giardia duodenalis*, and *Dientamoeba fragilis.* Haque and colleagues[109] demonstrated that the performance of the antigen-detection kit, TechLab (Blacksburg, VA) *E. histolytica* test, designed to specifically identify *E. histolytica* and differentiate it from *E. dispar* in stool specimens, was comparable in sensitivity to a nested PCR procedure run in parallel.

Other workers have found that PCR-based tests were more sensitive than antigen-detection procedures in the detection of *E. histolytica/E. dispar.* Katzwinkel-Wladarsch et al.,[138] using a simple DNA extraction method and PCR amplification, were able to detect the gene that encoded for an *E. histolytica*-specific antigen directly in stool specimens containing as few as one trophozoite per milligram of feces. Acuna-Soto et al.[2] demonstrated that PCR techniques could also be used to detect *E.*

histolytica in formalin-fixed stool specimens. Blessmann et al.[18] using a real-time PCR technique were able to detect as little as 0.1 parasites per gram of feces. Of note, the sensitivity of this PCR procedure indicated that both microscopic examination of stool specimens and culture underestimate the presence of *E. histolytica* infections. The disadvantages of PCR procedures are the amount of time consumed in performing the test, its relative complexity, the cumbersomeness of performance, the requirement for special equipment, and the relatively high expense. Newer, commercially available multiplex assays for enteric pathogens, some of which are parasite pathogens, are easier to use, more cost-competitive, and likely to be widely used.

Given that a high percentage of persons in whom morphologic forms resembling *E. histolytica/E. dispar* are observed in stool specimens may not require treatment, follow-up *E. histolytica*-specific antigen-detection or PCR-based procedures should be performed in the future to correctly identify the nonpathogenic *E. dispar* subgroup. The reagent kits currently available for transport of specimens to a reference laboratory require the processing of a fresh stool specimen for antigen detection. If therapy is not prescribed in patients in whom *E. histolytica/E. dispar* organisms are identified in stool specimens, the patients should at least be informed that they are carrying potentially virulent organisms requiring that they practice preventive measures accordingly. Metronidazole (Flagyl) is the treatment of choice when indicated.

Other Intestinal Amoebae. Two other commonly encountered intestinal protozoa, *Iodamoeba bütschlii* and *Endolimax nana*, warrant further consideration. The CDC still characterizes these as among the ameba that are generally not considered pathogenic. It is important from the laboratory perspective that these organisms are correctly identified in stool specimens, and not be confused with other protozoa that may cause disease. Therefore, the identifying criteria for each are as follows.

Iodamoeba bütschlii

- The trophozoite, which ranges from 6 to 25 μm, possesses a "ball-in-socket"-type nucleus. The smaller forms are difficult to distinguish from *Endolimax nana,* and a search for the more distinctive cysts should be made (Image Plate 22-2I).
- The cyst, which ranges from 6 to 15 μm, in addition to the "ball-in-socket" nucleus, possesses a unique, large, iodine-positive staining glycogen vacuole (the structure from which the genus name is derived) (Image Plate 22-2J).
- *Note:* Early precysts of *Entamoeba coli* and, less frequently, of *E. histolytica* may also have a cytoplasmic inclusion that appears similar (Image Plate 22-2G). However, more than one nucleus may be observed, each with a ring of chromatin on the nuclear membrane, characteristic of the so-called *Entamoeba*-type nucleus.

Endolimax nana

- Relatively small size, ranging from 5 to 8 μm.
- Observing trophozoites with a single nucleus devoid of peripheral chromatin, and a large "ball-in-socket" karyosome (Image Plate 22-2K).

- The cysts are also small (5 to 14 μm), and have up to four nuclei, each of which has a relatively large, blot-like karyosome (Image Plate 22-2L).
- *Entamoeba hartmanni*, although in the same size range as *E. nana*, can be distinguished primarily because both the trophozoite and the cysts possess an *Entamoeba*-type nucleus, in contrast to the "ball-in-socket" nucleus of *E. nana*.
- The cysts of *Entamoeba hartmanni* typically contain one or more chromatoid bars, with smooth, rounded end margins (Image Plate 22-2H).

Other Protozoa *Blastocystis hominis*. *Blastocystis hominis*, once thought to be a yeast, has been classified as a belonging to the phylum *Bigyra*. There is some debate regarding whether this phylum best resides in the kingdom *Protista* or in *Chromista*, which contains certain algae. Zierdt[286] considered this organism to have the following characteristics:

- Has no cell wall.
- Grows only in the presence of bacteria and not on fungal media.
- Has a preference for alkaline pH and a mildly hypotonic environment.
- Reproduces by binary fission and not by budding.
- Extends and retracts pseudopods and ingests bacteria.
- Is optimally active at 37°C, does not grow at 25°C, and is killed at 4°C.

Yet, Stenzel and Boreham[240] point out that little is known of the basic biology of the organism, and controversy surrounds its taxonomy and pathogenicity. Three morphologic forms (vacuolar, granular, and ameboid) have been described, although additional forms (cyst, avacuolar, and multivacuolar) have also been observed. Little is known about the biochemistry of the organism, and organelles and structures of unknown function and composition are present in the cells. Several life cycles have been proposed, but none have been experimentally validated, and the form involved in transmission has not been defined.

Morphologically, the forms of *B. hominis* present in human specimens have the following features:

- Appear as irregularly sized spherical cells ranging from 5 to 15 μm in diameter, although smaller forms may occasionally be found (Image Plate 22-2M).

- Possess a homogeneous staining (i.e., green in trichrome stains) central body that occupies[3] 70% of the cell.
- Have nuclear material either scattered in undefined fragments between the central body and the outer membrane, or
- Appear as one or two elongated masses in a bipolar distribution.

MacPherson and MacQueen[162] denote the morphologic variability in size, shape, nuclear detail, and central body characteristics between cells, which may account to some extent for the differences in the incidence of detection of *B. hominis* in various reported studies.

Early studies by Zierdt[286] and additional studies in the mid-1980s[81,150,251] were offered as evidence that *B. hominis* may be a cause of gastrointestinal disease. The potential pathogenic nature of *B. hominis* is supported by the instances of recurrent diarrhea associated with this organism as reported by Vannatta[251] and Lebar.[150] In the latter study, numerous *B. hominis* (i.e., 5 per high-power field) forms were seen in stool specimens in patients in whom cultures for bacterial pathogens and tests for rotavirus particles were negative. The symptoms subsided when these patients received antiprotozoal therapy. Recurrent diarrhea without fever, episodes of vomiting, and cramping abdominal pain have been the chief symptoms.

Blastocystis hominis may be a pathogen when no other agent is found, or when the parasite burden is high.[270] Garcia and associates,[82] in a review of more than 6,000 stool specimens, found that 289 (4.8%) were positive for *B. hominis.* This organism was the only parasite found in two-thirds of the patients. Within this group, gastrointestinal symptoms were noted in 24 patients, the majority of whom had underlying debilitating disease or were immunosuppressed. The clinical picture shows that patients with *B. hominis* consist of nonspecific abdominal pain, watery diarrhea, anorexia, vomiting, and weight loss. Rarely, a more invasive form of the disease with rectal bleeding can occur. Antonelli et al.[8] describe a 10-year-old girl who was hospitalized for diarrhea, abdominal pain, and fever. Many *B. hominis* forms were demonstrated in her stools, and she responded favorably to treatment with metronidazole. The authors, therefore, cite this incident as another in recognition of the fact that *B. hominis* should be considered a human pathogen.

In rebuttal, Markell and Udkow,[166] in a study of 32 persons with *B. hominis* detected in the stool in whom at least six stool examinations were performed, found that 27 had other recognized pathogens—*E. histolytica*, *Giardia*, or *Dientamoeba fragilis*. In all of these patients, symptoms subsided with therapy. In the five remaining patients in whom only *B. hominis* was found, treatment with iodoquinol eradicated the organisms, but symptoms more consistent with irritable bowel syndrome persisted. Nagler et al.,[180] in a study of 12 patients with exacerbated

inflammatory bowel disease in whom stool specimens were positive for *B. hominis*, also concluded that this organism is not a significant pathogen for this entity. All of their patients improved on medical therapy, three on treatment with corticosteroids alone and one on bowel rest without medication. Five patients failed to improve on metronidazole, but four responded to subsequent courses of corticosteroids. Keystone and Kozarsky,[142] applying antigen, isoenzyme, and DNA analyses, indicate that *Blastocystis hominis* may be more than one organism, with both virulent and avirulent strains identified. Such findings may account for the differences in apparent virulence as reported by various workers, similar to that which was discovered for *E. histolytica* and *E. dispar*.

Until the issue of pathogenicity is settled, clinical laboratory parasitologists should still indicate the detection of *B. hominis* in stool specimens in the final report. Some form of quantitation should be made (e.g., rare, few, moderate, many); specifically, the presence of five or more *B. hominis* forms per high-power field is considered in the moderate to many category, and is reported by most laboratories. The clinician must evaluate whether such reports have clinical significance and decide whether to treat based on the clinical presentation.

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Intestinal Flagellates

All of the organisms in this group are motile by the means of one or more flagella. Other structures also serve as an integral part of the locomotor organelle, namely, the kinetoplast to which the flagella are attached and the axostyle and parabasal bodies. Therefore, when any of these structures are identified in a parasitic form, the parasite can be tentatively grouped with the flagellates. Unlike the amoebae, which assume variable shapes, the flagellates are more rigid and tend to retain distinctive shapes, a feature often helpful in their identification. *Giardia intestinalis*, *Chilomastix mesnili*, *Pentatrichomonas hominis*, and *Dientamoeba fragilis* are the species of flagellates most commonly seen in human stool specimens submitted for examination.

***Giardia intestinalis* (Formerly *Giardia duodenalis*)**. By the late 1980s, *Giardia intestinalis* had become the most frequent cause of parasitic gastrointestinal disease in the United States,[202] and has become a major worldwide public health challenge.[70,127,149,228] In a past surveillance study, *Giardia* was found in 3.8% of 414,820 stool specimens submitted to state health laboratories.[27] There have been numerous outbreaks of giardiasis, with addition outbreaks reported each year.[77,100,176] In 2010, there were 19,927 cases of giardiasis reported, which is significantly less than the 27,778 cases reported in 1996; there has been a steady downward trend in the

incidence of disease year over year.[80,283] A number of outbreaks of giardiasis in children in day care settings have also been reported.[239,274] The high prevalence of giardiasis is worldwide, with endemic areas reported in England, Russia, several countries in Eastern Europe, and many coastal areas of the Mediterranean.[228] *Giardia intestinalis* was found in 10% of children residing in the Ain-Shams and El-Mowassa orphanages in Cairo[222]; it was the most common parasitic disease (11.63%) found among Muslims coming for the annual "Hajj and Omra" pilgrimages to Mecca.

The life cycle of *Giardia intestinalis*, representative of the intestinal flagellates, is shown in Figure 22-4. The clinical syndromes associated with *Giardia* infections are presented in Clinical Correlation Box 22-2.

Giardia intestinalis is readily identified in microscopic mounts of fecal specimens. The key identifying features are as follows:

Trophozoite

- The size ranges between 9 and 21 μm long by 5 to 15 μm wide, is bilaterally symmetrical, and has two nuclei, one on either side of a central axostyle (giving the appearance of a "face") (Image Plate 22-2O).
- Cytoplasm is finely granular. Two median parabasal bodies, simulating a "mustache," are located on either side of the axostyle. Sucking disks occupy half of the ventral surface.
- Organisms attach to the surface of epithelial cells, a feature important for the pathologist (Image Plate 22-2N).
- In wet preparations, has a graceful "falling leaf" motility is seen, albeit this is not frequently performed. If ever needed, this feature is useful in distinguishing it from *C. mesnili*, which has a slower, stiff motion, and from *T. hominis*, the movement of which is quick, jerky, and darting.

Cyst

- Cysts measure from 8 to 12 μm, is oval in outline, and may be lightly bile-stained.
- When mature, the cysts possess four nuclei, with a small karyosome that is eccentrically placed.
- There is no peripheral chromatin on the nuclear membrane.
- There is a clear space beneath the thin cyst wall, producing an easy-to-recognize "halo" effect (Image Plate 22-2P).
- Ill-defined longitudinal fibrils and four median bodies are present.

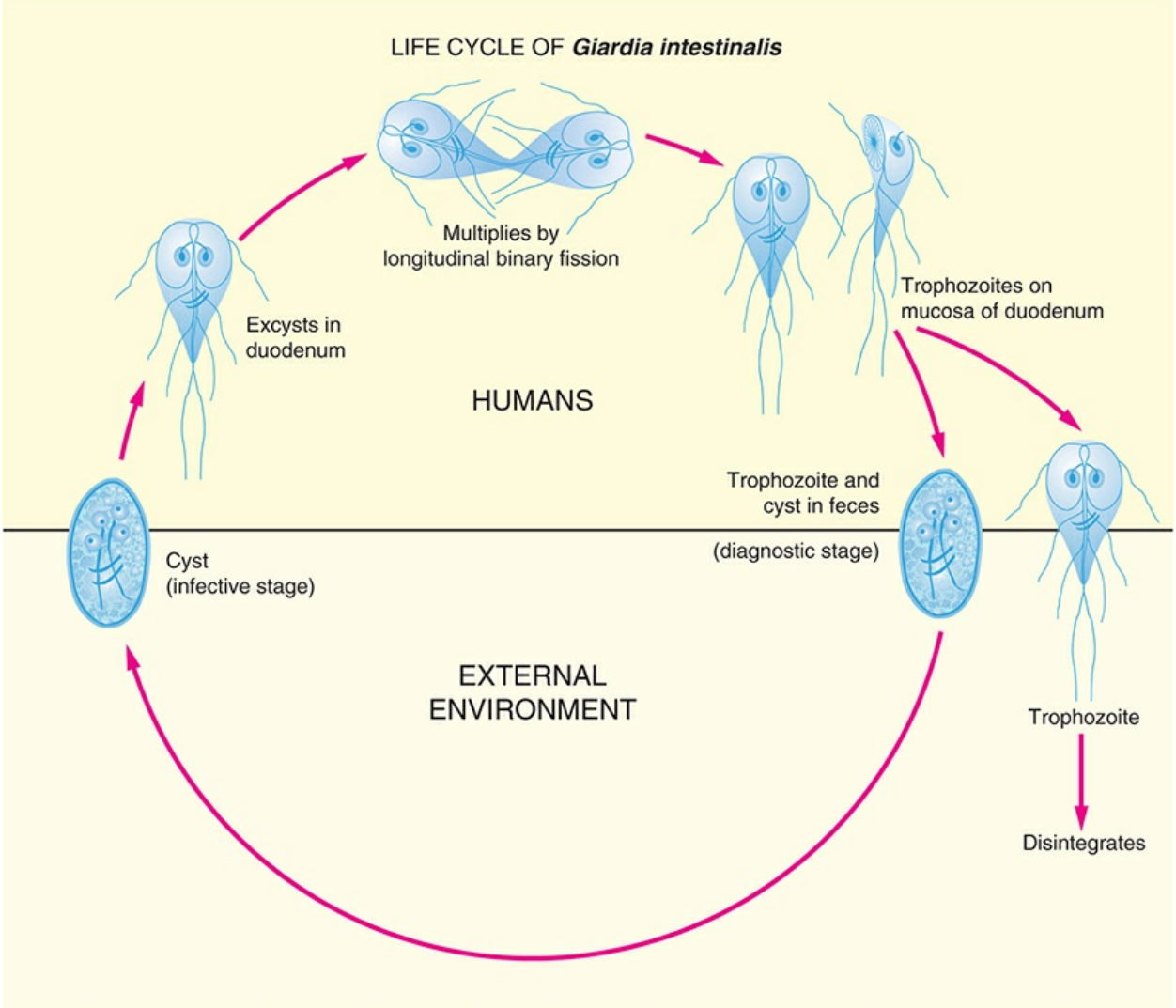
Because of irregular shedding of organisms in the feces, several specimens should be obtained on nonsuccessive days and may be required to establish a diagnosis in suspected cases, particularly in chronic disease. In a study reported by Heymans et

al.,[120] 53% of the patients had symptoms referable to giardiasis that existed for 6 months or more. The yield can be improved by preparing stained smears from concentrated fecal specimens, as organisms may often be missed if only saline mount procedures are performed.[86,231] Heymans and associates[120] also found that the examination of three stool specimens on nonconsecutive days had a sensitivity of 95.7% in detecting organisms in patients known to have giardiasis. Organisms were found in only three of 109 duodenal biopsies in patients whose stool examinations were negative (false-negative rate of 2.8%). Similarly, McHenry et al.[172] found only one positive aspirate in 144 duodenal aspirates in patients with nonspecific gastrointestinal symptoms. The conclusion can be drawn, therefore, that extraordinary procedures need not be performed to establish the diagnosis of giardiasis if there have been negative stool examinations.

The string test is an alternative to duodenal aspiration or biopsy procedures for patients highly suspected to have giardiasis in whom a diagnosis has not been established.[137] This procedure is described above, but is rarely used in practice.

Immunofluorescent Detection. Garcia et al.[84] reported 100% sensitivity and specificity in the detection of *Giardia duodenalis* cysts and *Cryptosporidium* oocysts in fecal specimens, using the MERIFLUOR (Meridian Diagnostics) direct immunofluorescence detection system. *Giardia* cysts appeared as oval, apple-green fluorescing forms measuring 11 to 14 μm in diameter, whereas the *Cryptosporidium* oocysts measured 4 to 6 μm in diameter and displayed the same fluorescence.[260] An advantage of this technology is the ability to screen smears under low-power magnification and the ability to detect forms in low concentration as major advantages of the direct fluorescence system.[80] It is not uncommon to find organisms by the fluorescence procedure that are not observed by routine ova and parasite examination.

Antigen Detection. The detection of antigens on the surface of organisms or shed from organisms in stool specimens is the current test of choice for diagnosis of giardiasis.[133] It provides increased sensitivity over more common microscopy techniques. There are multiple commercial products to choose from for the immunodiagnosis of giardiasis. These are either plate-based colorimetric immunoassays or lateral flow immunoassays. Most can be used on fresh or frozen stool samples, and those preserved in formalin, merthiolate-iodine-formaldehyde (MIF), and sodium acetate-acetic acid-formalin (SAF) fixatives, but not in PVA. The sensitivity and specificity of these kits approach 100% when compared with microscopy.



■ **FIGURE 22-4** The life cycle of *Giardia intestinalis* is also simply completed by a fecal–oral route of transmission, with the cysts providing for a long-term survival in the external environment under adverse environmental conditions. Cysts commonly find their way into streams and wells from inadequately controlled sewer systems, and mountain streams downriver from residential areas are commonly contaminated. Some animals also harbor *Giardia*. Humans become infected by ingesting fecally contaminated water or poorly washed fresh foods. In the human intestine, trophozoite multiplication occurs via binary fission. Trophozoites and cysts are passed in the feces to complete the life cycle.

CLINICAL CORRELATION BOX 22-2 Giardiasis[272]

Symptoms

Giardia intestinalis, also known of as *Giardia duodenalis,* is a known cause of acute

diarrhea, abdominal pain, and in some cases, constitutional symptoms such as weight loss and lassitude. In patients with chronic infections, malabsorption and steatorrhea simulating sprue may be experienced. The exact mechanism by which *Giardia* causes disease is not known. Physical occlusion of the mucosa, bile salt deconjugation, enterotoxin excretion, prostaglandin release, and injury to the mucosal epithelial cell brush border are several hypotheses reviewed by Smith and Wolfe.[238] The drugs of choice in the treatment of giardiasis when clinically indicated are metronidazole (Flagyl) or tinidazole.

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Epidemiology
Most infections are sporadic, contracted after exposure to contaminated food or water under a variety of circumstances. Waterborne outbreaks have been cited by Smith and Wolfe[238] in locales as diverse as Colorado, Russia, and New York and among campers in the western United States. In a follow-up on the Aspen/Snowmass outbreak, Lapham and associates[149] found only two cases of giardiasis among 225 visitors, a rate far lower than that reported during the previously cited outbreak. Giardiasis has been reported among individuals drinking water from an unfiltered municipal water supply in Dunedin, New Zealand[77]; in a chlorinated community water supply in Penticton, British Columbia[176]; among preschool children drinking from school water supplies in Lesotho[70]; and from use of a new hotel water slide that had been cleansed by both Bromide and sand filtration.[100] Although Holtan[127] and others have cited campers as a high-risk group for contracting giardiasis, Welch[264] found such risk as minimal at best, based on a review of 104 manuscripts relative to backcountry water as a potential source for acquiring infection. The author placed greater emphasis on observing careful hand washing techniques over water purification to decrease the risk for infection. There is a zoonotic potential that giardiasis is also carried by domestic animals such as calves and lambs (and perhaps wild rodents as well), which may serve as a reservoir of infection. Children attending day care centers have in the past also been placed in a high-risk group for contracting giardiasis, as evidenced by three reported past outbreaks in Wisconsin,[239] with attack rates of 47%, 17%, and 37%, respectively; and in Southern Ontario, Canada,[274] where almost 10% of children between the ages 2 and 5 years old were found to harbor *Giardia cysts*.

Several of these rapid immunochromatographic (ICT) assays are available in a combined format that includes the detection of either *Cryptosporidium* or *Giardia.* At least one product also offers the detection of *Cryptosporidium parvum*,

Giardiaduodenalis, and *Entamoeba histolytica,* but this assay requires fresh stool. These offer the advantage of short test time and multiple results in one reaction device with excellent sensitivity and specificity.

Since the early 1990s, the diagnosis of giardiasis had been established from the use of immunologic methods designed to directly detect *Giardia duodenalis* antigen in fecal specimens.[147] EIAs using antibodies prepared against a variety of *Giardia* antigens developed as the technology most often used.[241] Knisley and colleagues,[147] using rabbit and goat antisera following immunization with *Giardia* trophozoites, reported sensitivities of 92% and 87% and specificities of 87% and 91%, respectively. Carlson and coworkers[26] reported a 97% correlation between enzyme immunoassay and direct microscopic examination on 353 specimens from human subjects in the United States. Stibbs,[241] using a monoclonal antibody-based antigen-capture enzyme immunoassay for the detection of *Giardia duodenalis* antigen in human stool specimens, also found a 97% correlation with microscopic examinations, including formalin-fixed specimens.

Rostoff and associates[216] have evaluated a commercially available enzyme immunoassay (ProSpecT/Giardia; Alexon, Mountain View, CA) that detects *Giardia*-specific antigen 65 (GAS 65). All of 93 specimens obtained from symptomatic patients who had a positive microscopic stool examination for *Giardia* gave strongly positive visual and spectrophotometric results. Of 232 randomly collected specimens, only six with a negative microscopic stool examination had positive EIA results. A review of the clinical evidence strongly suggested that these six patients in fact had giardiasis. With these results taken into account, the EIA test was performed with a sensitivity of 96% and a specificity of 100%. Similarly, Scheffler and Van Etta[224] also demonstrated that antigen detection is also highly sensitive and specific when testing formalin-preserved stool samples. The ProSpecT/*Giardia* kit had a sensitivity of 95%, a specificity of 100%, positive predictive value of 99.5%, and a negative predictive value of 100%relative to conventional microscopy in the study of 223 formalin-preserved stool specimens. In summary, the EIAs for *Giardia* are simple to use and reliable methods for the rapid assessment of stool specimens for the more prevalent intestinal parasite in North America.

Other Intestinal Flagellates

Chilomastix mesnili. *Chilomastix mesnili* is a flagellate of cosmopolitan distribution, more commonly found in persons living in warm climates. It is considered to be a nonpathogen, and treatment is not required when forms are found in fecal specimens. Infections are acquired through ingestion of feces-contaminated food and water; improved personal hygiene and sanitation are paramount to reduce the incidence of colonization with this organism.

The key features for identification are as follows:

Trophozoites

- These are pear-shaped (i.e., pyriform) in outline, measuring 6 to 24 µm in length and 4 to 8 µm in width (Image Plate 22-3A).
- There is a single large nucleus immediately beneath the outer membrane.
- A prominent cytosome is adjacent to the nucleus.
- There are three anterior flagella immediately adjacent to the nucleus, which are often difficult to visualize, but possibly seen by reducing the amount of condenser light and sharpening the focus.

Cysts

- These are pear- to lemon-shaped and range from 6 to 10 µm long, by 4 to 6 µm wide (Image Plate 22-3B).
- There is a distinctive hyaline knob on one side (Image Plate 22-3C).

- These possess a single nucleus with a small central karyosome.
- Characteristically there is curved cytostome, appearing as a "shepherd's crook," which is diagnostic when observed.

Dientamoeba fragilis. *Dientamoeba fragilis* is a flagellate,[279] even though flagella are not observed with the light microscope. In practice, this organism may appear as an amebic trophozoite, rather than as flagellate in microscopic mounts. It is pathogenic, producing a syndrome characterized by persistent diarrhea, abdominal pain, loss of appetite, weight loss, flatulence, and anal pruritus.[269] Microbiologists in clinical laboratories should be aware that *D. fragilis* is being recovered from stool specimens with increasing frequency. It has been reported in 1.4% to 19% of specimens submitted for routine examination and in up to 47% of defined populations, such as patients in mental health facilities.[279] In some settings, identification of *D. fragilis* in stool specimens is as frequent as *Giardia duodenalis;* in fact, in laboratories where *G. duodenalis* is being identified to the exclusion of *D. fragilis*, a review of collection and diagnostic methods may be in order. Because the organisms are fragile and a cyst is not produced, *Dientamoeba fragilis* may be difficult to identify in wet mounts and concentrates. A thorough review of the permanently stained preparation is virtually mandatory for detection of this parasite.

Grendon et al.[101] warn that failure to use recommended stool fixation and permanent staining techniques virtually precludes identification of *D. fragilis*. To increase probability, they strongly recommend that all stools submitted for examination should be fixed in PVA, SAF, or Schaudinn's fixatives, and that all specimens, regardless of consistency, should be permanently stained prior to microscopic

examination. Chan et al.[30] successfully used an indirect fluorescence antibody assay to improve screening of preserved fecal specimens for *D. fragilis*, and further suggested that the development of ELISAs to detect *D. fragilis* antigens in fecal specimens would improve detection.

The identification of *D. fragilis* is made in fecal preparations by observing the trophozoite. A cyst stage has not convincingly been identified. There has been a claim that a cyst form has been discovered, but this has not been independently corroborated. Regardless, cysts are not readily seen in clinical specimen, so even if they are rarely produced, they are not of diagnostic value. The diagnostic morphologic features of *D. fragilis* are as follows:

- The appearance of an asymmetrical "ameboid" form, measuring 5 to 12 μm in dimension.
- The presence of two nuclei (Image Plate 22-3D). Approximately 20% of forms may possess only a single nucleus, and these forms are difficult to differentiate from the trophozoites of *Endolimax nana*. Scanning of the smear will usually reveal the more diagnostic, double-nucleated forms.
- Prominent karyosomes that are fragmented into four to eight granules may be seen and when present are diagnostically useful.
- Relatively broad-lobed, clear pseudopods that provide purposeful motility when observed in wet mounts; this is of limited utility, given the rarity of performing direct wet mounts on fresh stool.

Because *D. fragilis* does not have an identified or well-characterized cyst state, direct food-borne or waterborne transfer from host to host is less likely. The nine times higher incidence of *D. fragilis* in patients with pinworm infection suggests that the *Enterobius vermicularis* eggs may be infected with the flagellate and/or serve as the vector for transfer between humans. This possibility may also explain why almost 50% of reported cases of dientamoebiasis occur in patients under 20 years of age. The treatments of symptomatic patients have included tetracycline and metronidazole, among others.

Pentatrichomonas hominis. *Pentatrichomonas* (formerly *Trichomonas*) *hominis* has been encountered in all parts of the world. Although recovered from diarrheal stool specimens, it is not thought to be pathogenic. It may be somewhat more difficult to definitively identify this organism in stained fecal preparations because the trophozoites are fragile and do not stain well. There is no cyst stage.

Identification is made by detecting trophozoites, which possess the following features:

- Measure 7 to 15 μm long by 4 to 7 μm wide, are teardrop-shaped, and possess a single anterior-placed nucleus, which is slightly displaced from the outer

membrane, helping to distinguish it from *Chilomastix mesnili* (Image Plate 22-3E).

- These possess an undulating membrane that extends the full length of the organism. This is helpful in differentiating this organism from *Trichomonas vaginalis*, the undulating membrane of which extends only half or slightly more than half the distance of the body. In practice, this is not particularly useful, as the undulating membranes are not seen in regular trichrome-stained smears; rather, mounts of anoxic cultures are needed to demonstrate this feature.
- In wet mounts, which again are not common, have a somewhat stiff, rotary motility, from the action of a single flagellum that is located along an undulating membrane that extends the full length of the body.
- Typical forms when observed in stool specimens are most likely *T. hominis*. It is important not to mistake *T. hominis* for *T. vaginalis*, as the latter is a sexually transmitted disease.

Ciliates: Balantidium coli

Balantidium coli is the only member of the ciliates known to infect humans. The mode of transmission from humans to humans is via a simple fecal to oral route, and an intermediate host is not required for completion of the life cycle. The life cycle of *Balantidium coli* is shown in Figure 22-5. Although *Balantidium coli* is found primarily in swine and less commonly in other animals, it has a worldwide distribution. Human balantidiosis is most prevalent where pigs are raised and slaughtered.[86] Reports of infection are uncommon, with most coming from Latin America, the Far East, and New Guinea. Infestation in humans is generally noninvasive, asymptomatic, and self-limited. In debilitated patents carrying a heavy load of trophozoites, bloody dysentery, severe dehydration, or, in rare instances, death may result. These form "flask-shaped ulcers" similar to those produced by *Entamoeba histolytica.* Image Plate 22-3F demonstrates several *B. coli* trophozoites invading the submucosa of the bowel. This photomicrograph was prepared from a rare fatal case of *Balantidium* infection. Intestinal ulcers, mesenteric lymphadenitis, and rare extraintestinal extension to liver, lung, and other organs have been reported in isolated patients.[146] Tetracycline, metronidazole, and iodoquinol have all been used effectively.

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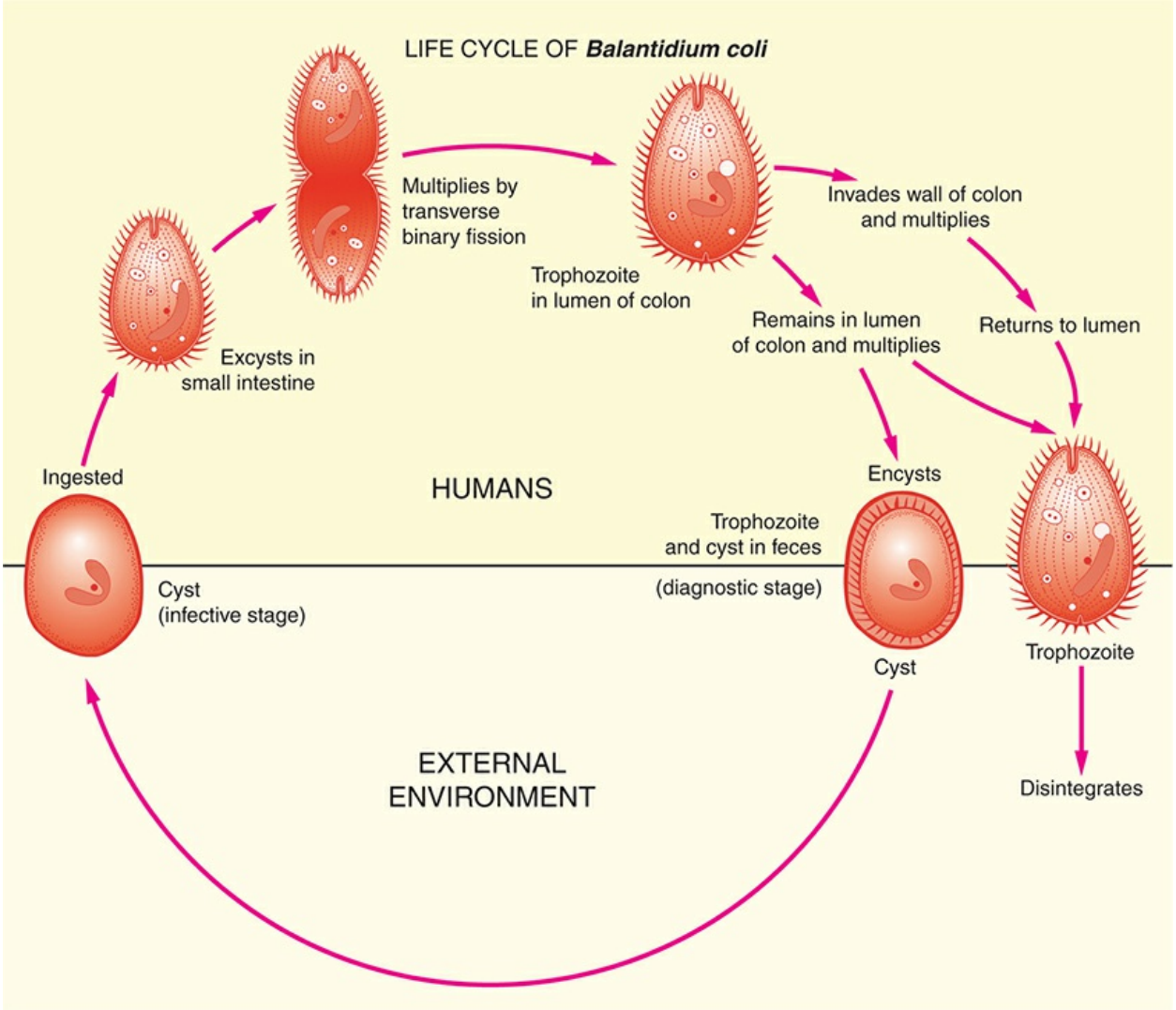


■ **FIGURE 22-5** The life cycle of *Balantidium coli* is simple, requiring no intermediate host. Cysts pass in feces into the external environment. On ingestion of cyst-contaminated food or water, excystation takes place in the small intestine. Then, there is multiplication by binary fission and trophozoites occupy the colon. On encystations, cysts are again passed in the feces to complete the life cycle.

Balantidium coli is readily recognized in stool specimens because of its large size (100 µm or greater in diameter). Additional diagnostic features include the following:

Trophozoite

- The cell membrane is covered throughout the circumference with short, delicate cilia.
- There is a large, kidney-shaped macronucleus. This is most visible in direct iodine-stained mounts (Image Plate 22-3G).

- A small, spherical micronucleus is present adjacent to the macronucleus. This may not always be readily apparent in every organism.
- A rotary, boring motility when observed in wet mounts.

Cyst

- Cysts are spherical to ellipsoid in shape, measuring 50 µm to 65 µm.
- A single kidney-shaped macronucleus, often with a small, spherical micronucleus (Image Plate 22-3H).
- Small vacuoles persist in the cytoplasm.
- Cilia are retracted within a thick, tough cyst wall.

Coccidia

The organisms that are commonly called coccidia are members of the phylum *Apicomplexa*, class *Coccidia*, and order *Eimeriida*. These are obligate tissue parasites with sexual and asexual stages in their life cycles. Included in this group are the malarial parasites (i.e., *Plasmodium* species), the tissue

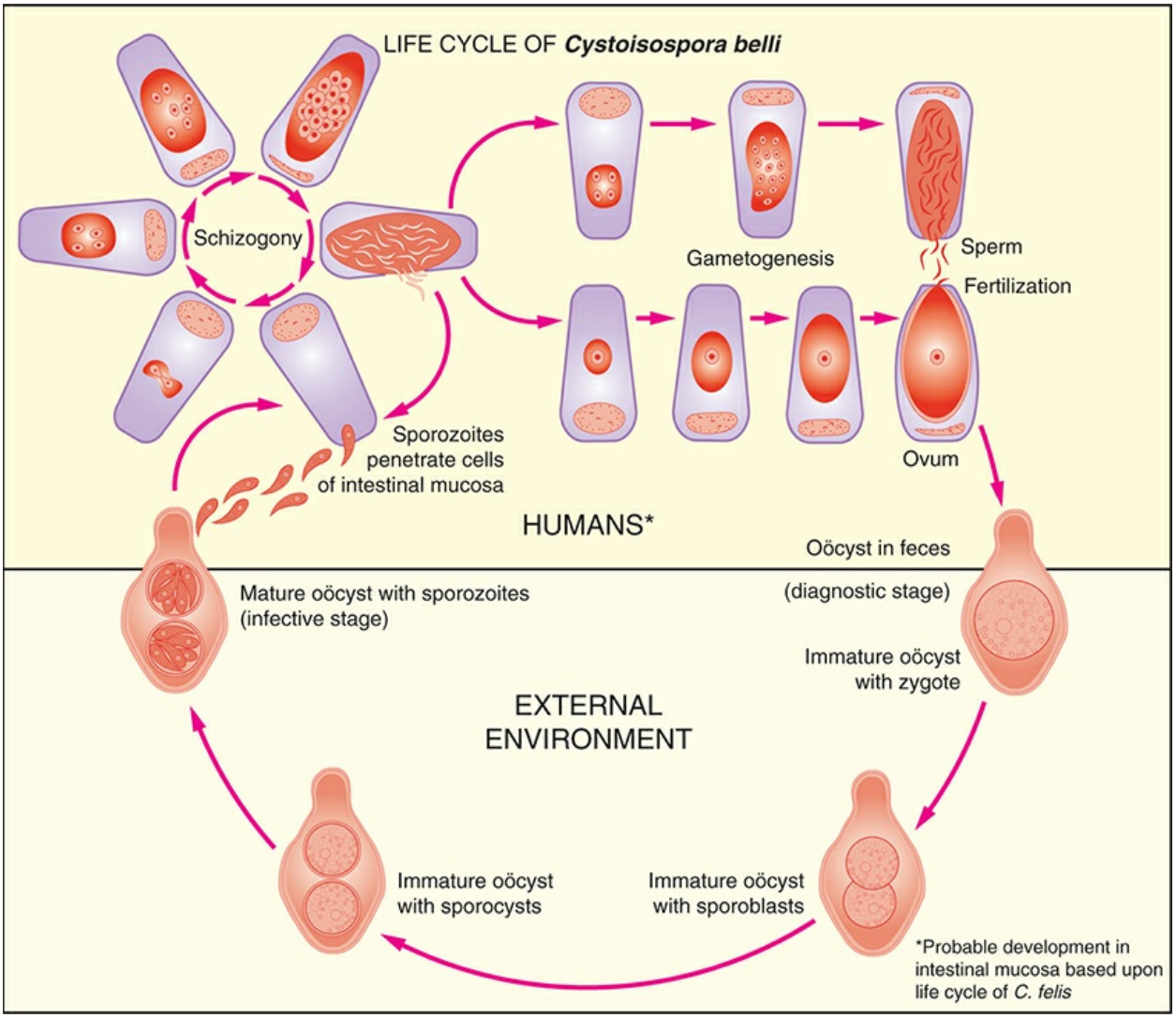
protozoan *Toxoplasma gondii*, and the intestinal coccidia—*Cystoisospora* (*Isospora*) *belli, Cryptosporidium* species, *Cyclospora* species, and *Sarcocystis* species.

The life cycle of most coccidia requires an external intermediate host, such as a cat, calf, human, or other animal, in which sporogenesis and oocyst formation takes place. The life cycle of *Cystoisospora belli* differs only in that both the sexual and asexual stages of gametogenesis occur in the same host without the need for a second host. The life cycle of *Cystoisospora belli*, as representative of the coccidia, is shown in Figure 22-6.

***Cryptosporidium* Species.** Cryptosporidia are minute coccidian protozoa that have been known to be associated with enterocolitis in a variety of domestic animals, including calves, pigs, and chickens.[7] Cryptosporidiosis came to the forefront of human medicine as an opportunistic infection seen in patients with AIDS through the 1980s.[248] At that time, it was appropriately characterized as an emerging infection.[49,184] It continues to cause significant morbidity and mortality in this patient population in resource-poor parts of the world wherein there is not access to highly active antiretroviral therapy. The detection of *Cryptosporidium* or *Cystoisospora belli*, which also infects HIV patients, should prompt testing for HIV infection and, if negative, for another immunocompromising condition.

Humans become infected either from direct contact with infected animals or from ingestion of fecally contaminated food or water. Curtailing the waterborne transmission

of



■ **FIGURE 22-6** Life cycle of *Cystoisospora belli*. Immature oocysts containing one sporocyst are released in the feces into the external environment. Under ideal environmental conditions, the oocyst matures and develops two sporozoites that serve as the infective form. On ingestion of infective oocysts in contaminated food or water by a human host, excystation takes place in the intestine. The released sporocysts invade the mucosal epithelial cells in which schizogony takes place. Mature sporozoites are released from the intestinal cells. In the process of gametogenesis, male and female gametocytes are formed. After fertilization, an oocyst with a thick cell wall develops. Release of these immature oocysts into the external environment completes the life cycle.

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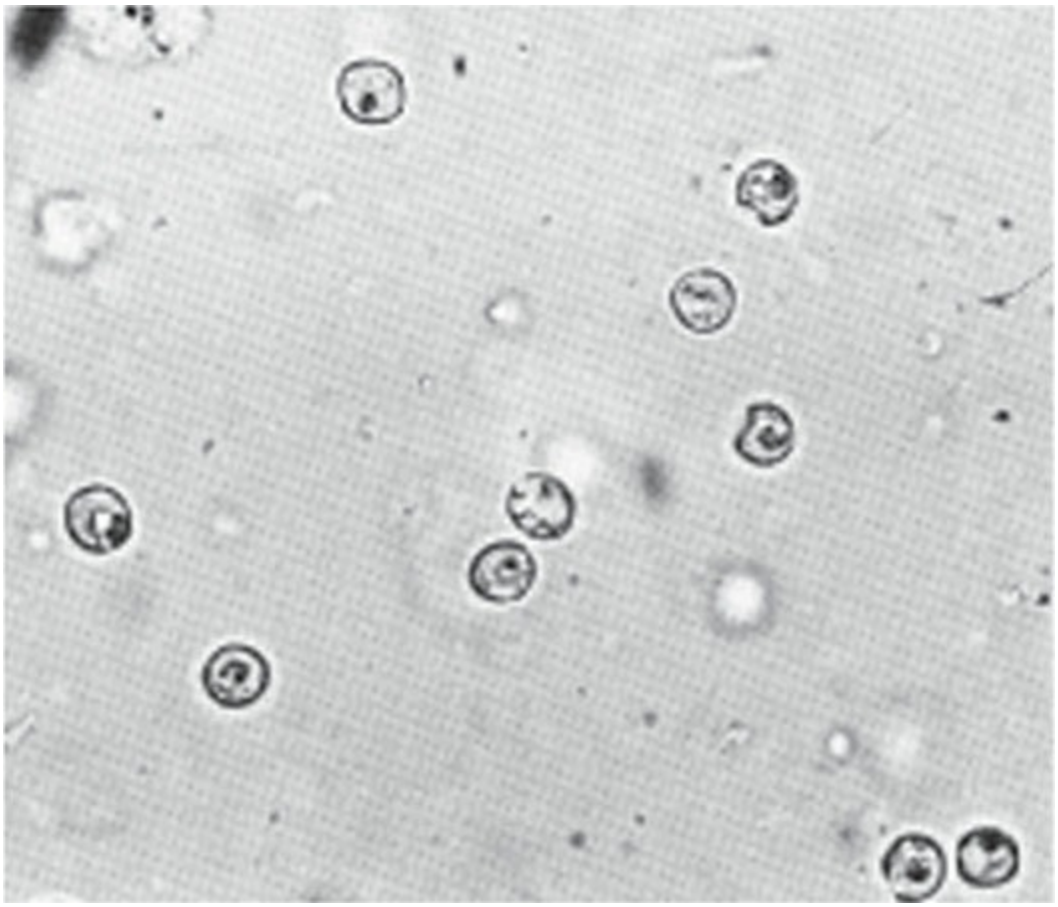
Cryptosporidium is particularly challenging, since the oocysts are resistant to chlorination and may persist in posttreatment water supplies.[237] Two major outbreaks of gastroenteritis and diarrheal disease have been reported in immunocompetent individuals, one in Milwaukee,[161] involving over 300,000 people, and the other in Carroll County, Georgia,[114] with an estimated 90,000 documented people contracting acute cryptosporidiosis. The only collective risk factor determined in each of these outbreaks was exposure to the public water supply, which was filtered and chlorinated in compliance with Environmental Protection Agency guidelines.

The organism during all stages of development is confined to the microvilli of intestinal epithelial cells. Thus, the diagnosis can be made in hematoxylin and eosin (H&E)–stained sections of small bowel by observing the tiny oocysts, often in huge numbers, attached to the surface of the epithelial cells lining the villi (Image Plate 22-4A). Oocysts have a propensity to adhere to the brush border of the epithelial cells with loss or degeneration of the microvilli at the attachment zone.[184] The loss of microvilli may result in impaired digestion, malabsorption, and diarrhea that make up the clinical syndrome. Substantial volumes of diarrhea that occur in the immunocompromised host with a severe infection cause electrolyte abnormalities and may be life threatening. The clinical manifestations of cryptosporidiosis are presented in Clinical Correlation Box 22-3.

The diagnosis is commonly made by identifying oocysts in fecal specimens.[136] These are extremely difficult to identify in the standard iodine wet mounts and modified trichrome-stained permanent smears. Effective detection relies upon the use of EIAs/immunofluorescent assays or the use of modified acid-fast staining. Cryptosporidia have the following characteristics:

- Cryptosporidia measure 5 to 6 μm in diameter, are ovoid to spherical, and appear highly refractile when observed in flotation preparations (Fig. 22-7).[156]
- Small granules may be observed internally, or, with phase contrast microscopy, up to four slender, bow-shaped sporozoites may be seen in each oocyst.[49,184] This is not commonly observed in standard preparations.



■ **FIGURE 22-7** Oocysts of *Cryptosporidium* as observed in a fecal flotation preparation (original magnification ×1,280). (Courtesy of Bruce C. Anderson.)

- These organisms appear as homogeneous, red-staining spherical oocysts, 4 to 6 µm in diameter, when observed in acid-fast–stained stool preparations (Image Plate 22-4B).

With heavy infections, the concentration techniques routinely used for the recovery of eggs and parasites in most laboratories are adequate for the recovery of *Cryptosporidium* oocysts. With light infections, Sheather's sugar flotation method may be performed as described by Garza[89] (Box 22-4), although this is rarely used in current practice. A modified formalin-ethyl acetate (FEA) stool concentration technique to detect *Cryptosporidium* oocysts in stool specimens is in common use. Following the usual FEA procedure, the sediment was layered and floated over a hypertonic saline solution to separate parasites from stool debris. This technique showed the most significant improvement in the recovery of oocysts from stool specimens that were formed and not fatty.

CLINICAL CORRELATION BOX 22-3 Cryptosporidiosis

The clinical syndrome of cryptosporidiosis includes a cholera-like, watery or mucous diarrhea, persistent gastroenteritis with varying degrees of vomiting and abdominal cramping, malabsorption, and low-grade fever. In immunocompetent patients, the syndrome is self-limited, with symptoms diminishing over 7–14 days. Cryptosporidiosis in patients with AIDS or other profoundly immunocompromising conditions, however, often is particularly troublesome because of the prolonged and progressively severe watery diarrhea that may last for months. In these patients, treatment must be aggressive, particularly if the CD4 count is below 200 per cubic millimeter.

Following infection, oocyst-positive stools occur between 7 and 28 days of infection, with a mean incubation period of 7.2 days. Conversely, Shepherd and coworkers,[229] in a study of 49 patients, found that most patients stopped shedding oocysts by 20 days, with symptoms related to shedding in 25 of the 49 patients. Thus, duration of diarrhea and the onset and length of time of oocyst shedding is patient-dependent.[136] Therapeutic regimens have largely been unsuccessful in severely immunocompromised patients, possibly because of the unique parasitophorous vacuole that is formed within the host cell, sheltering the parasite from antimicrobial drugs.

Currently, treatment with azithromycin,[267] nitazoxanide, and paromomycin[73] is partially active, but reversal of immunosuppression, if at all possible, is important to achieve parasite clearance.[40] Excellent reviews of the clinical, epidemiologic, and parasitic aspects of cryptosporidiosis have been published.[184,267]

BOX 22-4

Sheather's Flotation Technique for Recovery of *Cryptosporidium* Oocysts in Fecal Specimens

1. A heavy suspension of feces is made in physiologic saline solution and strained through gauze into a centrifuge tube to one-half full.
2. Add an equal volume of Sheather's sugar solution (500 g of sucrose, 320 mL of distilled water, and 615 g of melted phenol) to bring the surface of the liquid

slightly above the top of the tube. Gently mix the suspension with an applicator stick.

3. Place an 18 mm^2 or 22 mm^2 coverslip on the surface of the suspension, and let stand undisturbed for 45 minutes.
4. Gently remove the coverslip, and mount it on a glass slide. Observe under phase contrast microscopy for the spherical, highly refractile 5μm diameter oocysts.[87]

A modified acid-fast stain is commonly used to detect *Cryptosporidium* oocysts in air-dried, methanol-fixed smears prepared directly from a fecal sample.[89] The carbol fuchsin stain is applied to the smear in the same manner as the routine acid-fast stain; however, 1% H_2SO_4 is used as the decolorizer instead of acid alcohol. The oocysts appear pink-red against the light green background of the counterstain.

Several immunoassay kits are commercially available for the detection of *Cryptosporidium* oocysts in fecal specimens.[88] In a comparative study, Garcia and Shimizu[85] found a sensitivity of 98% to 99% and a specificity of 100% for the detection of *Cryptosporidium* oocysts using the ProSpecT (Alexon), the Meridian Premier *Cryptosporidium*, and the Meridian MERIFLUOR *Cryptosporidium/Giardia* EIA kits (Meridian Diagnostics). This affirms similar results previously reported by Kehl et al.,[140]who found that the Color Vue *Cryptosporidium* assay (Seardyn, Indianapolis, IN), the ProSpecT *Cryptosporidium* microtiter assay, and the MERIFLUOR *Cryptosporidium* kit, previously mentioned, were equally sensitive and specific for the detection of *Cryptosporidium*, although in their experience, the immunofluorescence procedure was considered easiest to read and required the least hands-on technologist time.

The extreme sensitivity and specificity make PCR-based methods for *Cryptosporidium* oocyst detection quite attractive, particularly in the analysis of drinking water. These PCR assays may detect as few as one oocyst in a sample, making them the most sensitive methods available.[79,177] The PCR detection of oocysts may also be affected by numerous substances found in stool specimens, for which special nucleic acid extraction techniques have been developed.[52,285] This has necessitated the optimization of methods to avoid amplification inhibition. More recently, several multiplex assays have been released, which take a syndromic approach to the diagnosis of infectious gastroenteritis. These are FDA-approved or pending approval, easy to use, and detect a wide variety of pathogens, such as the most common causes of bacterial, viral, and parasitic gastroenteritis, including *Cryptosporidium.* Clark has reviewed the biology, diagnosis, procedures for laboratory identification, and treatment of cryptosporidiosis for those who desire further

reading.[40]

Cyclospora cayetanensis. The organisms now included in the genus *Cyclospora* were originally thought to be cyanobacteria or blue-green algae-like organisms.[155,187,192,236] *Cyclospora* species have morphologic characteristics similar to *Cystoisospora* and *Cryptosporidium,* except that they have two sporocysts per oocyst and two sporozoites per sporocyst (*Cystoisospora belli* has two sporocysts per oocyst and four sporozoites per sporocyst; *Cryptosporidium* species have four nonencysted sporozoites per oocyst.)[75] The species name, *Cyclospora cayetanensis,* honors the Universidad Peruana Cayetano Heredia in Lima, Peru, the institution where much of the original epidemiologic and taxonomic research was performed.

In unstained, unconcentrated stool specimens, the oocysts of *Cyclospora* species appear as nonrefractile, spherical to oval, and slightly wrinkled bodies, measuring 8 to 10 μm in diameter. This is almost twice the size of the oocysts of *Cryptosporidium* species and an important differentiating feature. There is an internal cluster of membrane-bound globules within the cell.[16] These parasites are acid-fast and stain with a faint pink or pink-red color with darker staining internal structures (Image Plate 22-4C). Older cells may fail to stain. Therefore, the presence of both staining forms and non-staining forms is common, and is also a useful differentiating feature. The oocysts stain orange-red with safranin. They do not stain with iron hematoxylin, Grocott-Gomori methenamine silver, iodine, or periodic acid–Schiff (PAS).[155] The oocysts autofluoresce strong green (450 to 490 DM excitation filter), or intense blue (365 DM excitation filter) under epifluorescence. Directors and supervisors of microbiology laboratories must decide whether stool specimens are to be routinely examined for the presence of *Cryptosporidium* and *Cyclospora* organisms. In many settings, the performance of an acid-fast stain on a stool specimen requires a separate order. This additional stain could readily be added to the routine stool studies in outbreak situations. One of the recently released FDA-approved multiplex assays includes reagents that target *Cyclospora*.

The life cycle of *Cyclospora*, which is also a member of the apicomplexan, is similar to that of *Cryptosporidium* species. Sporozoites excyst from oocysts in the digestive tract soon after ingestion in contaminated food or water. The sporozoites enter the epithelial cells in the small intestine. The mechanism by which diarrhea is produced is not known; endoscopy reveals moderate to marked erythema of the distal duodenum. Duodenal and jejunal biopsies reveal blunting of jejunal villi, villous atrophy, and crypt hyperplasia of varying degrees. The absence of fecal leukocytes and erythrocytes indicates that the process is not invasive; impaired D-xylose absorption implies a malabsorption-type syndrome.

Illness usually follows ingestion of contaminated food or water and occurs primarily in the warm summer months. There are numerous outbreaks that have been reported, a review of which is instructive. Only a few will be mentioned here. Powdered milk diluted with water before consumption has been implicated.[277] In a Chicago outbreak at Cook County Hospital, diarrhea developed in house staff and employees in a dormitory following failure of the dormitory's water pump.[276] *Cyclospora*-linked disease often occurs as traveler's diarrhea, with several literature citations of diarrhea occurring in travelers returning from Haiti, Mexico, Guatemala, Puerto Rico, Morocco, Cambodia, Pakistan, India, the Solomon Islands, and particularly Nepal.[125,205]Specifically, outbreaks in the United States and Canada during the spring months of 1996 and 1997 were related to the ingestion of raspberries imported from Guatemala.[117] There have been 27 reported outbreaks of cyclosporiasis in the United States from 2000 to 2013. Although raspberries were suspected in at least four of these outbreaks, other implicated foods included basil, blackberries, snap peas, snow peas, and bagged salad mix (http://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html). Sifuentesosornio et al.[232] reviewed and compared cyclosporidiosis in patients with and without AIDS. The incubation period is 2 to 7 days. The onset of diarrhea may be abrupt (68% of cases) or gradual (32%).[225] Watery diarrhea, which is self-limited in immunocompetent patients (rarely lasting more than 12 days), may be accompanied by mild nausea, abdominal cramping, fatigue, and malaise.[229] In immunocompromised patients, the diarrhea may be prolonged, lasting 4 to 6 weeks, may simulate tropical sprue, and may be associated with biliary disease. The voluminous diarrhea that may occur in these patients leads to life-threatening dehydration and electrolyte abnormalities. In a review of stool specimens from 450 patients with AIDS, Pappe et al.[192] found *Cryptosporidium* species in the stool specimens of 135 (30%), *Cystoisospora belli* in 12%, *Cyclospora* in 11%, *Giardia* in 3%, and *Entamoeba histolytica* in 1%.

Specific pharmacotherapy has proven to have limited effectiveness. Oral trimethoprim–sulfamethoxazole has been effective in the treatment of some patients; metronidazole, norfloxacin, quinacrine, nalidixic acid, and diloxanide furoate have been used with varying success in others.[126,192] In profoundly immunocompromised patients, treatment is often limited to supportive care with hydration and nutritional supplements, although Pappe et al.[192] found trimethoprim–sulfamethoxazole effective in some patients, particularly when increased doses were prescribed. In a study conducted in Nepal, the shedding of oocysts and disappearance of symptoms occurred after 7 days of therapy.[125] All efforts should be made in reverse the immunosuppression, if possible, as the restored immune system will assist in the elimination of the infection.

Cystoisospora belli. Cystoisosporiasis, an illness characterized by diarrhea and malabsorption, was formerly only rarely encountered and was considered to be of limited clinical significance. Severe disease may be experienced in immunocompromised hosts, in which heavy concentrations of organisms may be seen in the intestine. Fever, headache, steatorrhea, and weight loss may occur in protracted cases; deaths due to dehydration and electrolyte imbalance have been reported in overwhelming infections. The disease may persist for months in these patients. It is recovered with increased frequency in patients with AIDS, and the detection of this organisms should prompt questioning regarding high-risk behaviors, HIV testing or other immunocompromising conditions. Garcia[87] reports several patients with AIDS who had debilitating intestinal and extraintestinal disease caused by this organism. It is further recommended that patients with HIV who plan to travel to Central and South America or resource-limited countries be advised of the waterborne and food-borne transmission of *C. belli,* and consider chemoprophylaxis. Although immunocompromised individuals are at risk for severe disease, cystoisosporiasis, immunocompetent individuals may also be infected.[199] The drug of choice is trimethoprim–sulfamethoxazole; pyrimethamine is an effective alternative.

Human *C. belli* infections differ from those of other coccidians in that both the sexual and asexual forms inhabit the human intestine. Therefore, a second host is not required to complete the life cycle. Human-to-human transmission can occur after ingesting infective oocysts in fecally contaminated food and water; thus, the disease is not a zoonosis.

The oocysts are the diagnostic forms observed in human fecal specimens. They measure 25 to 30 µm in diameter, have a thin, smooth wall, and are nonmotile in fresh stool. Immature oocysts contain a single sporocyst (Image Plate 22-4E); more typically, the mature oocysts that contain two sporocysts are observed in fecal specimens (Image Plate 22-4F).

***Sarcocystis* Species.** *Sarcocystis* species use two mammals for the sexual and asexual phases of their life cycles. Humans may serve as either the intermediate or the definitive host. As the definitive host, humans become infected by ingesting poorly cooked infected beef or pork; the sexual cycle of the organism develops in the subepithelial portion of the small bowel mucosa. Intestinal infections are associated with *S. hominis* or *S. suihominis,* are often asymptomatic, but may on occasion result in a diarrheal syndrome similar to that produced by *Cystoisospora belli*. The diagnostic forms in stool specimens are broadly oval oocysts measuring between 10 and 20 µm. They contain two sporocysts, appearing similar to those of *C. belli*. Single sporocysts measuring 13 to 17 µm may also be seen.

Acting as intermediate host, which occurs when humans inadvertently ingest oocysts from other animal stool sources, sarcocysts develop in the skeletal

musculature and may be observed in biopsies of skeletal muscle, or have been seen in skeletal muscle at autopsy.[14] The sarcocysts in skeletal muscle measure approximately 100 × 300 µm in dimension (Image Plate 22-4G). This form is usually asymptomatic, although a history of polymyositis and eosinophilia has been reported in rare instances. Arness et al.[9] reported an outbreak of acute eosinophilic myositis among a 15-man US military team working in rural Malaysia. Varying degrees of fever, myalgia, bronchospasm, fleeting pruritic rashes, transient lymphadenopathy, and subcutaneous nodules developed in seven soldiers; all of these symptoms are common for sarcocystosis. Laboratory abnormalities included eosinophilia, elevated erythrocyte sedimentation rates, and elevated levels of creatine kinase in muscle. *Sarcocystis* spp. were recovered from a

skeletal muscle biopsy specimen obtained from the patient who was identified as the index case. Symptoms persisted in some soldiers for as long as 5 years. In such prolonged cases, chronic myositis, fasciitis, and myonecrosis with calcification may be seen histologically.

Nematodes

The nematodes are helminth roundworms; the adults of which are characterized by a tapered, cylindrical body with longitudinally oriented muscles and a triradiate esophagus. The species of intestinal nematodes (roundworms) that most commonly infect humans include the following:

Ascaris lumbricoides
Trichuris trichiura
Hookworms: *Necator americanus* and *Ancylostoma duodenale*
Strongyloides stercoralis
Enterobius vermicularis

It has been estimated that the number of people worldwide infected with intestinal nematodes is in the billions, and this number increases as the world population grows, particularly in resource-poor areas.[20,24,31] The magnitude of these figures translates into considerable human morbidity and mortality from the effects of this parasite, from a variety of mechanisms, such as blood loss, malnutrition, and intestinal blockage. Therefore, it is highly likely that clinical laboratories will detect the eggs, larvae, or even an adult of one of these parasites.

The life cycles of this group of helminths vary in complexity and modes of infection. None of these nematodes, however, utilize an intermediate host in their life cycle (Fig. 22-8). However, most require a stage outside the human host for the ova to develop into an infective form.

The egg or ovum is a mature gamete produced by adult females residing in the intestine and passed with the feces. This form is the latent stage, serving as the infective form in most instances. It is also the form that will be seen in the microscopic examination of the stool and will be used to make the definitive diagnosis. The ova of most nematode species require intermediate stages of development in the external environment to develop into a viable larvae. This development is dependent on the temperature, moisture, and nature of the soil into which they are passed. Life cycle drawings with legends are included for several of the nematode species to be presented in this chapter.

Ascariasis and Ascaris lumbricoides

It has been estimated that *Ascaris lumbricoides* alone affects approximately 25% of the world's population, which is 1.7 a billion people.[24] The highest prevalence of disease occurs in often malnourished people residing in resource-poor countries. Areas with modern water and waste treatment have a low incidence of disease. *Ascaris lumbricoides* undertakes what has been termed an *indirect* life cycle. Following the ingestion of a fertilized egg and excystation, the larva penetrates the bowel wall and begins a migration to the lungs. It penetrates the lungs, enters the bronchioles, and is eventually swallowed. The second exposure to the gastrointestinal tract triggers the development of the adult form. These nematodes are highly adapted to their human host, and so minimal tissue damage occurs with migration. The transient eosinophilic pneumonia or pneumonitis that occurs is termed Löeffler's pneumonia. The migration of numerous worms from exposure to multiple eggs would be associated with more extensive tissue damage. This indirect life cycle will become very medically important for another nematode discussed below, *Strongyloides stercoralis.* Although infections with few worms may go medically unnoticed, mechanical obstruction of the intestine may occur with massive infections.[12] A life cycle drawing of *Ascaris lumbricoides* is found in Figure 22-8.

The clinical manifestations of ascariasis[245] are presented in Clinical Correlation Box 22-4.

Laboratory Identification. The laboratory diagnosis of ascariasis is made either by observing the adult worms as they protrude or are passed from body orifices or by visualizing in situ in the intestine or in contiguous duct systems such as the biliary or pancreatic ducts at endoscopy, surgery, or autopsy.[107,163] In some instances, these may be detected radiologically. Most commonly, the identification is made by detecting

the characteristic ova in stool specimens. The key identifying characteristics of *Ascaris lumbricoides* are as follows:

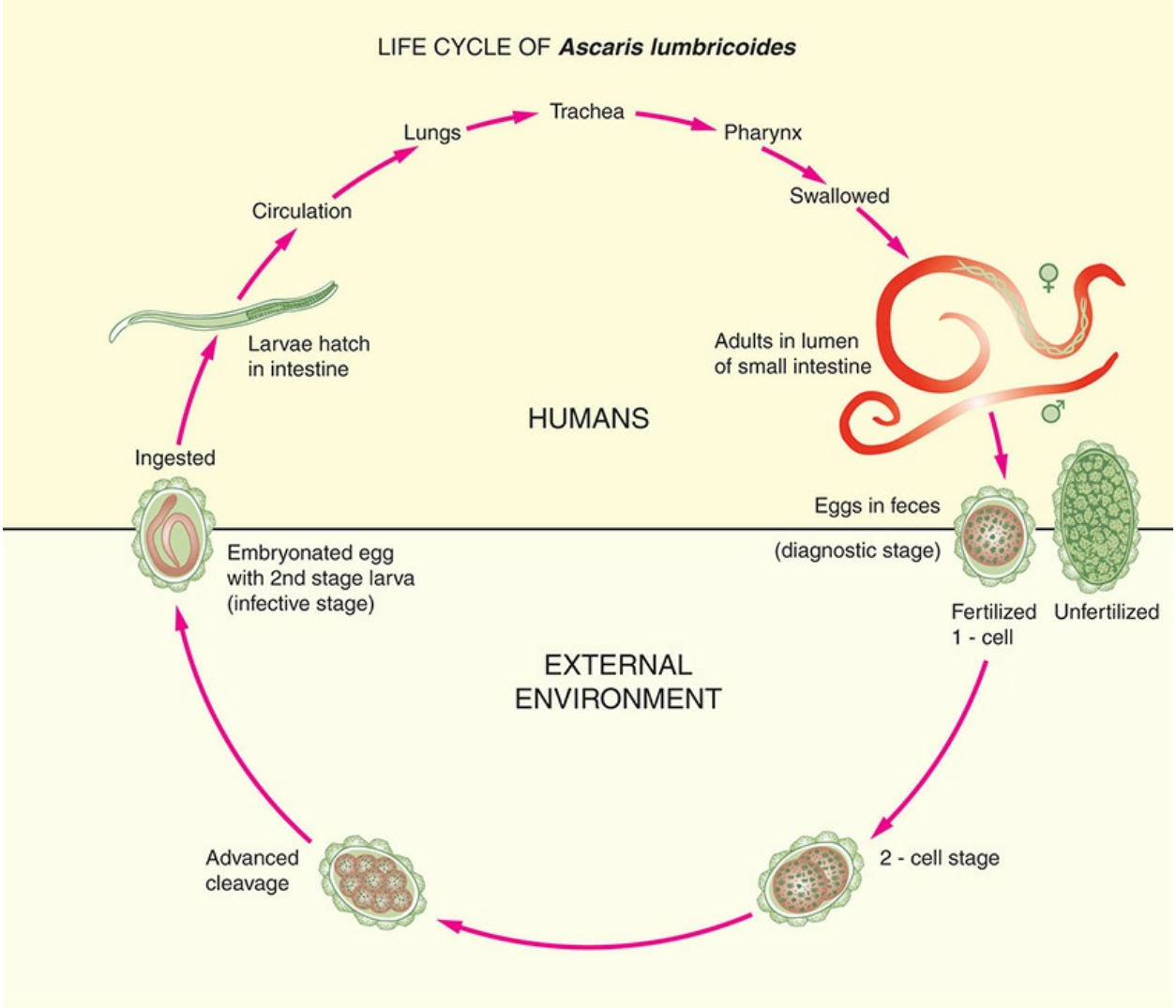
Adult Worms

- The adult worms measure between 15 and 35 cm in length.
- Male worms are smaller, and can be identified by their curved tail (Image Plate 22-5A).
- The cuticle is smooth and lacks the annular muscular striations characteristic of earth worms. It is important that parasitologists are aware of the morphology of earth worms, as occasionally these are submitted to the laboratory for identification.

Ova

- Fertilized eggs measure between 45 and 60 µm; unfertilized eggs average 90 × 40 µm.
- They are yellow-brown (bile-stained), oval, or spherical, and characteristically have a thick, transparent, hyaline shell covered by an albuminous coat (Image Plate 22-5B).
- Eggs that have had prolonged exposure to pancreatic secretions may be devoid of the albuminous coat (decorticoid).
- Fertilized eggs can be recognized by the cleavage of the internal yolk. Conversely, internal organization is lacking in unfertilized ova. Embryonated eggs in later stages of development may contain the larva (Image Plate 22-5C).
- Unfertilized, decorticate ova may resemble vegetable cells and can be extremely difficult to recognize in stool specimens (Image Plate 22-5D). In most instances, examination of additional fields will usually reveal the characteristic thick, outer mammillated albuminoid eggs.

Unfertilized *Ascaris lumbricoides* eggs are too heavy to float in the zinc sulfate flotation procedure and may be



■ **FIGURE 22-8** Life cycle of *Ascaris lumbricoides*. The life cycle does not involve an external intermediate host. It is somewhat complex, requiring a period for egg maturation in the external environment and a transpulmonary larval migratory phase in humans, which may result in transient asthma-like symptoms in heavy infections. Fertilized eggs passed into the soil require 2 to 3 weeks under ideal conditions of moisture and temperature to develop from the initial two-cell stage to advanced cleavage and the final infective embryonated stage. Upon ingestion of food or water contaminated with infective eggs by a human host, the larval forms hatch in the intestine and enter the circulation. After passage through the lung capillaries, the larvae break out into the pulmonary alveoli, then are coughed up and swallowed. In the intestine, they mature into male or female adult worms, where they may remain confined to the lumen or migrate to various ducts or orifices. Gravid females lay a large number of eggs that are passed with the feces to complete the life cycle.

CLINICAL CORRELATION BOX 22-4 Ascariasis

Patients with light infections may be asymptomatic. In heavy intestinal infections, abdominal pain, discomfort, and diarrhea are common findings. In contrast to hookworms, for which a heavy worm load is prerequisite to the presence of disease, a human intestinal infestation with only one *A. lumbricoides* worm, because of its large size, may be important. Ascarid adults have the propensity to migrate and wander into the bile ducts, pancreatic duct, or lumen of the appendix, or may rarely penetrate the bowel. Maddern et al.[163] reported a fatality caused by acute pancreatitis wherein a single *Ascaris lumbricoides* worm was found impacted within the ampulla of Vater. Appendicitis, pancreatitis, biliary obstruction, and hepatic abscess formation are all potential complications, even in light infections.[107]

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This propensity for ascarids to wander from the lumen of the intestine, particularly during drug therapy, is one reason why inadequate or incomplete courses of treatment can be particularly dangerous. In heavy infections, adult worms may protrude from the rectum or be coughed up. Intestinal obstruction, intussusception, volvulus, and bowel perforation are other potential complications of heavy infection. Intestinal obstruction occurs in approximately two of every 1,000 individuals infected, resulting in a fatality rate of six per 100,000 children.[245] Baird et al.[12] report an ascariasis hyperinfection in a 2-year-old South African girl from whom 796 *Ascaris lumbricoides* worms, weighing an aggregate of 550 g, were removed at autopsy. Torsion and gangrene of the heavily worm-infested ileum was the cause of death. Worms were also recovered from the stomach, esophagus, intrahepatic and extrahepatic bile ducts, and the gallbladder.

Treatment is with albendazole, which is also effective against *Trichuris trichiura*, a commonly found coinfecting organism. Mebendazole is an alternative treatment. Side effects include abdominal pain and diarrhea, particularly if infection is heavy and many worms are expelled. Use of the drug is contraindicated during pregnancy, wherein pyrantel pamoate is the alternative. There also is little evidence that drugs are helpful during the larval migratory stage of infection, so additional therapy may be needed.[87]

missed if the sediment is not also examined. The presence of only unfertilized ova in stool specimens may indicate infection with a single female worm. One adult female *Ascaris lumbricoides* can produce about 200,000 ova per day; therefore, the

enumeration of ova in fecal specimens using egg counts, a valuable procedure in evaluating the magnitude of hookworm infections, does not necessarily reflect the worm load in ascariasis.

Trichuriasis and Trichuris trichiura *(Whipworm)*

The overall incidence of *Trichuris trichiura* infections is not known, although a 90% prevalence has been cited in certain populations in Cameroon, Malaysia, and Caribbean countries. Vermud et al.[253] found that *T. trichiura* was the most common nematode recovered from 41,958 stool specimens submitted to the Columbia-Presbyterian Medical Center in New York.

The life cycle of *Trichuris trichiura* follows a simple fecal–oral route of transmission without an intermediate phase in an external host. This parasite has a so-called "direct" life cycle, since following ingestion of the egg the adult eventually develops in the intestinal tract without transpulmonary migration. The life cycle is illustrated in Figure 22-9. The clinical manifestations of trichuriasis are presented in Clinical Correlation Box 22-5.

Laboratory Identification. The laboratory diagnosis of trichuriasis is most commonly made by observing characteristic barrel-shaped ova in fecal specimens. The adults measure 30 to 50 mm in length, with the males on the average being slightly smaller. The males can be recognized by their long, thin tapering morphology that forms a 360-degree coil, from which the colloquial term *whipworm* is derived. In intestinal infections, the head is buried in the mucosa of the large bowel; therefore, adults are rarely seen in stool specimens. A magnified photograph of an adult worm is shown in Image Plate 22-5G, left.

Ova

- The eggs measure in the range of 54 × 22 μm.
- They are among the more readily recognized nematode eggs in microscopic preparations by their distinct barrel shape and the refractile, convex, hyaline polar plugs at either end (Image Plate 22-5F).
- These eggs may be confused only with the eggs of *Capillaria philippinensis.* However, the polar plugs of *C. philippinensis* are less prominent, are flat, and the shell is thicker and pitted (Image Plate 22-5P).

Enterobius vermicularis

"Pinworm" infections have been known since ancient times, and possibly represent the most common of all nematode infections. A well-known phrase is, "you may have had an infection as a child, or, if not, may likely get it when you have children." The life cycle of *Enterobius vermicularis,* like *Trichuris trichiura,* is a direct life cycle.

The eggs are spread by a fecal–oral route. Importantly, the eggs are infectious when they are deposited in the perianal area, meaning that direct human-to-human transmission occurs, similar to that of the intestinal amoebae.

Laboratory Identification. The diagnosis of pinworm infection may be made by observing adult worms either visually on the anal opening or microscopically in transparency (tape) mounts. These mounts are prepared by pressing the sticky side of a short segment of tape on the perianal folds or perineal skin, then sticking the tape to the surface of a glass slide for microscopic study (Image Plate 22-4F). The characteristic eggs are uncommonly seen in stool specimens (i.e., the diagnosis is not achieved using an O&P examination). Specimens collected from children early in the morning when worm migration is maximal have the greatest yield. The clinical manifestations of enterobiasis are illustrated in Clinical Correlation Box 22-6. The identifying characteristics are as follows:

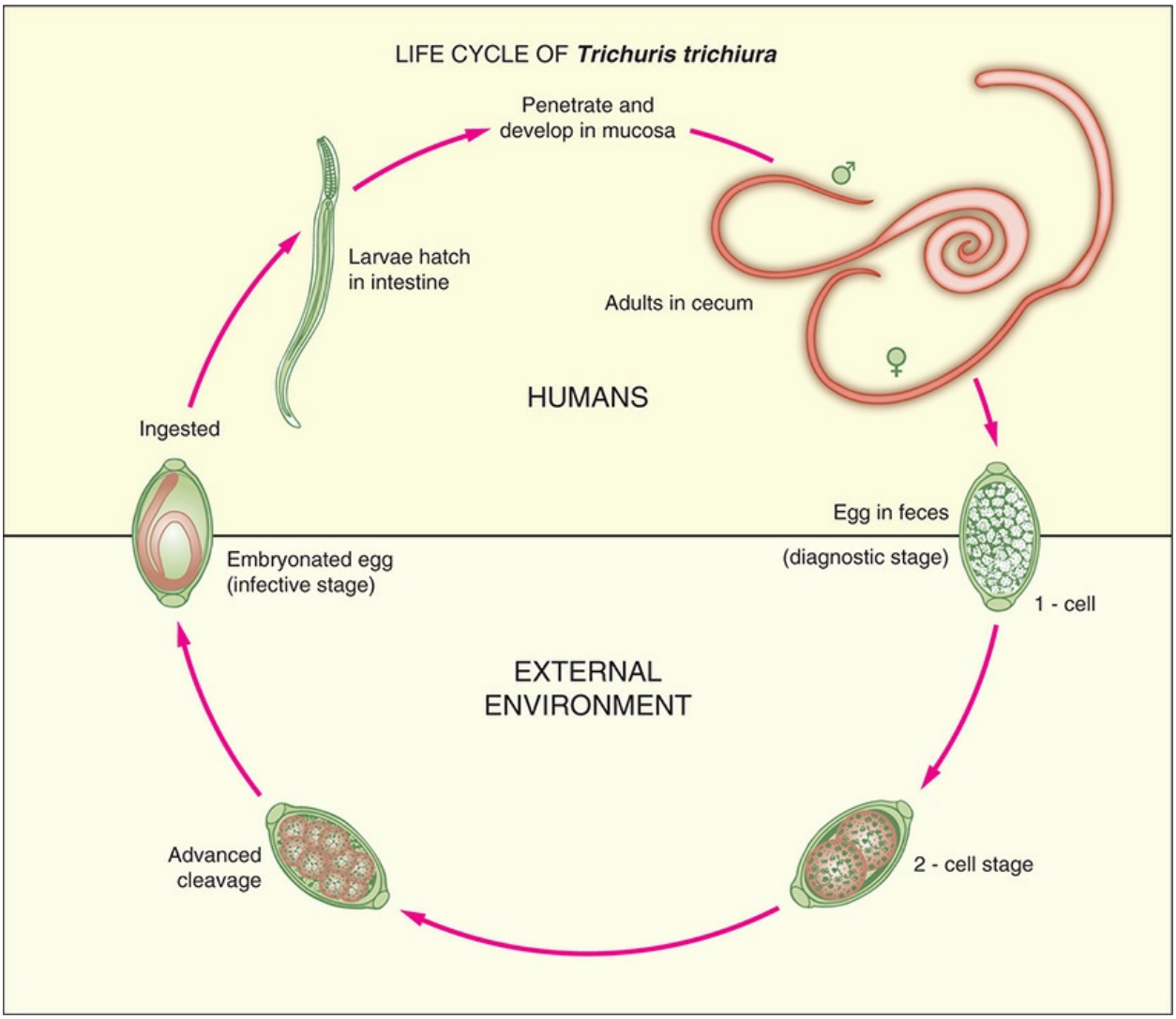
Adult Worms

- The adult female worm measures approximately 8 to 13 mm long × 0.4 mm in diameter.
- It can be recognized by the cuticular, winglike alar expansion at the anterior end (Image Plate 22-5E) and the long pointed tail, which makes the organism resemble a "pin."

Ova

- Measure approximately 30 × 50 µm in size.
- Have a thin, smooth, transparent shell.

1449



■ **FIGURE 22-9** The life cycle of *Trichuris trichiura* is what has been termed a direct life cycle, since after ingestion development occurs directly in the intestinal tract, without transpulmonary migration. The eggs, after passage into the external environment, also require a period of about 21 days under favorable conditions before they become infective. Upon ingestion of embryonated eggs by a human host, larvae hatch in the intestine. However, in contrast to the ascarids, these larvae penetrate into the mucosa of the intestine, primarily in the cecum, where they become anchored and develop into adult male and female worms. Gravid females pass eggs into the feces to complete the life cycle.

CLINICAL CORRELATION BOX 22-5 Trichuriasis

In light infections, patients are generally asymptomatic. The adult worms inhabit the large intestine. In heavy infections, diarrhea, dysentery, and abdominal discomfort,

resulting largely from mechanical damage to the intestinal mucosa, may be experienced. Anemia, growth retardation, and intestinal leakiness have been identified as predictable consequences of heavy infection and may serve as an indication of the intensity of infestation.[41] Varying degrees of malabsorption may also be observed, and because the diarrhea tends to be watery, sodium and potassium electrolyte imbalances may be a problem. Rectal prolapse in children is one of the complications of heavy infection with *Trichuris*.[254] Because the tiny adult worms are visible with the naked eye, the term *coconut cake* prolapse has been used to describe this condition.

Mebendazole is the drug of choice, but more than one dose may be necessary to effect a cure. Albendazole is an less effective alternative.

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CLINICAL CORRELATION BOX 22-6 Enterobiasis

The adult worms reside in the cecum and rectum. The diagnosis on occasion is made by observing worms in stained histologic preparations of the appendix or other portions of bowel. Symptoms occur when the gravid female deposits eggs in the folds of the perianal skin during the night. Nocturnal *pruritus ani* is the usual presenting symptom due to the irritation caused by the deposited eggs. Infections occur most commonly in children, and females are more prone to infection than males. In heavy infections, vaginitis with a mucoid discharge and/or urethritis may occur in young females.[87] Nervousness, insomnia, and nightmares are other presenting symptoms.

Pyrantel pamoate or mebendazole is effective, but more than one dose may be necessary as immature forms may be less susceptible to the drug. As infections may recur, repeat treatment after 1 or 2 weeks may be necessary if symptoms recur.

- Are oval in outline and asymmetrical, with one side flattened. Sometimes a groove is visible in the egg on the flattened side (Image Plate 22-5F).
- Each egg usually contains a well-developed larva (Image Plate 22-5E, right).

Hookworms

Ancylostoma duodenale is the Old World hookworm, and *Necator americanus* is the New World species, as defined by the predominant areas of endemic disease. Because the life cycles of these two species are essentially the same and cannot be differentiated by the appearance of their eggs, the general term *hookworm* is

commonly used for both species. An estimated 700 million to 900 million people worldwide are infected with hookworm (mostly *Ancylostoma duodenale*), 0.2% of whom suffer from severe anemia.

Unlike *Ascaris,* which gains entry to the host by the ingestion of eggs, the hookworm is one of the few parasites that has the ability to penetrate intact human skin. Following penetration, an indirect life cycle ensues, which results in an adult worm. This worm attaches to the intestine and feeds on the blood from the capillary-rich lamina propria of the bowel.[41] The life cycle is illustrated in Figure 22-10. The clinical manifestations of hookworm disease are presented in Clinical Correlation Box 22-7.

Laboratory Diagnosis. The laboratory diagnosis is made most commonly by observing the characteristic eggs in stool specimens, although may rarely be made by observing adult worms in the intestine as they are anchored to the mucosal lining of the intestine. It is important that the eggs of the hookworms are identified in stool specimens, and perhaps quantitative counts be made, because potentially severe disease may be associated with this nematode in heavy infections. Even light infections have been linked to serious sequelae, secondary to chronic anemia. The ova of both *A. duodenale* and *N. americanus* are identical; they also cannot be distinguished from the eggs of *Strongyloides stercoralis*, which usually are not seen in the stool, as this organism passes larvae rather than eggs.

Adult Worms

- Measure up to 1.5 cm in length, and reside in the upper intestine, where they are firmly attached to the mucosa by the biting action of cutting mouth parts.
- Observation of the mouth parts can be used to distinguish between the two species.
- *Ancylostoma duodenale* has two pairs of chitinous teeth (Image Plate 22-5I).
- *Necator americanus* is fit with a pair of cutting plates (Image Plate 22-5J).
- The male worms are distinguished by their frayed, posterior bursa.

Ova

- Eggs measure approximately 60 × 40 μm, and are distinctly oval.
- The shells are thin, smooth, transparent and nonpigmented.
- The yolk cells retract, leaving a clear space beneath the shell (Image Plate 22-5K).

Rhabditiform Larvae

- Rhabditiform larvae are rarely seen in the stool, since eggs are passed in hookworm infections. Nevertheless, it is important to be able to differentiate these from the larvae of *Strongyloides,* which are superficially similar.

- The rhabditiform larvae of the hookworms possess a long buccal cavity (Image Plate 22-5L, left), which distinguishes it from *Strongyloides stercoralis,* which has a short buccal cavity (Image Plate 22-5L, right).
- The rhabditiform larvae of the hookworms lack a genital primordium, which is present in the rhabditiform larvae of *Strongyloides stercoralis*.

Strongyloidiasis and Strongyloides stercoralis

Peculiarities in the life cycle set infections with *Strongyloides stercoralis* apart from those of other nematodes. Most important among these is that the laboratory diagnosis of strongyloidiasis is usually made by observing the rhabditiform larvae rather than eggs in stool specimens (Image Plate 22-5M). As with the hookworms, *Strongyloides* is able to penetrate intact human skin whereafter it undergoes an indirect life cycle, as described. The life cycle is illustrated in Figure 22-11. Interestingly, *Strongyloides* also survives in the environment in a nonparasitic cycle; ergo, parasitosis is not necessary for the propagation of *Strongyloides.*

Worldwide, the magnitude of strongyloidiasis is similar to that of the prevalence of hookworm infections, with upward of an estimated 800 million people being infected, involving up to 10% of the population in some locales.[129] The incidence of infection in any given region is variable as the filariform larvae require considerable moisture and survive best where

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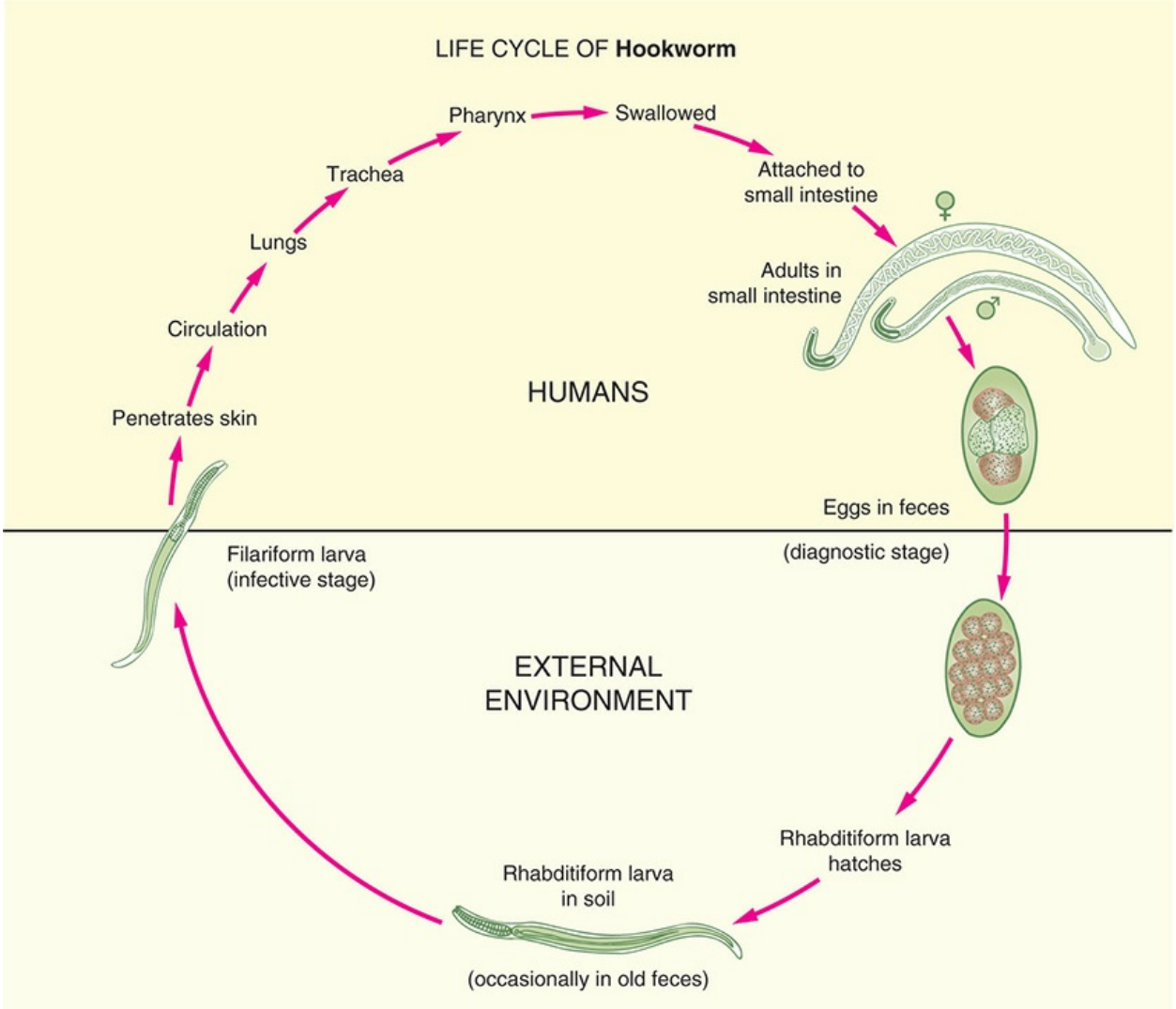

■ **FIGURE 22-10** Life cycle of hookworm. Hookworm eggs are usually passed in early stages of cleavage into the feces from gravid female worms. In approximately 24 hours in soil of ideal temperature and moisture content, the eggs hatch into the first larval stage, the free-feeding rhabditiform larva. About 5 to 7 days later, the rhabditiform larva transforms into the third-stage filariform larva, the infective form for humans. Depending on the temperature and moisture content of the soil, filariform larva can remain infective for up to 6 weeks. Humans become infected by penetration of the filariform larva on skin contact, either of the hands when digging into larva-infested soil or walking barefoot in soil contaminated with human feces. Similar to the ascarids, the hookworm larvae also penetrate the intestinal mucosa, enter the circulation, break out into the pulmonary alveoli, are coughed up, and swallowed. The larvae again burrow into the intestinal mucosa, where they develop into adult male and female worms. These adult worms remain firmly attached to the mucosa and access the blood supply of the host by cutting either plates or pairs of teeth depending on the species. Female worms lay large numbers of eggs that pass in the feces to complete the life cycle.

CLINICAL CORRELATION BOX 22-7 Hookworm Infections

Symptoms may occur relative to various stages of the life cycle. Skin infections may be experienced at the penetration sites of filariform larvae, particularly when humans are infected with nonhuman hookworm species ("ground itch").[254] Loeffler's syndrome in lungs and eosinophilia may be seen during the pulmonary larval migratory stage. Adult female hookworms produce only about 2,500–5,000 eggs per day; thus, the fecal egg counts may reflect the number of adult hookworms and, in turn, indicate the severity of infection. The presence of more than 2,000 hookworm eggs per milliliter of feces in women and children, and more than 5,000 per milliliter in males, is usually associated with anemia. Egg counting techniques are described by Garcia.[87]

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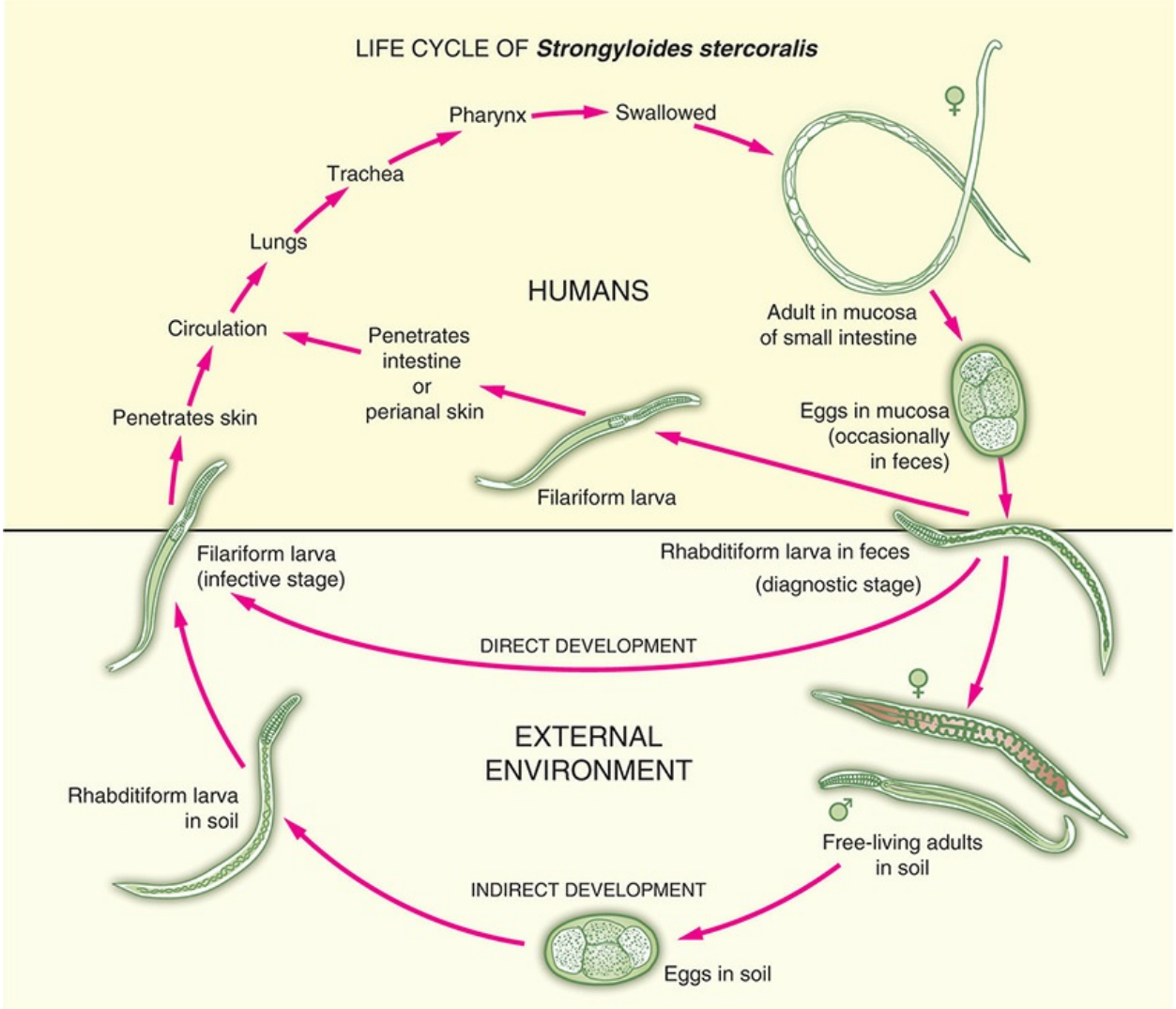
In heavy infections of 500 or more worms, the host could lose the equivalent of 1/2–1 pint of blood per week, resulting in severe hypochromic, iron deficiency anemia, and marked erythroid hyperplasia of the bone marrow, which in turn may lead to osteoporosis and the formation of bone cysts. Weakness, fatigue, growth retardation, peripheral edema, and congestive heart failure may be complications in severe infections. Of major concern worldwide is the lack of mental development from anemia-associated hypoxia in millions of children infected with hookworms. Diarrhea, abdominal pain, and nausea are manifestations of the intestinal phase of infection. Kelley et al.[141] reported that hookworm infections developed in 35 of 684 soldiers who took part in the Grenada military operations; they were most commonly associated with ground exposure near homes where sanitation practices were minimal.

Albendazole and other benzimidazoles are effective therapy; iron replacement should be given to patients with iron deficiency anemia.



■ **FIGURE 22-11** Life cycle of *Strongyloides stercoralis*. The life cycle of *Strongyloides stercoralis* is similar to that of the hookworms, except that the majority of eggs hatch into rhabditiform larvae while still in the intestinal lumen. Some of the rhabditiform larvae may transform into filariform larvae while still within the intestinal lumen. These can directly invade the mucosa or perianal skin, resulting in a cycle of chronic reinfection.151 This occurs unbeknownst to the immunocompentent host and occurs at a relatively low level (i.e., the reinfection cycle is not eliminated, but rather kept at a low steady-state by the immune system). However, once the host becomes immunocompromised, the rate of reinfection increases, resulting in what might be a fatal hyperinfection syndrome.[132,145] In cases of diarrheal disease, eggs, indistinguishable from those of hookworm, may occasionally be observed in the feces. Under ideal conditions of moisture and temperature in the external environment, rhabditiform larvae hatch, and quickly transform into infective filariform larvae. Humans become infected by direct penetration of filariform larvae into the skin on direct contact. The migration of these invasive larvae into the circulation is similar to that of hookworms, ultimately resulting in

the development of adult worms in the intestine.

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the water table is high. There have been outbreaks among military personnel stationed in endemic areas.[141] In the United States, strongyloidiasis is endemic in the rural south and southeast, with 3% to 5% of people being infected in some locales.[254] Infections also have high prevalence among patients in mental institutions, inmates in prisons, and in immigrants who formerly resided in endemic tropical regions.

Unlike other nematodes, *Strongyloides* commonly reinfects the same host, establishing a chronic parasitic infection. This is due to the fact that some of the larvae that are passed in the stool may penetrate either the bowel wall or the perianal skin. Chronic infections are usually unapparent, unless the host becomes immunocompromised.[97,110] This may occur through age or therapeutics (e.g., immunosuppressives given to control transplant rejection).[53] In such an instance, *Strongyloides* hyperinfection syndrome ensues, which is often fatal.[36,100,207] Less commonly, complications such as perforation and hemorrhage have been seen.[17] The clinical manifestations of strongyloidiasis are presented in Clinical Correlation Box 22-8.[112]

CLINICAL CORRELATION BOX 22-8 Strongyloidiasis

Skin irritation and pruritus in the form of low-grade chronic dermatitis may be seen at the portal of entry.[34] The full-blown ground itch characteristic for cutaneous infections from nonhuman hookworm infections usually does not occur. Leighton and MacSween,[151] however, reported a 74-year-old woman with a 65-year history of urticarial-like eruptions since childhood. These symptoms were finally correlated with chronic or long-term strongyloidiasis. The case for making a diagnosis of disseminated strongyloidiasis through recognition of filariform larva in skin biopsies was also made by Gordon et al.[97]

The intestinal manifestations of *Strongyloides* infections vary from few to no symptoms in light infections to severe necrotizing bowel disease in heavy infections. Symptoms may suggest peptic ulcer disease in some patients; in others, involvement of the small intestine may radiographically mimic Crohn's disease. A massive upper gastrointestinal hemorrhage involving a 29-year-old black immigrant from Africa caused by a heavy infection of the duodenum with *S. stercoralis* has been reported.[17]

With a heavy infective dose, pulmonary disease suggestive of Loeffler's

pneumonia, manifested as wheezing and eosinophilia, may occur; or in cases of hyperinfection syndrome to be described later in this chapter, full-blown pneumonia, cough, and shortness of breath may develop. Harris and associates[112] report two patients with disseminated strongyloidiasis in which the diagnosis was first made by observing larva in the sputum. They suggest that immunosuppressed patients in whom nonbacterial pneumonia develops, particularly in the presence of eosinophilia, should have both stool and sputum samples checked for infection with *S. stercoralis*.

Long-term corticosteroid therapy may also predispose to disseminated *Strongyloides* infections. Chu et al.[36] describe a 65-year-old man with steroid-dependent chronic obstructive pulmonary disease who developed pneumonia secondary to *Strongyloides*. The diagnosis was made based on larvae that were detected in an expectorated sputum specimen. In general, the pneumonitis caused by nematode larvae is transient and characterized by cough and fever; in more severe cases, it is characterized by chest pain, dyspnea, and hemoptysis.

Immunosuppressed hosts are particularly vulnerable to disseminated *Strongyloides* infections. The propensity for *S. stercoralis* eggs to hatch quickly and to produce intraintestinal filariform larvae (Image Plate 22-5N) makes patients vulnerable to autoinfection, producing a condition known as hyperinfection syndrome.[132] Purtilo and associates,[207] after observing the absence of granulomatous tissue response to larvae in several autopsy cases of fatal strongyloidiasis, concluded that an intact cell-mediated immune system is necessary to keep the organism in check. Once immunity is compromised or abrogated, hyperinfection from direct invasion of the intestinal mucosa (Image Plate 22-5O) with dissemination of larvae to many organs and tissues is liable to occur in patients harboring the parasite. Genta[90] offers the alternative argument that corticosteroids, rather than working through suppression of immunity, may in fact act directly on the worm as "molting hormones" that directly promote organism proliferation, making disseminated disease a more likely possibility. Although *Strongyloides* hyperinfection syndrome has not been specifically associated with HIV positivity and AIDS, a few instances have been reported.[110]

Because *S. stercoralis* can be harbored by humans as a subclinical infestation for many years following initial contact, patients in whom disseminated strongyloidiasis develops need not have had a recent exposure. Klein and associates[145] reiterate the need to screen stool specimens for strongyloidiasis before treating patients living in endemic areas with a course of immunosuppressive therapy. Recipients of organ transplants represent another high-risk group. This group should have serologic screening prior to beginning immunosuppressive therapy. Some have advocated a stool examination, which may be performed but is an insensitive method of detection.[53] Hyperinfection syndrome is further complicated because of the increased risk of gram-negative sepsis developing, presumably because intestinal bacteria accompany the penetrating *Strongyloides* larvae.

Thiabendazole and ivermectin are commonly used antihelminthics for strongyloidiasis, and are superior to albendazole. Ivermectin is usually used in

patients with hyperinfection syndrome.

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Laboratory Identification

Rhabditiform Larvae

- The rhabditiform larva of *S. stercoralis* has a buccal cavity which is short (Image Plate 22-5L, right), in contrast to the hookworms which have a long buccal cavity (Image Plate 22-5L, left).
- *Strongyloides stercoralis* rhabditiform larvae also have a prominent, oval genital primordium about one-third the distance from the tail.
- Often chronic infections are associated with very few parasites, making an objective diagnosis difficult. Examination of several stool specimens on successive days may be required, or the concentration procedure of Baermann may be indicated. In this procedure, active larvae are induced to migrate out of a fecal mass into a water reservoir through a wire screen covered with a pad of gauze, as described by Garcia.[87] DeKaminsky,[57] in a study of 427 stool samples, found that an additional 33 patients were diagnosed using a modified Baermann technique, and 28 additional patients were diagnosed using the agar plate culture method.

Trichostrongylus *Species*

Uncommonly detected in the United States, *Trichostrongylus* species are small adult nematodes similar to the hookworms that reside with heads buried in small intestinal epithelium. Adult worms typically inhabit the gastrointestinal tracts of sheep, cattle, goats, and other herbivores. Infection occurs through the ingestion of third-stage larvae that lie on grass and other vegetation. These parasites do not possess the special mouthparts characteristic of hookworms, and so the leaching of blood does not occur. Heavy infections may produce abdominal pain, diarrhea, and mild eosinophilia, but for the most part symptoms are minimal. The eggs of *Trichostrongylus* resemble the eggs of hookworms, but are longer (78 to 98 μm × 40 to 50 μm), with more pointed ends. It is important to recognize these subtle differences in egg morphology so that an incorrect diagnosis of hookworm infection is not made. Pyrantel pamoate is commonly used for treatment, with albendazole and mebendazole as alternatives.

Capillaria philippinensis

Capillaria philippinensis is an extremely small nematode; the adults measure 1.5 to 3.9 mm in length, and their width ranges from 5 mm at the filamentous head to 30

mm in midbody. After considerable research to find the reservoir hosts, it is now believed that fish-eating birds are natural hosts within a fish–bird life cycle.[44] Humans become infected when they eat poorly cooked fish commonly consumed by birds.

The disease is endemic in the Philippines, Thailand, and regions adjacent to the South China Sea. Local eating habits include ingestion of raw animal organs and the use of intestinal juices from animals to season rice and other foodstuffs. Additionally, eating uncooked crabs and small freshwater fish is considered a delicacy in many indigenous populations. Infection with *C. philippinensis* always causes illness and may lead to death if untreated. From 1967 to 1990, 1,884 confirmed cases of intestinal capillariasis were documented, from which 110 people died.[44]

The gradual onset of abdominal pain, borborygmus (i.e., stomach gurgling), and intermittent diarrhea over a 4- to 8-week period is the common presenting clinical picture. Severe protein-losing enteropathy, malabsorption of fats and sugars, and low xylose excretion are commonly seen. Fluid loss and electrolyte imbalances characterized by low plasma levels of potassium, sodium, and calcium are seen. Increased IgE levels and depressed IgG, IgM, and IgA levels occur. Postmortem examinations of patients who have died of this disease disclosed heavy infection in the jejunum, with focal mucosal atrophy at sites of organism invasion.[44]

Laboratory Identification

- The eggs of *C. philippinensis* may be confused with the eggs of *Trichuris trichiura* because of a similar morphology.
- In contrast to *T. trichiura,* the eggs of *C. philippinensis* have less conspicuous polar plugs and a thick, striated, or pitted eggshell (Image Plate 22-5P).
- A misidentification could lead to serious sequelae, unnecessary morbidity, or even the death of patients infected with *C. philippinensis*, if this infection proceeds untreated.

Cestodes

The cestodes are a subclass of helminths comprising true tapeworms, which have a scolex and a series of hermaphroditic, egg-producing body segments called proglottids. The cestodes of human importance to be presented are as follows:

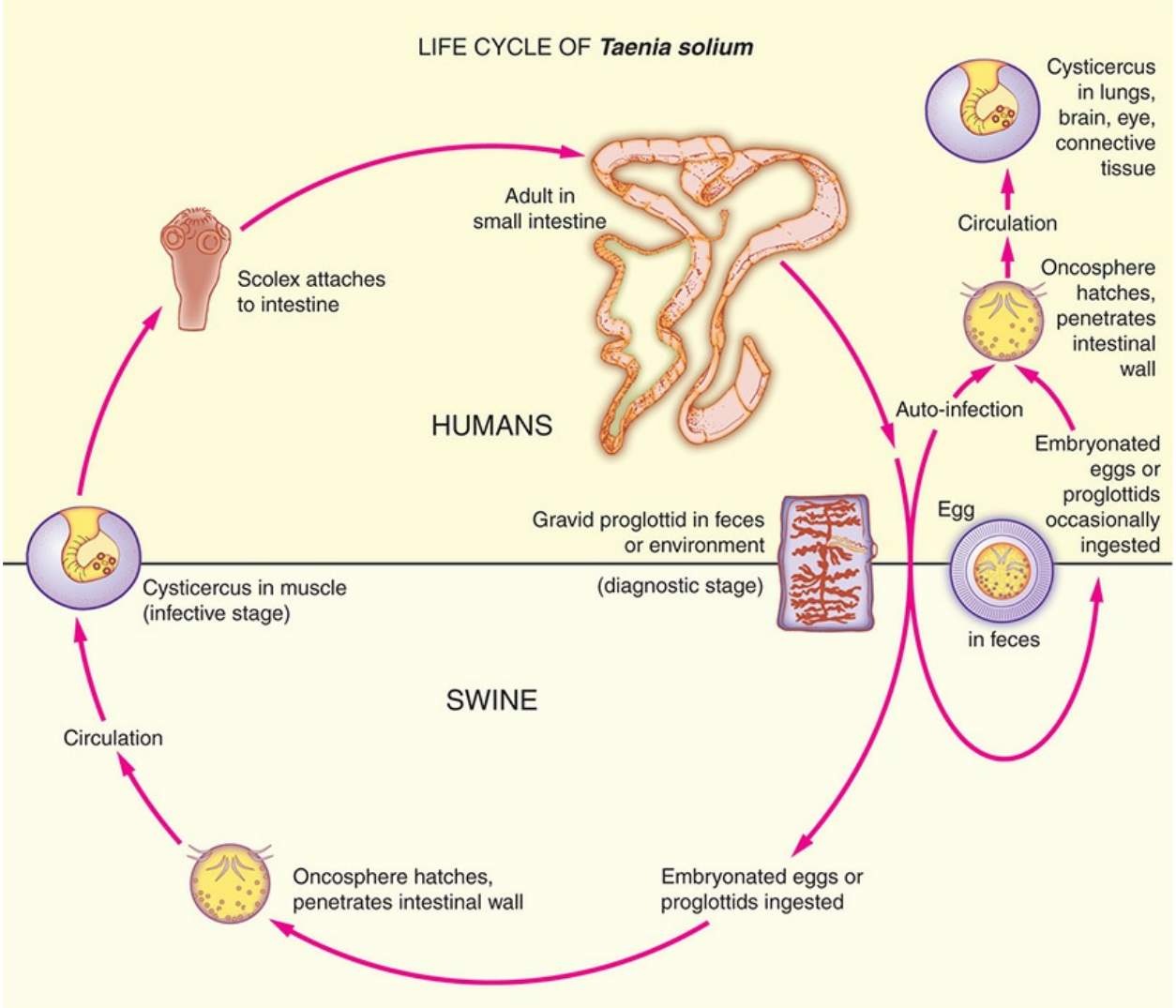
Taenia saginata
Taenia solium
Diphyllobothrium latum
Hymenolepis nana
Hymenolepis diminuta

Dipylidium caninum

The body of an adult cestode or tapeworm, called the strobila, consists of two parts, a scolex and the proglottid (Image Plate 22-6A). The scolex is the anterior portion with hooklets and/or suckers by which the worm attaches and anchors to the intestinal mucosa. The crown of the scolex, called the rostellum, may be fit with hooklets (i.e., armed) (Image Plate 22-6B) or may be devoid of hooklets and smooth (i.e., unarmed). These morphologic differences are helpful in establishing a species-level identification.

The major portion of the body of a tapeworm consists of a long series of segments called proglottides. Each proglottid possesses male and female reproductive organs; the uterine branches of which are packed with eggs when mature (Image Plate 22-6C). The eggs or proglottides filled with eggs are passed in the feces. Subtle differences in the size, shape, and internal structures of proglottides serve as aids in establishing a species-level identification. The evaluation of stool in microscopic mounts for the detection of eggs is the most common means of establishing a laboratory diagnosis.

With the exception of *Hymenolepis nana*, in which human-to-human transmission of infections may occur through ingestion of fecally contaminated food or water that



■ **FIGURE 22-12** Life cycle of *Taenia solium*. A human infected with an intestinal tapeworm passes proglottids and/or mature eggs into the external environment, where soil, water, and vegetative matter become contaminated with fecal matter. In the case of *Taenia solium*, the embryonated eggs are ingested by a pig, within the intestine of which an oncosphere hatches and penetrates the mucosa. The released larvae enter the circulation and migrate to the skeletal muscle to encyst in the form of a bladderlike cyst called a cysticercus. On ingestion of such cysticercus-infested pork by an unsuspecting human, the scolex evaginates in the intestine and the scolex attaches to the intestinal mucosa. In time, the tapeworm grows through the proliferation of hundreds of proglottids, until a length of several meters is reached. Eggs and proglottids are again released into the intestinal lumen and passed with the feces to complete the life cycle. The right side of the figure demonstrates that humans may also act as an intermediate host for this parasite, if an egg is ingested. This disease is cysticercosis.

contains infective eggs, the life cycles of the cestodes require one or more intermediate

hosts to support larval development. The life cycle of *Taenia solium,* as a representative of cestodes, is illustrated in Figure 22-12.

Taenia solium *and* Taenia saginata

Taenia infections have been known since biblical times, primitive knowledge of which may be related to the Judaic prohibition against the ingestion of pork. Humans acquire the intestinal infections with an adult *Taenia* tapeworm through the ingestion of encysted larval in poorly cooked pork (*T. solium*) or beef (*T. saginata*) (Image Plate 22-6D). The intestinal infections with the adult tapeworms of these two species produce similar symptoms in humans, as discussed below. Of greater medical importance is the fact that humans may also serve as an intermediate host for *T. solium.* This would occur if a human ingested the egg of *T. solium*. In this type of infection, called cysticercosis, the larvae travel widely throughout the body and may encyst in any tissue, but have a propensity to encyst in the brain[266] (Image Plate 22-6E). Laboratory workers must be careful when handling the ova-packed proglottides of *T. solium* to avoid inadvertent hand-to-mouth transmission. Human cysticercosis does not

occur with *T. saginata*; however, as the true identity of the infecting organisms may not be immediately known, care must be taken when handling any proglottides.

Cysticercosis has high prevalence in Mexico and Latin America, where an estimated 350,000 persons are infected. The disease has been found in 2% to 3% of individuals autopsied in Mexico City, accounting for 25% of all intracranial masses found by computed tomography scans.[204] In the past, neurocysticercosis had been uncommon in the United States, with only a few hundred cases recorded since surveillance began in 1957. However, more recently, there has been a marked increase in disease, which has, in part, paralleled the increase of immigration of people from endemic areas.[42] In 1989, cysticercosis became a reportable disease in California.[68] During the first year, 134 cases were reported, nearly all (117) from people of Hispanic background and most of whom had immigrated from countries where *T. solium* is endemic. In addition to the higher incidence of disease in California, several sporadic infections have also been reported from various areas in the United States, including Texas, Colorado, Pennsylvania, and Missouri.[171] Perhaps because of this experience, Roman et al.[213] propose that neurocysticercosis be declared an international reportable disease. Several publications reflect high rates of incidence of symptomatic cysticercosis in many regions throughout the world, as exemplified by endemic areas in Andean communities and in Burundi, as reported by Cruz et al.[45] and by Newell et al.,[186] respectively. The clinical manifestations of taeniasis and cysticercus infections are presented in Clinical

Correlation Box 22-9.

Laboratory Identification. The laboratory diagnosis of *Taenia* tapeworm infections (i.e., taeniasis) is usually made by observing the characteristic eggs in microscopic mounts of fecal material; the adult worms or portions thereof are required to make a species-level identification, since the eggs for the two species are morphologically indistinguishable.

The identifying characteristics of *Taenia* species are as follows:

Ova

- Spherical in shape, measuring approximately 30 by 45 µm in diameter.
- Characteristic smooth, thick shell with radial striations (Image Plate 22-6H). A photograph of these eggs in situ within the uterus of a gravid female worm is illustrated in Image Plate 22-6G.
- Three pairs of hooklets may be observed internally, in a structure called an oncosphere.

Adult Worms

The features by which *T. saginata* and *T. solium* can be differentiated are as follows:

	Taenia saginata	***Taenia solium***
Strobila:	4–10 m long Up to 2,000 proglottids	2–4 m long 800–1,000 proglottids
Scolex:	Four suckers Unarmed rostellum	Four suckers Armed rostellum (Image Plate 22-6B)
Proglottides:	More than 13 lateral uterine branches	Less than 13 lateral uterine branches (Image Plate 22-6C)

CLINICAL CORRELATION BOX 22-9 Taeniasis and Cysticercosis

Taeniasis

Intestinal symptoms are usually insignificant. Passage of proglottides in stool may be the first indication of disease. Epigastric pain, vague abdominal discomfort, nervousness, and increase in appetite may be experienced. Weight loss is minimal. Eosinophilia is usually moderate.

Cysticercosis

Cysticercosis refers to the development of extraintestinal encysted larval forms of *T. solium* in various organs following the ingestion of gravid eggs in fecally contaminated food or water. Although most human infections are caused by *T. solium*, other species of animal tapeworms can on rare occasions also produce morphologically similar cysticerci (e.g., coenurosis). The CNS is involved in 60%–96% of patients, a condition known as neurocysticercosis (Image Plate 22-6E). The majority of patients with neurocysticercosis have more than one cyst, with as many as 200 found in one autopsy case.[171]

Symptoms vary considerably from patient to patient and are dependent on the anatomic location of the cysticercus. Lesions in the cerebral cortex may result in seizures or localizing neurologic deficits. Cranial nerve palsies, in particular involving the fifth and seventh nerves, and abnormal reflexes are commonly found. Cysticercosis is the most frequently identified cause of epilepsy in young adults living in endemic areas, and it is the sole manifestation in up to one-third of patients.[221] Focal seizures occur in up to three-fourths of those infected. Intraventricular brain cysts may obstruct the flow of cerebrospinal fluid, leading to symptoms of acute intracranial hypertension (headache, vertigo, nausea, vomiting, papilledema, and visual disturbances); changes in personality and mental status are present in 40% of patients.[171]

The invasive larvae (onchospheres) are susceptible to circulating antibody and complement in the human host[266]; however, significant titers develop only after the larvae have transformed into the antibody-resistant metacestodes. In fact, antibody may bind via Fc receptors to the worm, which may use it as a source of protein.

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Taeniaestatin and other parasite molecules may interfere with lymphocyte proliferation and macrophage function, thus paralyzing the cellular immune response.

Praziquantel and albendazole have been extensively tested and successfully used for the treatment of neurocysticercosis, usually in combination with corticosteroids.[259] There is debate concerning the treatment of extensive neurocysticercosis because dying cysticerci provoke an enhanced inflammatory response, which may lead to cerebral edema, herniation, and death. Corticosteroid use may also be needed. In some instances, surgery is necessary.

A mass effort to treat cysticercosis in a rural community in Mexico was undertaken giving all participating citizens a single dose of praziquantel of 5 mg/kg; human taeniasis was reduced by 56%, and late-onset seizures by 70% over a 42-month period.[221] In this same study, anticysticercus antibody levels were reduced in humans by 75%, and by 55% in treated pigs. Vaccines, using recombinant antigens, have been developed and found in experimental studies to induce over 90% protection for challenge infections for hydatid disease in sheep, providing encouragement for

potential vaccine use in humans as well.[154]

Chapman et al.[32] have developed a species-specific DNA probe that differentiates *T. solium* eggs from those of *T. saginata*. The authors describe the isolation and characterization of recombinant clones that contain repetitive DNA sequences (a 158-bp sequence) for *T. solium* and an unrelated *T. saginata* sequence that encodes a portion of the mitochondrial *cytochrome c oxidase I* gene, each specifically hybridizing with genomic DNA from respective species. In the absence of such probes, it is not possible to differentiate *T. solium* from *T. saginata,* based on egg morphology alone. This should be clearly conveyed to the clinician who ordered the stool examination, since individuals, particularly children, in the home of a patient shedding *T. solium* eggs are at elevated risk for neurocysticercosis. Sloan et al.[235] examined the use of an enzyme immunoassay for the serologic diagnosis of cysticercosis. An immunoblot assay is available at the CDC for confirming the diagnosis of cysticercosis; acceptable specimen types include serum and cerebrospinal fluid (CSF).

Diphyllobothrium latum: *The Giant Fish Tapeworm*

D. latum, the human giant fish tapeworm, uses two intermediate hosts in the development of its larval forms. Endemic areas include the cold clear lake regions of Scandinavia, northern Europe, northern Japan, the upper Midwest of the United States, Canada, and Alaska. Figure 22-13 is an illustration of the life cycle of *D. latum*. The clinical manifestations of diphyllobothriasis are presented in Clinical Correlation Box 22-10.

Laboratory Identification

Adult Worm

Although the proglottides are rarely passed in the stool, but when found they are distinctive.

- An intact strobila measures 3 to 10 m in length and has over 3,000 proglottides.
  - Only a part of the strobila is recovered in most instances.
- Individual segments (i.e., proglottids) are characteristically broader than they are long (*latum* is Latin for *broad*) (Image Plate 22-6I).
- The scolex is rarely recovered from stool specimens. If recovered the features include the following:
  - It is almond-shaped and measures 2 to 3 × 1 mm.
  - It is distinctive for two deep dorsoventral suctorial grooves (*bothria* = pit), demarcated by lateral lip-like folds (*phyllon* = leaves) (Image Plate 22-6J).

- Each proglottid includes a nondescript coiled uterus in the form of a compact rosette (Image Plate 22-6K).

Ova

The laboratory diagnosis of human *D. latum* infection is commonly made by identifying the characteristic operculated eggs in stool specimens. This is the only operculated egg that is from a cestode; all other operculate eggs that will be covered are from trematodes.

The eggs of *D. latum*:

- Measure approximately 55 to 75 × 40 to 55 μm and are elongated.
- Have a smooth shell with an inconspicuous non-shouldered operculum at one end and a knoblike thickening at the other end (i.e., the abopercular end) (Image Plate 22-6L).
- These are differentiated from the operculate eggs of *Paragonimus westermani* (discussed later) by the lack of a distinctly shouldered operculum and lack of an abopercular knob (Image Plate 22-7P).

Hymenolepis *Species*

Hymenolepis nana, known as the dwarf tapeworm, has a worldwide distribution and is one of the more common causes of cestode infections in humans, particularly in children. In contrast, *Hymenolepis diminuta* is primarily a parasite of rats and mice, and is uncommonly found in humans. Various species of arthropod (e.g., "meal beetles"), which harbor the infective larval forms, serve as intermediate hosts. A photograph of an *Hymenolepis* cysticercus as it may appear within the insect is illustrated inImage Plate 22-6F. Humans become infected through the ingestion of these larval-infected insects. Such an intermediate host is not obligatory for human-to-human transmission of *Hymenolepis nana*. Most commonly, humans become infected through the ingestion of food or water contaminated with *Hymenolepis* eggs.

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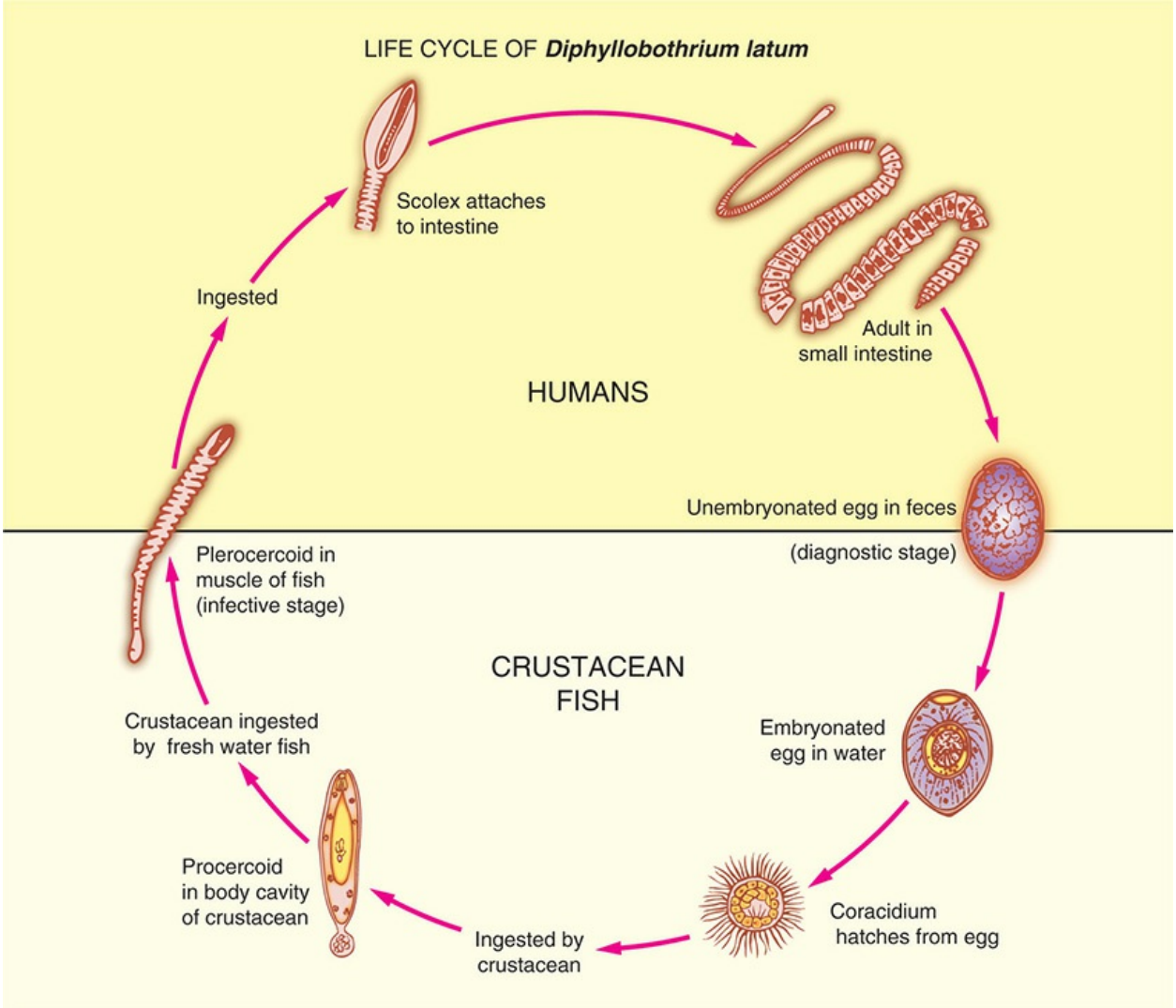

■ **FIGURE 22-13** The life cycle of *Diphyllobothrium latum* involves two intermediate hosts. Unembryonated eggs, passed with the fecal material from an infected human into a freshwater estuary, undergo embryonation during a period of several days and hatch, releasing a free-swimming coracidium. These coracidia in turn are ingested by a crustacean (copepods or cyclops), within the body cavity of which develop procercoid larvae. Copepods serve as one of the major food sources for a variety of freshwater fish in North America. Following ingestion of the copepod by the fish, the plerocercoid larvae of the parasite (spargana) develop within the flesh of the fish. Humans ingest these plerocercoid larvae in raw or poorly cooked fish, where they develop into adult worms in the intestine. On maturation, the adult worm passes eggs into the feces, to complete the life cycle.

CLINICAL CORRELATION BOX 22-10 Diphyllobothriasis

Patients may harbor an adult worm for up to 20 years. Intestinal symptoms are minimal. Large adult worms may cause mechanical bowel obstruction, accompanied by abdominal pain and diarrhea. In a minority of patients infected with *D. latum*, particularly in northern Europe and specifically in Finland, megaloblastic anemia secondary to vitamin B_{12} deficiency occurs, secondary to the selective competition of the parasite for this essential vitamin. Praziquantel is an effective therapy, with niclosamide as an alternative.

Humans may also become infected with the larval plerocercoids of diphyllobothroid tapeworms closely related to *D. latum*, causing an infection known as sparganosis. Adult worms are found in dogs and cats; the larval form develops in humans following ingestion of larval-infected Copepoda or the raw flesh of amphibians and reptiles. The plerocercoid larvae (sparganum) develop into pruritic nodules in the subcutaneous tissues over a period of months. Peripheral blood eosinophilia is a nonspecific clue to the possibility of sparganosis; however, the characteristic larvae must be demonstrated in subcutaneous nodules before a definitive diagnosis can be made.

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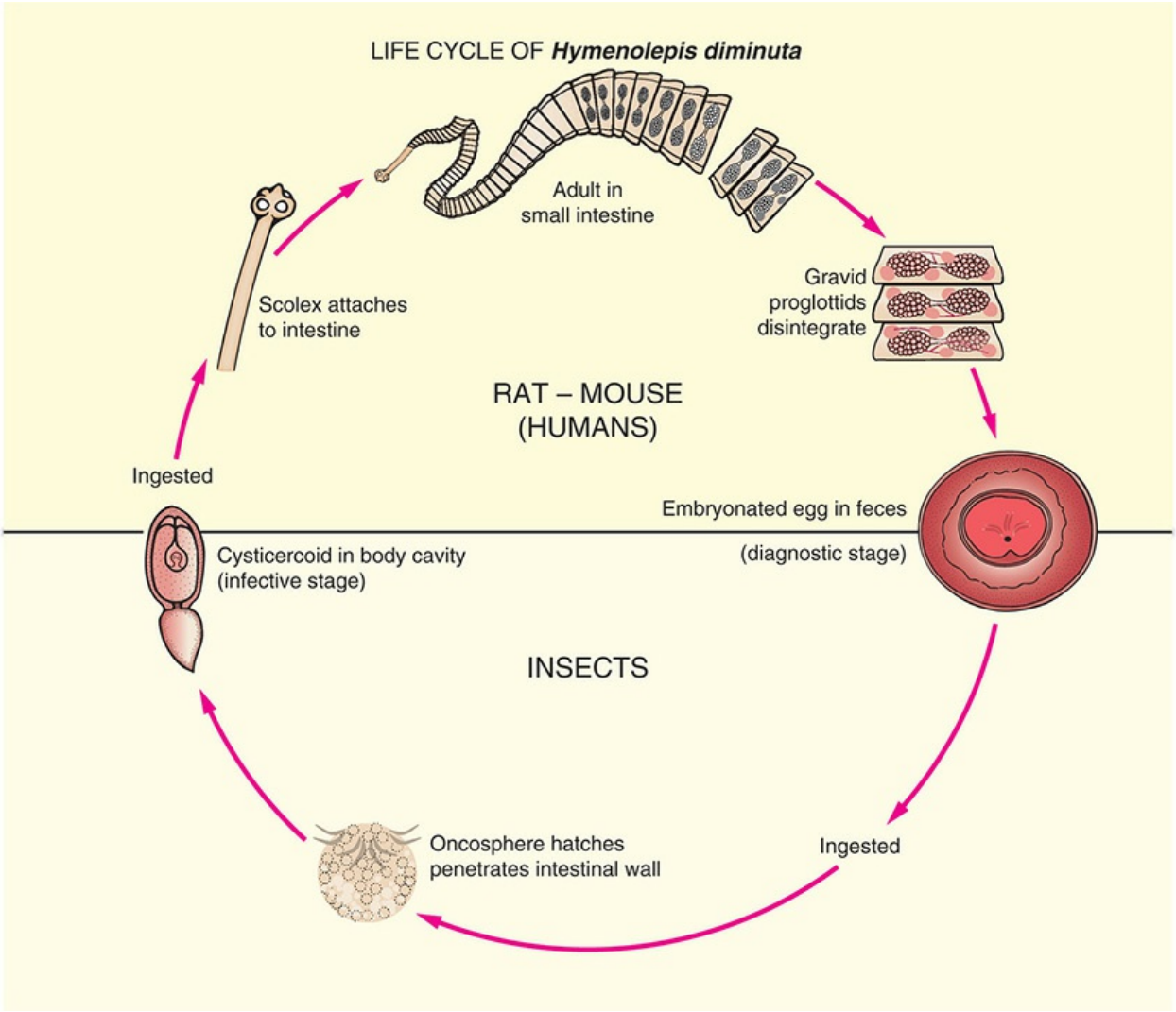

■ **FIGURE 22-14** Life cycle of *Hymenolepis diminuta*. Embryonated eggs, passed into soil or water from an infected human via the feces, in turn are ingested by an insect. An oncosphere hatches in the intestine of the insect and penetrates the intestinal wall. An infective cysticercoid then develops in the body cavity. On the accidental ingestion of the infected insect by a human, larvae are released and penetrate the villi of the upper part of the small intestine. The scolex attaches to the intestinal mucosa, where they develop into adult worms. On maturity, embryonated eggs are released from gravid proglottids and passed in the feces, to complete the life cycle.

Figure 22-14 is an illustrated life cycle of *Hymenolepis diminuta.* Infections with *Hymenolepis* are largely subclinical. The clinical manifestations of hymenolepiasis are presented in Clinical Correlation Box 22-11.

Laboratory Identification. The laboratory diagnosis of *Hymenolepis* infections is usually made by detecting the characteristic parasite eggs microscopically in mounts of

fecal specimens.

Ova

- The eggs are morphologically distinctive between *H. nana* and *H. diminuta*. Each has a smooth outer shell and an inner membrane, containing a hexacanth (six hooklets) embryo.
- The eggs of *H. nana* are oval, small (47 × 57 µm), and possess a characteristic pair of polar filaments that arise from thickenings on either side of the hexacanth membrane (Image Plate 22-6M).
- *H. diminuta* eggs are spherical, larger (58 × 86 µm) than those of *H. nana*, and are devoid of polar filaments (Image Plate 22-6N).

Adult Worms

- The adult worms of *H. nana* are small, measuring no longer than 1.5 inches when mature.
- They often simulate mucous threads; thus, they are not commonly seen in stool specimens.
- The tiny scolex has a protruding, armed rostellum with a row of 20 to 30 hooklets.

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CLINICAL CORRELATION BOX 22-11 Hymenolepiasis

In contrast to infections with *Taenia* and *Diphyllobothrium* species, in which only one worm most commonly inhabits the intestine, up to 1,000 *H. nana* worms may be present in a patient. Symptoms in light infections may be absent or limited to mild, diffuse lower abdominal pain and loose stools. Anorexia, abdominal pain, diarrhea, headache, nervousness, and pruritus ani may be seen in heavy infections. Peripheral eosinophilia is mild to moderate (4%–16%). Autoinfection, in which ova hatch in the intestine and reinfect via the normal life cycle described in the text, may result in a huge worm burden that can cause severe complications, particularly in immunosuppressed patients. The treatment options include praziquantel, niclosamide, and nitazoxanide.

Dipylidium caninum

The life cycle of *Dipylidium caninum* is similar to that of *Hymenolepis diminuta*.

Humans serve as an accidental host, and become infected following ingestion of dog or cat fleas that are infected with the cysticercoid form of the cestode. The adult tapeworm resides in the intestine of dogs or cats. The eggs, discharged in the feces, are ingested by several species of dog fleas or lice; the infective cysticercoids develop within the body cavity of the insect. Upon ingestion of a dog flea by a human, the cysticercoid larva penetrates the small intestinal mucosa and develops in situ into adult worms. Human infections most commonly occur in children, who have more intimate contact with household pets and, when very young, are likely to eat undesirable objects from the floor. Symptoms are usually minimal or absent. In heavy infections, varying degrees of indigestion, appetite loss, and vague abdominal discomfort may be experienced.

Praziquantel is the treatment of choice; niclosamide is also effective. Human infection is preventable by keeping pet dogs and cats free of tapeworms and fleas.

Laboratory Identification. The adult worms are not commonly seen. The laboratory-based identification is usually accomplished through the detection of characteristic egg packets in the stool.

Adult Worm

- The strobila ranges from 15 to 70 cm in length and possesses 60 to 175 proglottides.
- The scolex is rhomboidal in shape, possessing a retractile, conical armed rostellum.
- The proglottides measure 12 × 2.7 mm, and are distinctive for possessing a double genital pore (*Dipylos* means two gates) (Image Plate 22-6P).
- Proglottides may detach from the strobila and migrate singly or as short chains from the anus of cats and dogs, appearing as tiny grains of rice when deposited on the carpet or floor.

Ova

The laboratory diagnosis in human infections is usually made by observing the characteristic egg packets microscopically, each of which:

- Contain 15 to 25 globular eggs (Image Plate 22-6P).
- Each egg measures 35 to 60 µm in diameter.
- Each egg also contains an oncosphere with six hooklets.

Trematodes

The trematodes are also known of as flukes, several of which are parasites for humans. Flukes are leaf-shaped (i.e., there is dorsoventral flattening). All that are

human parasites are hermaphroditic, except for the schistosomes. These parasites possess two suckers—one oral sucker, through which the digestive tract opens, and the other is ventral for attachment. The trematodes of human importance to be presented in this chapter are as follows:

The Schistosomes: *S. mansoni*, *S. haematobium*, *S. japonicum*
Liver Flukes: *Fasciola hepatica* and *Clonorchis sinensis*
Giant Intestinal Fluke: *Fasciolopsis buski*
The Lung Fluke: *Paragonimus* species, most commonly *P. westermani*

The initial stages in the life cycles of trematode parasites are virtually identical, with free-swimming miracidia released from gravid eggs discharged into appropriate water estuaries in the feces from an infected human. All flukes include snails as an intermediate host. Many trematodes also require a second intermediate host. The type animal utilized as an intermediate host is dependent on the species of trematode. The developmental stages of the trematode species included in this chapter are depicted in the life cycles presented.

Schistosomes

The name schistosome is derived from the appearance of the adult male, the body of which has a longitudinal genital groove, or canal, serving as a receptacle for the female during copulation (Image Plate 22-7A). Three *Schistosoma* species, *S. mansoni*, *S. haematobium*, and *S. japonicum*, cause the majority of human infections. *S. mekongi* is a human parasite that is endemic along the Mekong river; the eggs of this are morphologically indistinguishable from *S. japonicum*. *S. intercalatum* is another less commonly encountered human pathogen. The egg of this parasite resembles those of *S. haematobium,* but it causes disease that clinically mimics that caused by *S. mansoni*. *S. intercalatum* is endemic in Central and West Africa.[242] The life cycle of the schistosomes is similar to that of other flukes except that a second intermediate host is not required to transmit disease.[54] The *Schistosoma* life cycle is illustrated in Figure 22-15.

Depending on the species, the schistosome adults are located in various parts of the portal vein system: (1) in the portal veins of the large intestine for *S. mansoni* and *S. intercalatum*, (2) in the small and large intestine for *S. japonicum* and *S. mekongi*, and (3) in the veins of the urinary bladder for *S. haematobium*. Adult worms, which measure about 2.5 to 3 cm in length and 0.5 mm in diameter when mature. Heavy infections cause portal vein obstruction at the sites where they reside, heavy infections lead to portal hypertension. Extensive tissue damage also results from the deposition of the myriad of eggs that are produced daily by the female.[242] The cutaneous manifestations of schistosomiasis have also been described.[55] A presentation of the

clinical manifestations of schistosomiasis is found in Clinical Correlation Box 22-12.

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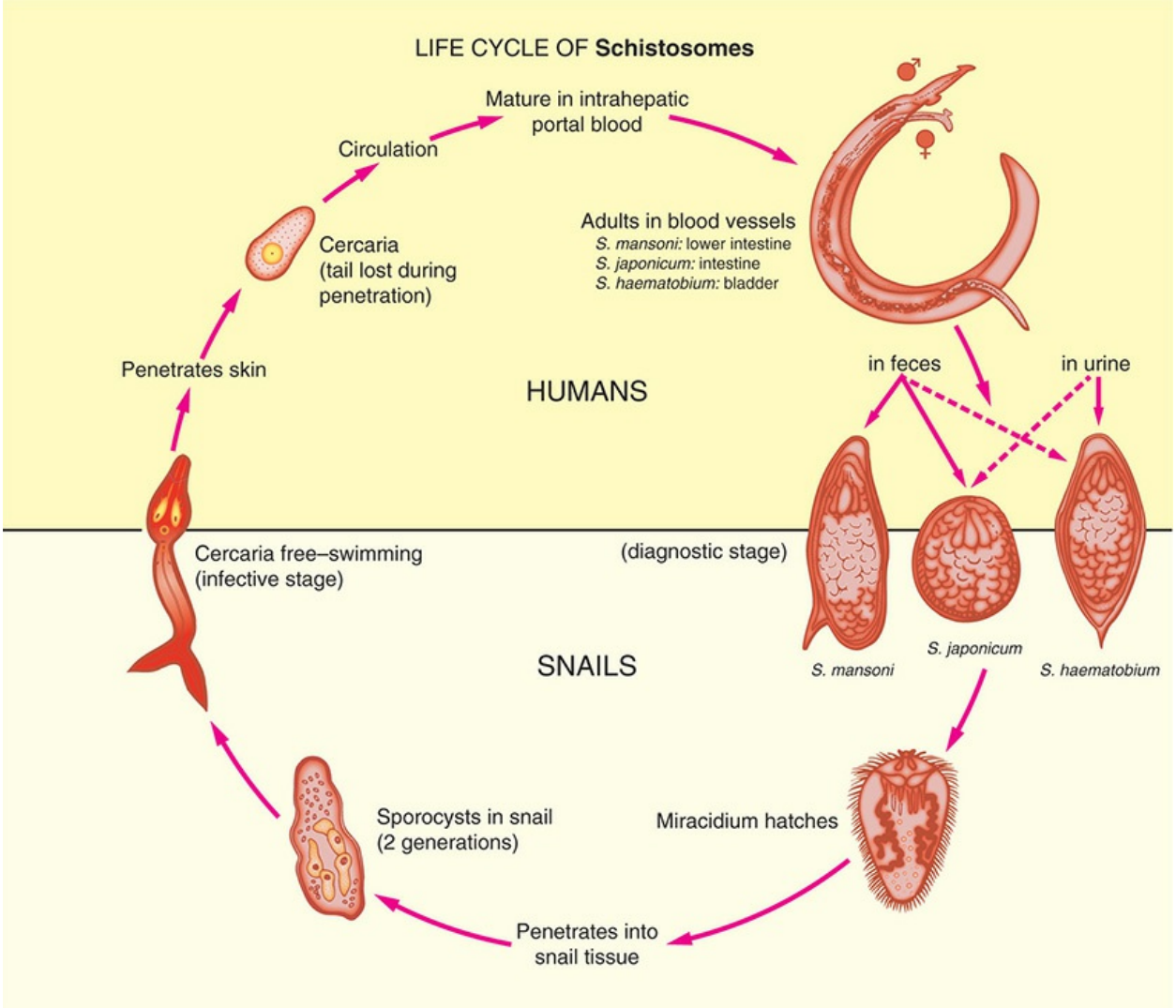

■ **FIGURE 22-15** Life cycle of the schistosomes. Eggs, passed in the feces into freshwater lakes, canals, and the like, hatch under suitable conditions, releasing a free-swimming miracidium. The miracidium penetrates the tissues of an appropriate species-specific snail, within which it undergoes maturation division to form hundreds of cercariae. The free-swimming cercaria, released from infected snails, have the capability to directly penetrate the water-softened skin of unsuspecting swimmers and waders in freshwater estuaries. Once in the subcutaneous tissues, the heads of the cercariae enter the circulation and migrate to the portal venous system, where they develop into adult male and female flukes. The females, occupying the gynecophoral canal of the male, produce a myriad of eggs that are released into an appropriate aquatic estuary to complete the life cycle.

Laboratory Identification. The laboratory diagnosis is made by detecting the characteristic eggs in stool or urine, depending on the species.

Ova

- The eggs are very large. Following are the range of dimensions for each of three commonly encountered human species:
- *Schistosoma mansoni*: 116 to 180 × 45 to 58 µm.
- *Schistosoma haematobium*: 112 to 180 × 40 to 70 µm.
- *Schistosoma japonicum*: 75 to 90 × 60 to 68 µm.
- The eggs of *S. mansoni* and *S. haematobium* are distinctly oval.
- The eggs of *S. mansoni* possess a prominent lateral spine (Image Plate 22-7B).
- The eggs of *S. haematobium* possess a terminal spine (Image Plate 22-7C).

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CLINICAL CORRELATION BOX 22-12 Schistosomiasis

Davis[54] has divided the clinical manifestations of schistosomiasis into four distinct stages, manifesting as symptoms that often overlap, particularly in individuals who are constantly reexposed to infection.

Stage 1: "Swimmer's Itch." Cercarial invasion early on produces a prickling sensation or itching of the involved skin. In nonimmune individuals, skin reactions ranging from minute petechiae to urticaria or a pruritic papular eruption may be seen. The signs and symptoms of these eruptions are generally more severe in reaction to cercariae of nonhuman (bird) schistosomes. Davis-Reed and Theis[55] have reported a patient with cutaneous *Schistosoma haematobium* infection wherein this appeared as the sole clinical manifestation 3 years after exposure. This is a quite unusual presentation, as most commonly symptoms appear relatively soon after exposure. These authors counsel that dermatologists should be reminded of schistosomiasis in patients who present with unusual skin lesions, particularly with a history of travel in endemic areas.

Stage 2: Katayama Syndrome. During the second, or maturation, stage, during which the schistosomes are migrating to their preferred anatomic sites and pairing preparatory to egg laying, symptoms may be minimal or develop into what has been referred to as the Katayama syndrome or Katayama Fever, characterized by varying degrees and combinations of fever, malaise, backache, arthralgia, anorexia, cough, headache, and toxemia. Mild hepatosplenomegaly may be detected, and peripheral

blood eosinophilia may be significant. Because at this stage eggs are often not yet excreted, the diagnosis may be dependent on eliciting a history of exposure, the presence of typical dermatitis, and one or more of the symptoms listed above.

Stage 3: Granulomatous Inflammation. The third stage involves the inflammatory reaction secondary to the deposition and migration of ova in the tissues. Egg deposition is approximately 300 per day for female *S. haematobium* and *S. mansoni* worms, and 3,000 per day for *S. japonicum*. After invading the wall of the veins in which the adult female resides, the eggs have a propensity to penetrate into the adjacent viscera, eliciting a severe suppurative and granulomatous inflammation, ultimately resulting in fibrosis and scarring. Marked thickening of the walls of the intestine or urinary bladder, with loss of function, results. Bloody diarrhea, vague abdominal pain, and in severe cases, bowel obstruction may be observed with *S. mansoni*, *S. japonicum*, and *S. mekongi* infections. *S. haematobium* produces inflammation of the urinary bladder, resulting in intermittent hematuria, lower abdominal pain, and ultimately, contraction.

Stage 4: Chronic Inflammation. In the fourth, or chronic, stage of disease, marked progressive fibrosis around the areas of granuloma occurs, with a marked decrease in the number of excreted eggs. Patients with chronic *S. haematobium* infection have persistent urinary frequency and dysuria as the bladder continues to contract, and are also at high risk for squamous cell carcinoma of the urinary bladder. In late-stage *S. mansoni* infections, hepatosplenomegaly, cirrhosis, and ascites are common complications, as the eggs are swept upstream in the portal veins and lodge in the liver. Intestinal polyposis and inflammatory masses in the colon simulating carcinoma of the bowel may also be observed. *S. japonicum* may produce similar symptoms; however, because of its greater egg-laying capacity, morbidity may be much greater.

Praziquantel is the recommended treatment. Corticosteroids may be needed in acute infection (i.e., Katayama syndrome) or with CNS involvement.

- The eggs of the less commonly encountered species, *S. intercalatum,* also possess a terminal spine.
- The eggs of *S. japonicum* are broadly oval to semispherical; a small, rudimentary, lateral, knoblike spine may be seen if the plane of focus is correct (Image Plate 22-7D).
- The eggs of *S. mekongi* are morphologically indistinguishable from *S. japonicum.*

A photomicrograph of a forked-tail cercaria, the infective form in the life cycle of *Schistosoma* species, is illustrated in Image Plate 22-7E.

Several immunologic techniques, including immunofluorescent antibody procedures, ELISAs, radioimmunoassays, and complement fixation, are used in reference

laboratories to help establish a diagnosis. Using a monoclonal antibody raised against a 15-kDa tegumental antigen of *S. mansoni* adult worms, Da Silva et al.[51] have developed a competitive ELISA that they report as 94% sensitive in the detection of *S. mansoni* antigen in infected patients. Considerable work has been done by De Jonge et al.[56]and others to improve the serodiagnosis of schistosomiasis. van Etten et al.[250] report 95.5% sensitivity and 96.7% specificity in the detection of schistosomal cathodic antigen in the urine of patients infected with *S. mansoni*, using a monoclonal antibody-coated nitrocellulose/polyvinyl reagent strip. Such a strip is purported to be of value in the qualitative diagnosis of *S. mansoni* infections in control programs.

Fasciola hepatica *and* Fasciolopsis buski

Fascioliasis is primarily a cause of zoonotic parasitic liver disease in sheep. Flukes may remain in the bile ducts for many years, and can cause much damage from mechanical irritation at sites of local invasion, and the production of toxic by-products. Conversely, the adults of *Fasciolopsis buski* remain in the intestine, where they attach to the mucosa of the duodenum and jejunum. Other herbivores, such as deer, rabbits, cattle, goats, pigs, and horses, among others, may also be infected. *Fasciola hepatica* and *Fasciolopsis buski* are morphologically similar in many ways. The life cycles of both these parasites are the same, both of which require two intermediate hosts.

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The life cycle of *Fasciola hepatica* is illustrated in Figure 22-16. The clinical manifestations of fascioliasis and fasciolopsiasis are presented in Clinical Correlation Box 22-13.

Laboratory Identification

Adult Fluke

The adult flukes are seen only through endoscopy or if removed at surgery. They can be recognized by the following:

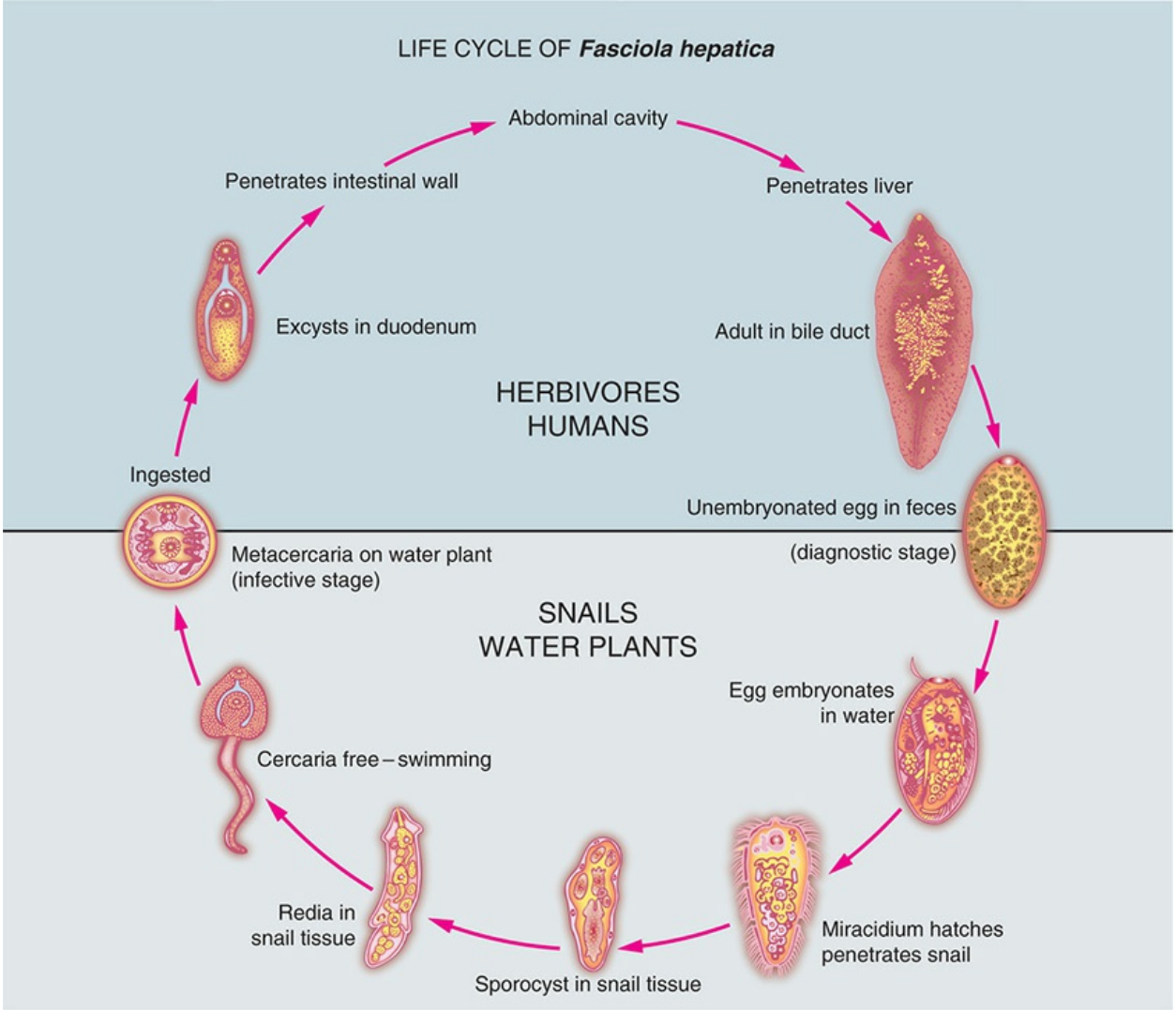
- The *F. hepatica* adults, which measure 20 to 30 × 8 to 13 mm, are flattened and leaflike in appearance, having a cone-shaped anterior protrusion (Image Plate 22-7F); the adult flukes of *F. buski* have a rounded cephalic end (Image Plate 22-7G).
- Each adult fluke has an anterior and a ventral sucker and are hermaphroditic, with a convoluted uterus seen anteriorly.

Ova

The laboratory diagnosis is most commonly made by detecting eggs in fecal preparations. The eggs can be recognized by their:

- Very large size (150 × 80 μm), ovoid shape, and yellow-brown staining.
- Thin, smooth shell, with an indistinct operculum (Image Plate 22-7H).
- The material within the egg is poorly organized and extends to the shell margin without leaving a clear space.

Because parasite eggs may be shed only after 8 weeks or more after infection or intermittently thereafter, serologic tests have been used in reference laboratories for the diagnosis of fascioliasis. Espino and Finlay[69] developed a mouse monoclonal antibody raised against *F. hepatica* excretory–secretory antigens, and a rabbit polyclonal antibody peroxidase



■ **FIGURE 22-16** Life cycle of *Fasciola hepatica*. On discharge of mature eggs via the stool into freshwater, embryonated eggs hatch and release free-swimming miracidia. After burrowing into the flesh of an appropriate snail, sporocysts are formed in the tissue. Within the snail, maturation/multiplication takes place in the redia, ultimately leading to the release of hundreds of free-swimming, straight-tailed cercaria. The cercariae attach to water plants and encyst as infective metacercaria. Humans become infected by ingesting raw or undercooked, metacercaria-infested water plants. Upon ingestion, the metacercariae excyst in the human duodenum with the release of larval forms. These penetrate into the intestinal wall, transmigrate the abdominal cavity, and enter the liver, where mature flukes develop in the bile ducts. These flukes are hermaphroditic and release eggs into the lumen of the bile ducts. The eggs in turn are carried into the intestinal tract, where they are discharged in the feces, completing the life cycle.

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CLINICAL CORRELATION BOX 22-13 Fascioliasis and Fasciolopsiasis

Upon ingestion of raw sheep or goat liver infected with adolescent worms, the flukes can attach to the pharyngeal mucosa and cause local laryngopharyngitis, called halzoun. Human fascioliasis is manifested by headache, chills, fever, and right upper quadrant pain. Hepatomegaly, jaundice, diarrhea, and anemia may occur in severe infections; hepatic biliary cirrhosis is a late complication. Bithionol is the most effective agent, but must be released from the CDC for treatment. A new therapeutic, triclabendazole, holds promise. Praziquantel, a formerly recommended drug, may have limited efficacy.

F. buski, a fluke similar to *F. hepatica* except that it is somewhat larger (20–75 × 8–20 mm) and has a rounded instead of a conical anterior portion (Image Plate 22-7G). These flukes reside in the intestine and by means of a small oral sucker, which they use to attach to the intestinal mucosa. Local mucosal ulcers produce varying degrees of epigastric pain, nausea, and diarrhea, especially in the morning. Ascites and intestinal obstruction may be seen in heavy infections. Laboratory diagnosis is made by identifying the large eggs in the feces, which are identical in appearance to those of *F. hepatica*.

conjugate detector to identify *Fasciola* ES antigen in stool specimens. The authors indicated that their antigen-detection test had the advantage over several other antibody-detection methods because serum titers may remain elevated even after cure. They also cite the advantages of detecting antigen in stool specimens over serum as (1) avoiding the problem of immune complex formation that decreases the potential rate of detection, (2) circumventing venipuncture in many regions of the world where the procedure is objectionable, and (3) having a test readily available for cure.

Bithionol is the most effective agent, and a newer agent, triclabendazole, holds promise. However, these are only available by release through the CDC. Praziquantel, a formerly recommended drug, may have limited efficacy, and should only be used if bithionol or triclabendazole is unavailable.

Clonorchis sinensis

Clonorchis sinensis, the Chinese liver fluke, is a relatively small fluke that varies in size (12 to 20 × 3 to 5 mm). When mature, it resides within the biliary ducts or in the

gallbladder of humans. The life cycle is similar to that of *Fasciola hepatica*, except that a freshwater fish rather than water plants are used as the second intermediate host. Humans become infected with *C. sinensis* by ingesting the raw or poorly cooked flesh of several species of freshwater fish. Figure 22-17 is an illustration of the life cycle.

The genus name (from the Greek *clon,* to divide, and *orchis*, testis) is derived from the freely branching testicular organ in the adult hermaphroditic fluke. The disease is most common in a wide area of Asia, particularly in Indochina, Japan, Korea, and Southern China. Importantly, this organism is the cause of cholangiocarcinoma.[226,230] The clinical manifestations of clonorchiasis are presented in Clinical Correlation Box 22-14.

Laboratory Identification. Adult flukes are rarely encountered. The laboratory-based diagnosis is usually achieved by the identification of the characteristic small, urn-shaped eggs in stool preparations.

Adult Fluke

- The adult fluke is flat and flabby, ranging in size from 12 to 20 × 3 to 5 mm.
- A bottle-shaped, protruding head is seen at the cephalic end (Image Plate 22-7I).
- An anterior sucker leads to an esophagus that branches into blind ceca that extend laterally to the posterior end (Image Plate 22-7J).
- A ventral sucker is located just anterior to a loosely coiled uterus (Image Plate 22-7K).
- Immediately posterior to the uterus is the ovary connected to delicate vitelline ducts (Image Plate 22-7L).
- A highly branched testes, from which the genus name is derived, extend into the posterior part of the body (Image Plate 22-7M). The blind termination of the ceca and an ill-defined excretory bladder are also seen in this photomicrograph.

Ova

- The typical egg is relatively small, urn-shaped, and measures 27 to 35 × 14 to 16 µm.
- A light yellow-brown pigmentation is evident from bile staining.
- A prominent convex, shouldered operculum is an identifying feature (Image Plate 22-7O).
- A small knob may be microscopically observed posteriorly when in the correct plane of focus.
- Note: This egg is morphologically similar to those of *Heterophyes heterophyes, Opisthorchis viverrini*, and *Metagonimus yokogawai*, three

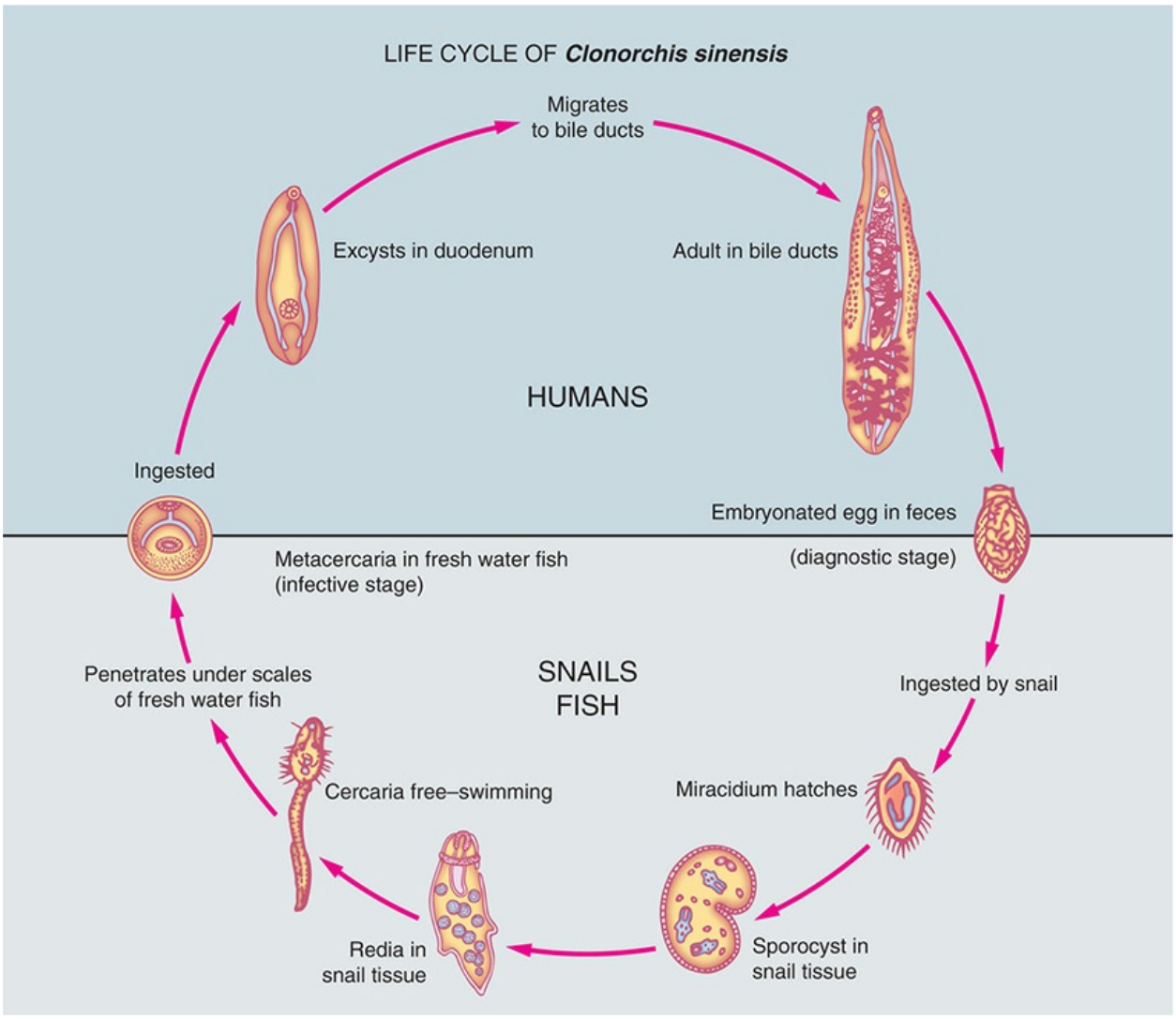
small intestinal flukes that are also prevalent in Asia and infect humans who ingest raw or pickled fish.

Paragonimus *Species, Most Commonly* P. westermani

Paragonimus species are present on all continents except Australia and Antarctica, with *Paragonimus westermani* being the most commonly encountered species. The adult worms measure in the range of 8 to 16 × 4 to 8 mm and are spoon-shaped, with one end contracted and the other elongated. The anterior and ventral suckers are of equal size.

The life cycle of these flukes begins when eggs from an infected host are passed into the environment. The miracidium infects the snail, which is the first intermediate host. Subsequent forms infect and develop within freshwater crabs or crayfish, which are the second intermediate host. Humans become infected following the ingestion of raw or poorly cooked crabmeat or crayfish, the flesh of which contains encysted metacercaria. Following ingestion, the metacercariae

hatch in the duodenum, releasing larvae that attach to the duodenal mucosa. The larvae penetrate the bowel wall, enter the abdominal cavity, and transmigrate the diaphragm to enter the pleural space. The larvae then invade the lung tissue, where maturation takes place. Figure 22-18 is an illustration of the life cycle. The clinical manifestations of paragonimiasis are presented in Clinical Correlation Box 22-15.



■ **FIGURE 22-17** Life cycle of *Clonorchis sinensis*. Upon discharge of mature eggs into freshwater, embryonated eggs hatch and release free-swimming miracidia. The miracidia are ingested by an appropriate snail, within which it hatches and forms a sporocyst in the snail tissue. The sporocyst in turn matures into redia, within which maturation/multiplication takes place, leading to the release of hundreds of free-swimming, straight-tailed cercaria. The cercariae attach to and penetrate under the scales of several freshwater fish species, forming metacercaria. Humans become infected by ingesting raw or undercooked, metacercaria-infested fish flesh. Upon ingestion, the metacercariae excyst in the human duodenum, with the release of larval forms that migrate into the bile ducts. These flukes are hermaphroditic and release eggs into the lumen of the bile ducts. The eggs in turn are carried into the intestinal tract, where they are discharged in the feces, completing the life cycle.

CLINICAL CORRELATION BOX 22-14 Clonorchiasis

In light infections, the damage to the liver is minimal, and cirrhosis does not occur. Slight leukocytosis and eosinophilia may be observed early in infections. Although the bile ducts in human infections may become thickened and dilated, particularly at the points where the flukes are attached to the inner lining, biliary obstruction and jaundice are rare except in extremely heavy infections (Image Plate 22-7N). In most infections, the disease tends to remain low-grade and chronic, with organisms persisting for four or five decades, producing only minor symptoms of abdominal distress, intermittent diarrhea, and abdominal pain or tenderness. Of concern is the potential development of cholangiocarcinoma in association with chronic *Clonorchis* infections.[226,230]

Laboratory Identification. The laboratory diagnosis is most commonly made by detecting the characteristics ova microscopically in stool preparations or respiratory specimens.

Ova

- The eggs range in size from 80 to 120 × 48 to 60 µm.

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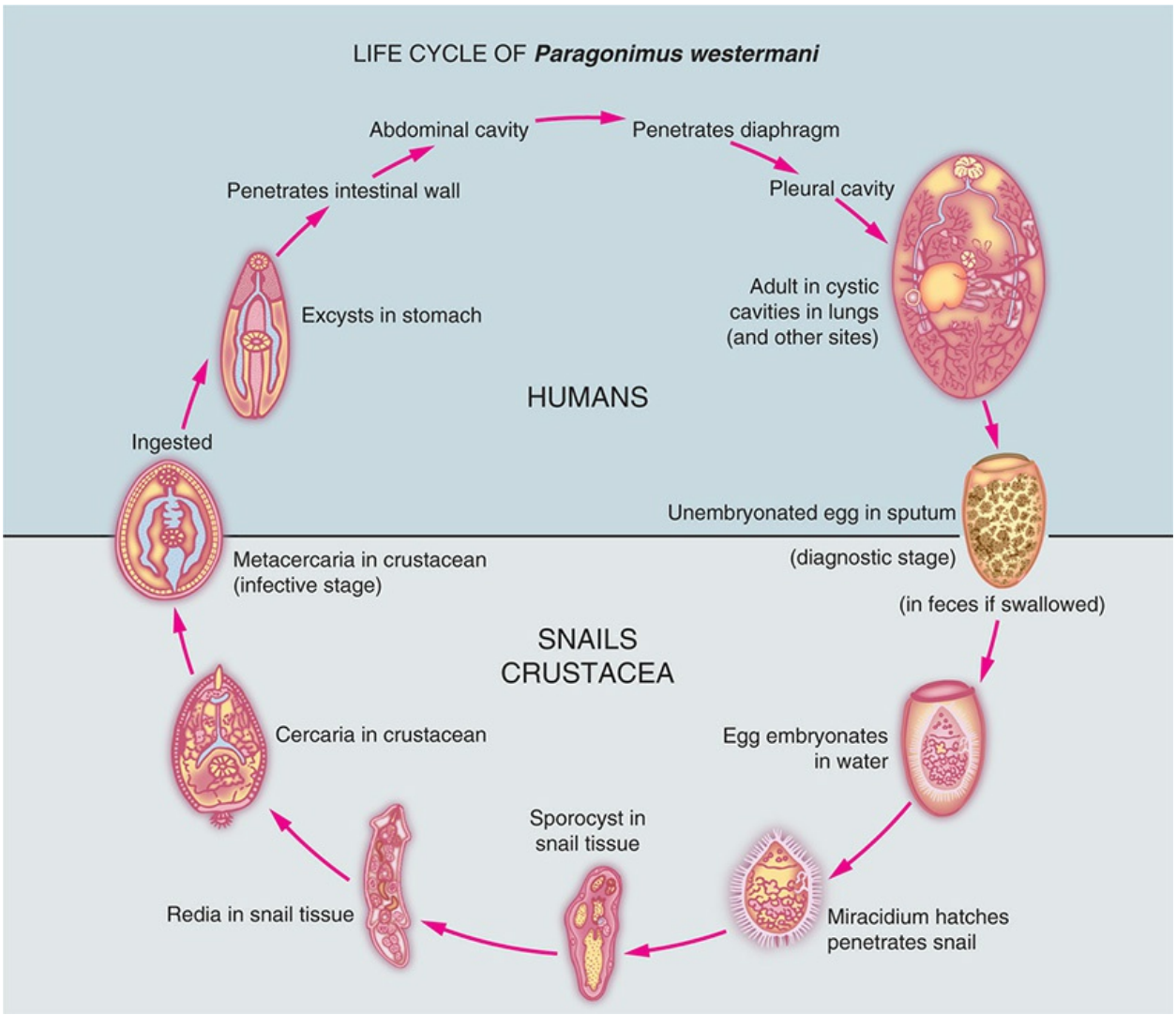

■ **FIGURE 22-18** Life cycle of *Paragonimus westermani*. Nonembryonated eggs are discharged either in the sputum or in the feces into a water environment. These eggs then embryonate in the water, releasing a free-swimming miracidium that penetrates the tissues of a species-specific snail. Sporocysts and redia develop in the snail, within which cercaria replicate. When mature, free-swimming cercaria break out of the snail and penetrate the gills, muscles, or viscera of a freshwater crustacean, within which they encyst as dormant metacercaria. Humans become infected following ingestion of raw or poorly cooked metacercaria-infested crabmeat or crayfish. Following ingestion, the metacercariae hatch in the duodenum, releasing larvae that penetrate the full thickness of the bowel wall, enter the peritoneal cavity, transmigrate the diaphragm, and enter the pleural space. The larvae then bore into the peripheral lung tissue, where they mature into adult flukes that reside within parabronchial cysts. Eggs are released into the sputum, where they are either expectorated or swallowed to ultimately pass in the feces, to complete the life cycle.

CLINICAL CORRELATION BOX 22-15 Paragonimiasis

Chills, fever, and marked eosinophilia may be seen during the migratory phase of the worm. In time, the adult fluke comes to reside within a small pseudocyst in the lung. The cyst enlarges as the adult fluke grows and may break into an adjacent bronchiole. When a pseudocyst ruptures into a bronchus, coughing and hemoptysis are common symptoms. Eggs produced by mature flukes are discharged into the bronchi and are ultimately swallowed by the patient from secretions coughed into the oropharynx. The disease tends to become chronic, leading to varying degrees of pulmonary fibrosis and scarring. Intermittent episodes of chest pain, fever, and chills may be late manifestations. Occasionally, ectopic migrations may occur. The worm may come to reside in the CNS. Unfortunately, this ectopic migration occurs more frequently in children. Praziquantel is the recommended treatment.

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- They are dark yellow-brown in color and have a thick, smooth shell with a prominent "shouldered" operculum.
- The shoulders serve to distinguish the *Paragonimus* ova from those of *Diphyllobothrium latum*, the opercula of which are smooth and devoid of shoulders (Image Plate 22-7P).
- *P. westermani* eggs also do not possess an antiopercular, knoblike protrusion characteristic of *Diphyllobothrium* eggs.

Blood and Tissue Parasites

Parasites found in the blood or in other organs are usually discussed separately from those inhabiting the gastrointestinal tract. The blood and tissue parasites covered here include protozoa, nematodes, and cestodes. Various parts of their life cycles are morphologically similar to their intestinal counterparts. For example, *Toxoplasma gondii* is a tissue coccidia, with many similarities to the coccidia covered above. Similarly, visceral and cutaneous larval migrans are caused by the larval forms of dog or cat nematodes. Echinococcosis is the larval form of a dog cestode that occurs in humans.

In general, the life cycles of the blood and tissue parasites are more complex than those of their intestinal counterparts, involving both sexual and asexual stages. Most of

the blood parasites are transmitted to humans by an arthropod vector within which the sexual phase of the life cycle develops. Alternatively some of these parasites use insects simply as a site for the development of the intermediate stages (i.e., molting) and as vectors (i.e., neither sexual or asexual replication occurs within the vector, simply development and transport).

Tissue parasites may be either intracellular or extracellular, depending on the species and the phase of the parasitic cycle. An outline of human blood and tissue parasites that will also serve as a guide to the discussion for the remainder of this chapter is as follows:

Blood and Tissue Protozoa	
Malaria	*Plasmodium falciparum* *Plasmodium vivax* *Plasmodium malariae* *Plasmodium ovale*
Babesiosis	*Babesia microti* and other species
Leishmaniasis	*Leishmania donovani* *Leishmania tropica* *Leishmania brasiliensis* *Leishmania mexicana*
Trypanosomiasis	*Trypanosoma brucei gambiense* *Trypanosoma brucei rhodesiense* *Trypanosoma cruzi* *Trypanosoma rangeli*
Toxoplasmosis	*Toxoplasma gondii*
Blood and Tissue Helminths	
Filarial Nematodes and Filariasis	*Wuchereria bancrofti* *Brugia malayi* *Loa loa* *Mansonella ozzardi* *Mansonella perstans* *Onchocerca volvulus* *Dirofilaria immitis*
Tissue Nematodes: Nonfilarial Worms	*Trichinella spiralis* *Toxocara canis* (visceral larva migrans) *Ancylostoma braziliensis* or *A. caninum* (cutaneous larva migrans) *Dracunculus medinensis* *Anisakis* species *Gnathostoma* species

Tissue Cestodes	*Echinococcus granulosus* *Echinococcus multilocularis* *Taenia multiceps* and *T. serialis* (coenurosis) *Spirometra mansonoides* (sparganosis)

Malaria

In the United States, the vast majority of patients with malaria are travelers, foreign-born students, or immigrants who have been exposed to mosquitos in endemic areas. In 2011, 1,925 cases of malaria were reported to the CDC, an increase from the previous years (i.e., 1,691 in 2010; 1,484 in 2009; and 1,298 in 2008).[47] These numbers are substantial increases over the 165 cases reported in 1988.[28] Although the vast majority of patients with newly diagnosed malaria in the United States have been foreign-born or recent returnees from endemic regions, anopheline vectors are found in many locales. For example, cryptic cases of malaria have been reported from San Diego County in California, and from Bay County in Florida.[91] None of the affected individuals gave a history of recent foreign travel, recent blood transfusions, or use of intravenous drugs. In some instances, infected patients had camped in mosquito-infested areas adjacent to encampments of migrant workers. Epidemiologic studies revealed that none of the migrant workers had a clinical history suggestive of malaria; however, the competent mosquito vector, *Anopheles hermsi*, was found along the San Luis Rey River in California. Similarly, two cases of indigenous malaria were reported from Suffolk County, New York, in persons who had no history of travel outside the United States.[29] Additionally, there have been reports of individuals contracting malaria who live in close proximity to international airports (i.e., airport malaria). The supposition is that infected mosquitos may occasionally be transported in incoming aircraft. Therefore, malaria should be included in the differential diagnosis of any patient with acute onset of cyclic fever, even if there is no history of recent foreign travel.

The worldwide incidence of malaria is astounding with infections estimated by the World Health Organization at 207 million infections in 2010, resulting in 627,000 deaths. It is estimated that 91% of the deaths were in Africa. Clinicians must, therefore, remain alert to the possibility of malaria in certain population groups. Although great progress in the elimination of malaria from certain locales has been made, malaria remains a worldwide challenge, with reemergence occurring in areas of previous control, particularly when control efforts are not maintained. Martens and Hall[167] attribute this resurgence in malaria to the relocation of poor people, often more likely to be infected with malaria, from endemic areas to malaria-free locales in search of a better life. The clinical manifestations of malaria are dependent on the infecting species, with infections caused by *P. falciparum* most likely to cause death. Clinical

manifestations are presented in Clinical Correlation Box 22-16.

CLINICAL CORRELATION BOX 22-16 Malaria

Fever is the constant presenting symptom of infections with all *Plasmodium* species. Temperature spikes commence 7–10 days following the bite of an infected *Anopheles* mosquito during a period when the organisms are undergoing preerythrocyte multiplication in the cells of the liver. Prodromal symptoms of headache, myalgia, malaise, and fatigue suggestive of a flu-like syndrome may be experienced during this period. However, once a brood of merozoites leaves the hepatic cells and invades the erythrocytes, the regularly spaced fever cycles begin in many instances. The least synchronous of the *Plasmodium* species is *P. falciparum.* The periodicity of each episode of high fever is related to the rupture of erythrocytes as merozoites are released into the circulation. The designations of this fever cycle "tertian" malaria, for *P. vivax, P. ovale,* and *P. falciparum* malaria, and "quartan" malaria for *Plasmodium malariae* malaria are somewhat confusing. The fever spikes for tertian malaria occur on an every-other-day schedule; however, because any given fever episode is counted as day 1, the next spike will not occur until the third day (thus, tertian). Similarly, the fever spikes of quartan malaria occur on an every three-day cycle; however, counting the first episode as day 1, the next spike will occur on the fourth day. Such classic fever patterns, however, may often be irregular and cannot be relied on exclusively to make a presumptive diagnosis.

Each fever episode is characterized by a short "cold" period lasting for approximately 1 hour, when the skin is cold and the lips and nail beds appear cyanotic because of peripheral vasoconstriction. This short period is followed by the sudden onset of the "hot period," when the skin feels warm and dry and fever spikes up to 105°F–106°F are experienced, lasting 3–6 hours. Headaches, chest and back pain, tachycardia, cough, vomiting, and delirium of varying degrees of severity accompany the fever spikes. Fatigue and sleep follow each febrile episode. Patients are essentially asymptomatic between febrile episodes.

Hemolytic anemia, splenomegaly, and tender hepatomegaly are found to varying degrees. Patients are highly susceptible to rupture of the spleen, and deep palpation of the left upper abdomen and flank should be avoided during physical examination. Lymphadenopathy is absent and always points to some other condition if lymph nodes are enlarged. Of greatest concern are the CNS complications that may occur secondary to *P. falciparum* infections. The erythrocytes infected with *P. falciparum* trophozoites undergo membrane changes in which "knobs" appear on the surface, causing them to become "sticky" and adhere to specific receptors on the endothelial

cell lining of capillaries.[37] The microcirculation of the brain is particularly vulnerable to blockage with *P. falciparum*-infected erythrocytes, resulting in small areas of cerebral infarction and hemorrhage.[4] Disturbances in consciousness ranging from somnolence to coma, behavioral changes, hallucinations, motor seizures, and occasionally tremors, focal muscle paralysis, and other localizing signs may be present. A rapidly progressive downhill course leading to death may occur in fulminant cases.

The life cycle of the *Plasmodium* parasite has two phases: a sexual cycle, known as sporogony, which takes place within the intestinal tract of the mosquito; and an asexual cycle, known as schizogony, occurring in the human host. The life cycle of *Plasmodium malariae*, as a prototype of all species of malaria, is shown in Figure 22-19.

Laboratory Identification. Laboratory identification of the malarial parasites in humans, as previously described, is made by analysis of thick and thin peripheral blood smears. More than one assessment of blood is necessary to exclude the diagnosis. Smears should be obtained at different times of the day from patients with suspected infections because parasitemia may be intermittent, and the number of circulating parasites may vary depending on the phase of the parasitic cycle.

There are now five species of *Plasmodium* shown to cause human disease. The three species of *Plasmodium* most commonly causing human malaria are *P. vivax*, *P. falciparum*, and *P. malariae*. A fourth species, *P. ovale*, is rare in much of the world but relatively common in western Africa. The most recently described species, *P. knowlesi,* is endemic in only certain parts of Asia; this parasite is so rarely encountered that it is not covered further here. The differential features of the three common species are outlined below. The microscopic morphology of the intraerythrocytic forms is illustrated in Image Plate 22-8.

The laboratory identification of the various *Plasmodium* species is not difficult if an orderly approach based on the observation of a few key morphologic structures is followed. Foremost, the detection of the presence of a blood parasite is paramount, which is achieved most effectively through the analysis of the thick blood film. Once a parasite is detected, the thin blood film is used to achieve species-level identification. It is quite important that infections with *P. falciparum* be recognized as early as possible because the disease can be particularly severe, rapidly progressing to a fatal outcome. The most important feature in the differentiation of *Plasmodium* species is the size of the infected erythrocytes compared with uninfected erythrocytes. The comparison should be concluded after the comparison of many infected and uninfected cells. If the infected erythrocytes are the same size as uninfected erythrocytes (i.e., normocytic),

then the infecting species is either *P. falciparum* or *P. malariae.* If the infected erythrocytes are enlarged compared with the uninfected erythrocytes, then the infecting species is either *P. vivax* or *P. ovale.* A suggested laboratory approach to the identification of *Plasmodium* species is as follows:

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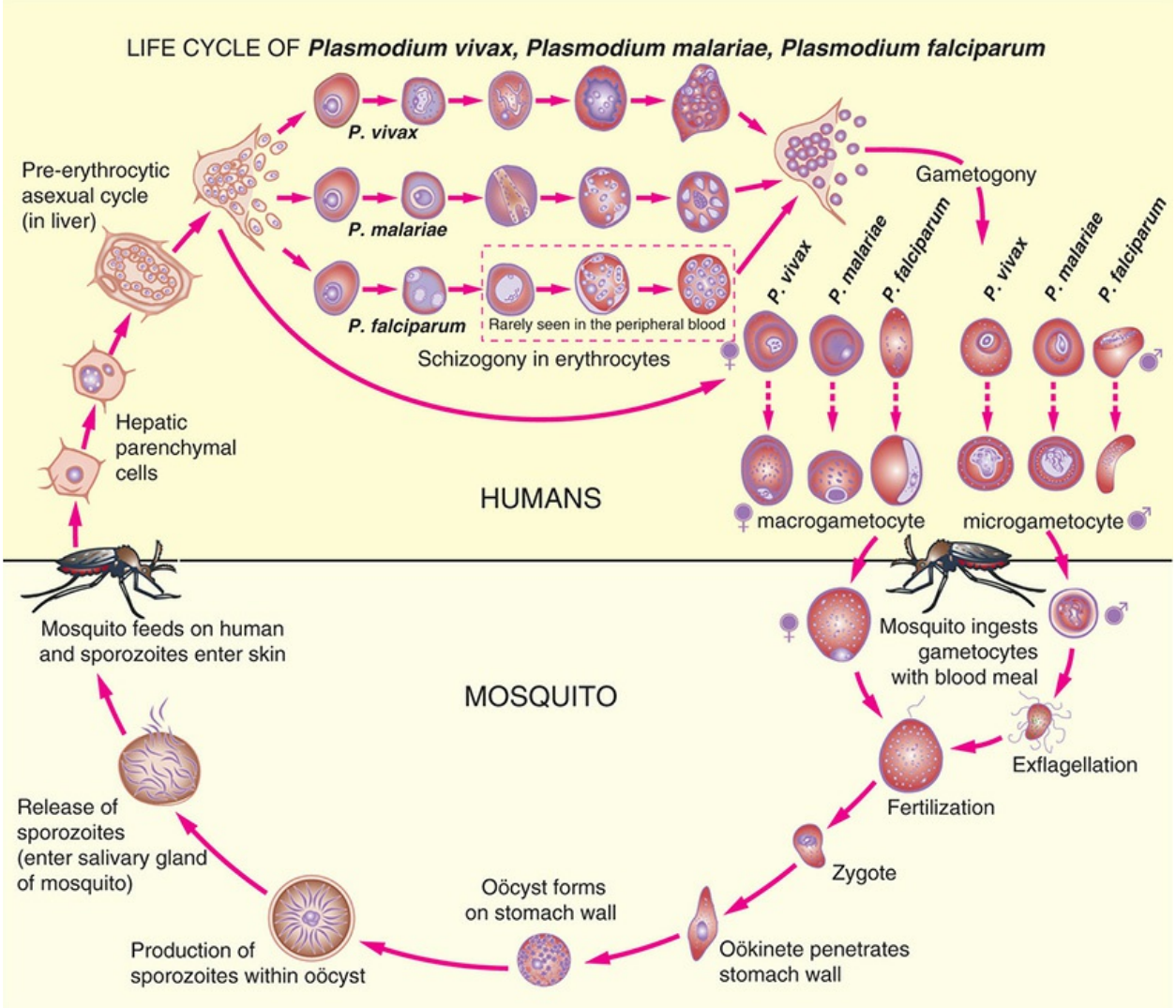

■ **FIGURE 22-19** Life cycles of *Plasmodium vivax, P. malariae,* and *P. falciparum* are illustrated. After several erythrocyte cycles have taken place, certain of the merozoites transform into sexual macrogametocytes (female) and microgametocytes (male). When a *Plasmodium*-free mosquito bites a human infected with malaria, these gametocytes are ingested along with the trophozoite-infected erythrocytes as part of the blood meal. In the stomach of the mosquito, the male microgametocytes develop six to eight flagella. The

microgametocytes penetrate the female macrogametocytes and produce fertilized zygotes. These zygotes then enter the wall of the mosquito stomach, where sporozoites eventually break out into the body cavity and migrate to the salivary glands. When the mosquito bites a new host, sporozoites are expelled from the salivary glands and injected through the proboscis.

Saliva containing infective sporozoites is injected into the bloodstream of humans through the mosquito proboscis. After circulating in the peripheral blood for 20 to 30 minutes, the sporozoites enter the parenchymal cells of the liver, where they begin to multiply (the exoerythrocytic cycle). In about 10 days, multiple small forms, called merozoites, are released from the liver cells into the circulation, where they infect erythrocytes. Within the erythrocytes (erythrocytic cycle), a series of developmental stages occur (Image Plate 22-8). These organisms develop into a "ring form" known as the trophozoite, which enlarges into an ameboid trophozoite. This subsequently divides into a segmented state known as the schizont. The individual segments of the schizonts are merozoites. When mature, the schizonts rupture the erythrocytes and merozoites are released into the circulation. These merozoites then seek out uninfected erythrocytes, and the erythrocytic cycle continues.

1. **First, rule out *Plasmodium falciparum*.** As mentioned above, this species is potentially the most dangerous, and infections should be diagnosed as early as possible. In the peripheral blood smear, look for the following:
   - The infected erythrocytes are normocytic compared to uninfected erythrocytes.
   - Tiny ring forms occupy less than one-third the diameter of the erythrocyte (Image Plate 22-8A).
   - Often, multiple forms are seen within one erythrocytes.
   - Often, two nuclei in the same ring.
   - Infections may be heavy, involving 20% or more of the erythrocytes, indicating a fulminant infection (i.e., hyperparasitemia).

   - The tiny rings often are plastered on the erythrocyte cell membrane, known as an "appliqué" form (Image Plate 22-8B).
   - Advanced forms (i.e., amoeboid trophozoites and schizonts) are rarely observed in stained peripheral blood smears. The only forms usually seen, except in terminal, fulminant infections, are early ring forms and gametocytes.
   - The presence of banana or crescent-shaped gametocytes is diagnostic (Image Plate 22-8C). Unfortunately, these may be absent in early stages of infection and usually begin to appear only after 7 to 10 days after onset of fever. Therefore, it is imperative that the parasitologist be able to identify *P.*

falciparum in the absence of these forms.

2. **Next, consider *Plasmodium vivax*.** Look for the following features in the peripheral smear:
   - Infected erythrocytes that are macrocytic compared with uninfected erythrocytes.
   - The infected erythrocytes may contain small, regular, punctate pink-red-staining granules in the erythrocyte membranes, called Schüffner's dots (Image Plate 22-8D).
     - Note: These dots are dependent on the pH of the stain and are not always present. The Wright's stain used in hematology, for example, is not pH balanced, and so Schüffner's dots will not be present. It should also be noted that *P. falciparum* very rarely contains larger, "comma-shaped" red dots called Maurer's clefts, so the presence of dots should not be the sole criteria for identification. It must be used in conjunction with the other morphology features present.
   - Ring forms in all stages of development may be present.
   - Young rings measure greater than one-third the diameter of the infected erythrocyte.
   - As the trophozoites mature, they transform from the ring trophozoite into the ameboid trophozoite.
   - Ameboid trophozoites undergo consecutive rounds of mitosis to form a cluster of merozoites called a schizont. The schizonts of *P. vivax* consist of 12 to 14 or more merozoites (Image Plate 22-8E).
   - Gametocytes are large and circular.
     - Note: Any single-celled nucleus occupying the majority of the cell diameter is likely a gametocyte.
   - Ameboid trophozoites can always be distinguished from gametocytes, because by the time an ameboid trophozoite reaches the size of half the diameter of the cell, it will undergo cellular division to form the schizont. Conversely, the gametocyte will continue to grow, occupying more than half of the cytoplasm, yet still remaining one nucleus; abundant hemozoin pigment is also often present (Image Plate 22-8F).
   - Malarial pigment in the form of finely granular, brownish pigment may be abundant in both the gametocyte and the schizont.
3. **Consider the identification of *P. malariae or P. ovale*.** The identification of these *Plasmodium* species is often by exclusion after the characteristics of *P. falciparum* and *P. vivax*, as above described, have been excluded. Look for the following features by which an objective identification can be made:

P. malariae

- Infected erythrocytes are normocytic compared with uninfected erythrocytes.

- The presence of ameboid trophozoites that form a "Band" across the infected erythrocytes (Image Plate 22-8G).
- There are no "dots" (e.g., Schüffner's Dots) ever present.
- Schizonts consist of 6 to 12 merozoites, which often form a "Rosette" (Image Plate 22-8H).
- Gametocytes are round and contained within normal-sized erythrocytes.

P. ovale

- The infected erythrocytes are macrocytic compared with the uninfected erythrocytes.
- Infected cells that are oval and have a fimbriated edge may be seen.
- The ameboid trophozoite is more compact than that of *P. vivax,* usually occupying only one-third of the cytoplasm of the infected cell.
- James' stippling, which are dots indistinguishable from Schüffner's dots, may be seen, but are also pH dependent.
- Schizonts contain 6 to 12 merozoites.
- The gametocytes are round, rather than crescent-shaped, which does not help differentiate them from *P. vivax and P. malariae,* but does differentiate them from *P. falciparum.*

The identification of *Plasmodium* species is usually not difficult if the principles described above are applied. Problems arise, however, in the interpretation of peripheral blood smears in patients who have been partially or inadequately treated when normal maturation of the parasites may be interrupted and only atypical forms are observed. The peripheral blood film interpretation may also be confusing if a given individual is infected with more than one *Plasmodium* species. Much progress has been made over the past decade in the molecular aspects of malarial infection. These advances, including an elucidation of the various genes encoding *Plasmodium falciparum* erythrocyte membrane protein 1, and those encoding the rosettin and rifin proteins on the surface of infected red blood cells, are reviewed in a publication by Chen et al.[35]

Individuals planning to travel to parts of the world endemic for malaria should consult the CDC Yellow Book (http://wwwnc.cdc.gov/travel/page/yellowbook) for complete advice on travel protection and prophylaxis. The type of prophylaxis recommended will be based on the prevalence of chloroquine-resistant malaria, previous reactions to antimalarial drugs, and the availability of medical care in the region to be visited.

Chloroquine prophylaxis should begin 1 to 2 weeks prior to anticipated travel and extend for 4 weeks after return. Chloroquine-resistant strains of *P. falciparum* have been found in all regions endemic for malaria. There are a variety of other prophylactic and therapeutic options for malaria, the scope of which is beyond this

text.[43]

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Babesiosis

Babesiosis in North America, which is the focus of this section, is caused by *Babesia microti*.[197] *B. divergens* causes a more severe disease in patients in Europe. *Babesia* is transmitted by the bite of a tick vector; *B. microti* is transmitted by the *Ixodes* tick, which is the same vector for *Borrelia burgdorferi*, the spirochete that causes Lyme disease. It has been shown that up to 40% of infected ticks are coinfected with both parasites. Perhaps this is not surprising, as both agents have the white-footed mouse,*Peromyscus leucopus*, as the main reservoir host. Most outbreaks have occurred in the northeastern regions of the United States, particularly in Connecticut, Nantucket Island, Long Island, and Cape Cod. The much lower incidence of babesiosis relative to Lyme disease in humans living in endemic areas may reflect the tendency of *B. microti* to produce subclinical infections. Serologic studies indicate that 10% of *B. burgdorferi*-seropositive Connecticut natives also have antibodies against *B. microti*, indicating that infections are far more frequent than previously suspected. Babesiosis may also be contracted through transfusion with contaminated blood or intravenous drug abuse via the sharing of contaminated needles. Transfusion-related infections have also been reported; specifically, a cluster of six transfusion-associated cases of babesiosis in New York State traced to a single asymptomatic donor.[62] It has been established that *B. microti* can remain infective at 4°C for 30 days, the normal storage time for donor blood.[67] A case of transfusion-related infection with the WA1 strain has also been recently reported from the state of Washington.[119] *Babesia*-like organisms have also been reported in Northern California.[198] Although many infections with *Babesia* are subclinical, severe-to-life-threatening disease occurs in patients without a spleen. In rare instances, exchange transfusion has been required.[64] The clinical manifestations of babesiosis are presented in Clinical Correlation Box 22-17.

The diagnostic intraerythrocytic forms described above are scarce during the early stages of infection and may be missed, even by experienced microscopists. Mattia et al.[168] used a buffy coat technique to improve the detection of parasitemia in patients with babesiosis. Garcia[87] has published a precautionary note warning of potential diagnostic problems with results derived from differential instruments. The number of fields routinely examined on instrument-read smears is quite small, and these instruments are not designed to detect intracellular parasites. Therefore, unless thick and thin blood film reviews are requested, patients with both babesiosis and malaria

may be missed.

Laboratory Identification. The laboratory diagnosis is most commonly made by detecting the intraerythrocytic parasitic inclusions in stained peripheral blood smears. They have the following identifying characteristics:

- The ring forms are tiny, ranging between 1.0 and 3.0 μm, resembling the early trophozoites of *P. falciparum* (Image Plate 22-9A).
- The nuclei are very small with usually only a single chromatin dot.
- Mature forms may appear as doublets, simulating rabbit ears; or, in tetrads resembling a Maltese cross.

CLINICAL CORRELATION BOX 22-17 Babesiosis

The symptoms in early babesiosis may be minimal: Malaise, anorexia, and fatigue are nonspecific symptoms that are easily confused with other infectious diseases.[197] Although the symptoms of infections incurred in the northeastern United States tend to be relatively mild, recent reports from case studies in California and other parts of the world indicate that fulminant, febrile, hemolytic disease presentations may be encountered.[198] Genetic studies reveal that these more severe infections may be caused by a strain more closely related to a known canine pathogen, *B. gibsoni*, or to *Theileria* species, rather than *B. microti*.[197] This strain, designated WA1 (Washington 1), and other such agents have been described. Dorman et al.[64] reported a 58-year-old man infected through a tick bite, in whom severe hemolytic anemia developed, followed by disseminated intravascular coagulation and acute renal and respiratory failure. Rapid clinical improvement was noted after treatment with clindamycin, 300 mg four times a day (started 2 days before admission), quinine (650 mg three times a day) on hospitalization, and an exchange transfusion, in which the proportion of parasitized red cells was reduced from 13.8% to 4.2%.

- The erythrocytes are not enlarged (i.e., are normocytic) and do not develop stippling.
- Extracellular forms may be seen, which is a distinctly distinguishing feature from *Plasmodium* species.
- Gametocytes and hemozoin pigment (i.e., malaria pigment) are not produced.

Hemoflagellates: Leishmania *Species and* Trypanosoma *Species*

The protozoan order *Trypanosomatida* includes members from the genera *Leishmania* and *Trypanosoma*, which are flagellates that inhabit the blood and tissues of humans. Three species/subspecies of *Trypanosoma* are associated with human disease;*Trypanosoma brucei* subspecies *gambiense* and *Trypanosoma brucei* subspecies *rhodesiense* cause African sleeping sickness, whereas *Trypanosoma cruzi* is responsible for South American trypanosomiasis. *Leishmania donovani*, the cause of visceral kala-azar in humans, and *Leishmania tropica* complex, the agents of cutaneous tropical sores, are the leishmanial species most commonly associated with human diseases.[195]

Four stages of development of the parasites may be observed. The leptomonas and crithidial stages of development are harbored within the insect vector, the latter representing the infective form for humans. The adult, or trypanosomal, forms have an elongated body with a posterior flagellum attached by a delicate undulating membrane that runs the

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length of the body. The tissue form of these protozoa is called an amastigote. It is devoid of flagella and is found as intracellular parasites. The amastigotes of *Trypanosoma* species infect somatic cells, whereas those of *Leishmania* infect cells in the reticuloendothelial system (i.e., monocytes and macrophages).

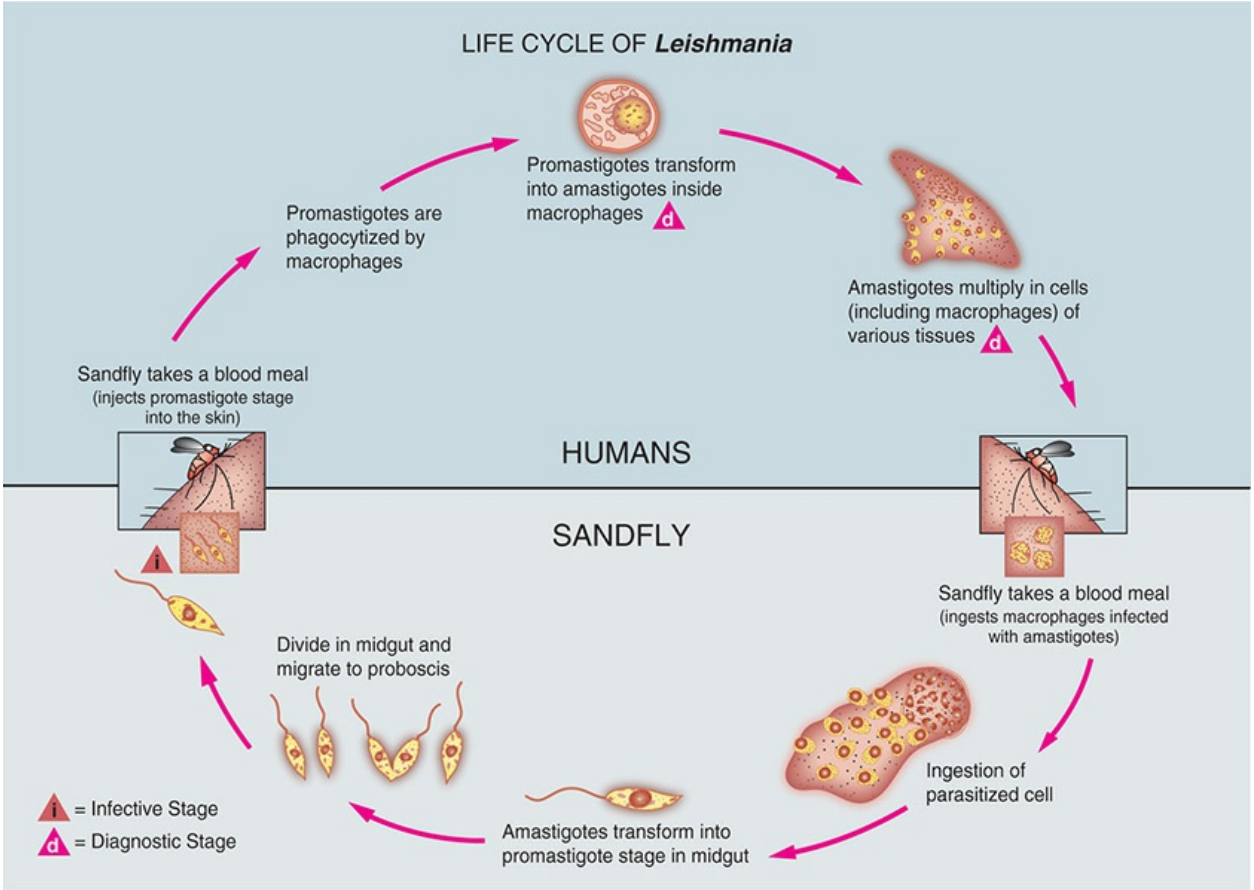
Leishmaniasis and *Leishmania* Species. Human leishmaniasis may take three forms, a disseminated disease, kala-azar, involving the liver, spleen, and other parts of the reticuloendothelial system, a primary cutaneous form, clinically manifesting as ulcers of the skin, and a mucocutaneous form (Image Plate 22-9B). Humans become infected through the regurgitation of the promastigotic infective forms of the parasite into the subcutaneous tissue as the *Phlebotomus* sandflies take a blood meal. Figure 22-20 depicts the life cycle of *Leishmania* species.

Although certain species are more likely to cause certain types of leishmaniasis, there is overlap. *Leishmania* is traditionally categorized into complexes and individual species. *Leishmania donovani* complex contains *L. donovani* and *L. infantum*, which is also known of as *L. chagasi*. *L. donovani* is an important cause of visceral leishmaniasis.[214] Important causes of leishmaniasis include the *L. mexicana* complex, *L. tropica, L. major,* and *L. braziliensis,* among others.

When the flagellates gain access to humans, they first invade inflammatory monocytes in the subcutaneous tissue at the site of the bite. When these inflammatory cells rupture, they release free parasites that in turn invade other monocytes and macrophages, within which they are disseminated throughout the reticuloendothelial system. The incubation period is in the range of 3 to 8 months. Disease progression or

cure is the result of the complex host/parasite interaction. The clinical manifestations of leishmaniasis are presented in Clinical Correlation Box 22-18.

Laboratory Identification. The laboratory-based diagnosis of leishmaniasis is made by demonstrating amastigotes in stained smears, in imprints, or in biopsies of infected tissues. This may be from a splenic aspirate in patients with visceral leishmaniasis. For cutaneous and mucocutaneous



■ **FIGURE 22-20** Life cycle of *Leishmania* species. A small skin papule develops at the site of the sandfly bite, but rarely progresses to an ulcer. The promastigotes transform into amastigotes, which proliferate locally in the subcutaneous tissue into amastigotic forms that disseminate throughout the reticuloendothelial system to involve the spleen, liver, bone marrow, and lymph nodes where the organisms are found as obligate intracellular parasites (Image Plate 22-9C). The simple life cycle is then completed when amastigotes are again taken up by a sandfly during a subsequent blood meal. The amastigotes transform into infective promastigotes in the midgut of the fly.

CLINICAL CORRELATION BOX 22-18 Leishmaniasis

Disseminated Leishmaniasis: Kala-Azar

Symptoms may be mild and self-limited. Conversely, there may be a sudden onset of symptoms, including spiking fevers (a pattern of two or three peaks per day), anorexia, malaise, and a feeling of ill health simulating typhoid fever or malaria. If the disease progresses and becomes chronic, low-grade fever, vague abdominal pain, enlargement of the abdomen from hepatomegaly and splenomegaly (which can become enormous), generalized lymphadenopathy, anemia, and leukopenia may persist. Recently, kala-azar has been recognized as an AIDS-related opportunistic infection.[179]

Primary Cutaneous Leishmaniasis

Disease is traditionally classified into Old World and New World forms. Old World disease, formerly called Oriental sore, is caused most commonly by members of the *L. tropica* complex (including *L. tropica, L. major*, and *L. aethiopica*). This form of disease is endemic in the tropical and subtropical regions of Asia Minor, China, the Southern Mediterranean, India, and Africa. At the site of insect bites on exposed parts of the body, the promastigotes injected into the skin are taken up by the reticuloendothelial cells, where they develop into the amastigotes. The species causing cutaneous leishmaniasis do not circulate except in very rare instances. An intensely pruritic papule develops at the site of the bite, which in the course of several weeks or months progresses to a chronic well-circumscribed ulcer with a raised, erythematous margin and a shallow bed (Image Plate 22-9B). The ulcers produced range from moist and multiple to drier with a tendency to crust over. Most lesions are benign and self-healing, and permanent immunity develops. The cutaneous lesions of *L. aethiopica* may not ulcerate but often result in deep, spreading subcutaneous infections, and this species also has the predilection for causing disseminated disease indistinguishable from *L. donovani* infections.[195]

New World cutaneous leishmaniasis is caused by the *L. braziliensis*, which is endemic in almost all countries in South America and in several regions in Latin America, and by the *Leishmania mexicana* complex, including several separate species endemic in various regions in Mexico, Guatemala, Venezuela, and in the Amazon basin of Brazil. *L. braziliensis* is the cause of aggressive, chronic cutaneous ulcers, with mucous membrane (oral, nasal, pharyngeal) spread, a clinical picture called espundia. Organisms of the *L. mexicana* complex are more likely to produce self-limiting cutaneous ulcers (60% of lesions occur on the ear lobes[87] producing what are known as "Chiclero ulcers"). The mucous membranes are less commonly involved, although *L. peruviana* is likely to produce a more diffuse cutaneous disease known as "uta" in Peru.

Stibogluconate sodium is the treatment of choice; treatment should be

individualized based on the available therapies, condition of the patient, and the extent of disease.

lesion, impression smears or tissue sections should be prepared from active lesions. Biopsies should be obtained from the elevated, inflamed margin of the lesion; aspirates should be obtained by extending the tip of the needle deep beneath the ulcer bed. The parasites at this stage have the following characteristics:

- The organisms are oval in shape, intracellular, and very small, averaging 2 to 4 µm in size.
- They simulate the yeast cells of *Histoplasma capsulatum* in many stained preparations, such as the H&E and Wright's stains.
  - Note: These parasites do not stain with the GMS stain, which is useful for differentiation.
- The *Leishmania* amastigotes possess a bar-like kinetoplast adjacent to the nucleus.
  - Note: This structure is helpful in distinguishing these organisms from the yeast forms of *H. capsulatum* (Image Plate 22-9C, D, and E).
- These parasites are largely within macrophages, which differentiates them from the amastigotes of *Trypanosoma*, with infected somatic cells.
  - Note: This difference is important, since the amastigotes of *Trypanosoma* also have a kinetoplast.

Culture methods, used primarily in laboratories serving practices in endemic areas, are described in detail by Garcia.[87] Lopez-Valdez et al.[159] also describe a culture method using buffy coat samples from peripheral blood that was successful in diagnosing leishmaniasis in patients with AIDS who presented with atypical clinical findings. Animal inoculation techniques are also used in these laboratories for diagnosing infections when parasite concentrations are low.

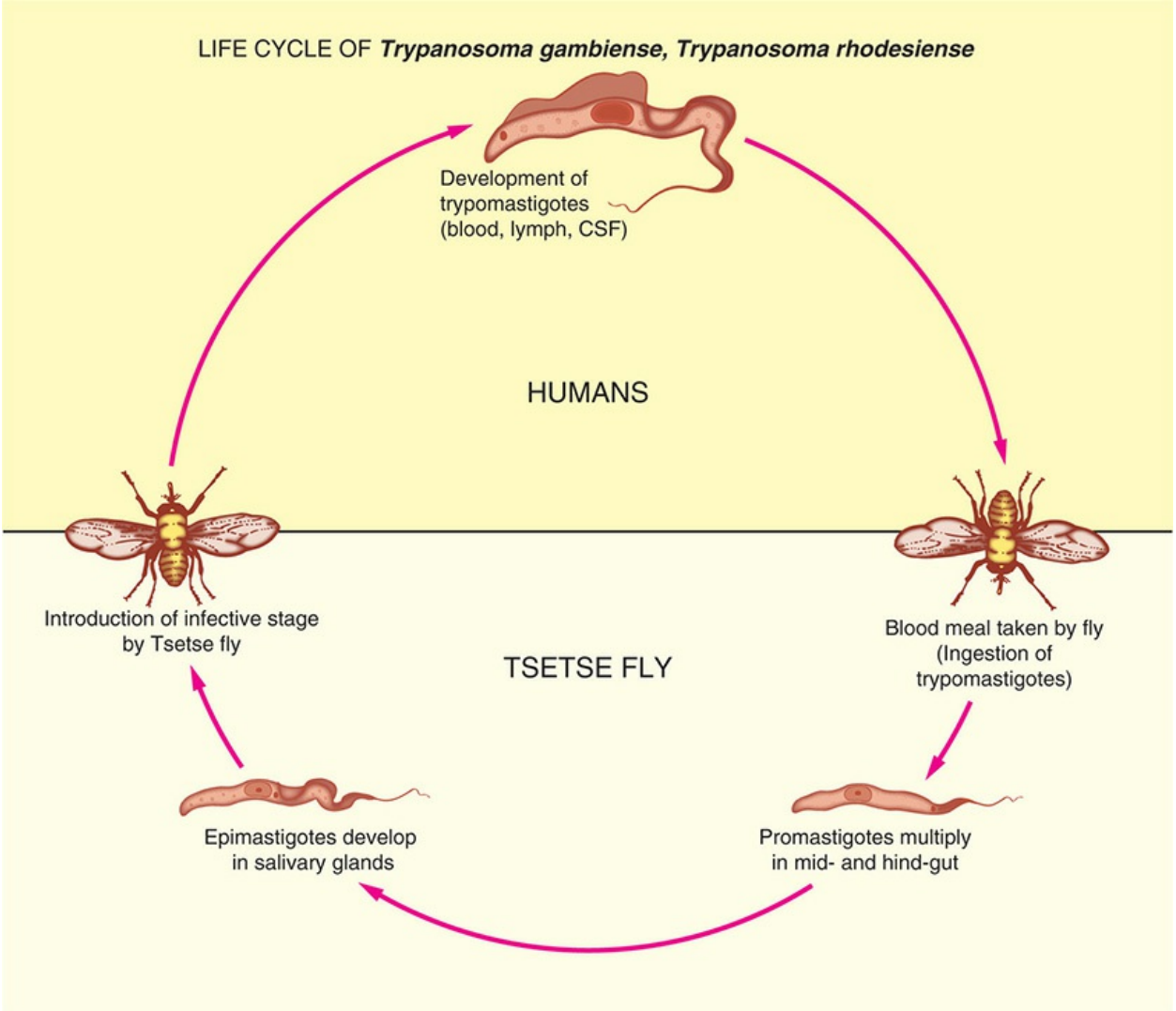
ELISA techniques, using species-specific monoclonal antibodies, immunohistochemistry, and nucleic acid amplification techniques, have been used successfully in the direct detection of *Leishmania* in extracts of tissue samples in laboratories where reagents are available. For example, Piarroux et al.[201] used a PCR assay amplifying a repeated sequence from the *Leishmania infantum* genome in a study of 73 patients with a working clinical diagnosis of visceral leishmaniasis; they demonstrated superior sensitivity of the method (82%) in making the diagnosis compared to examination of bone marrow aspirates (55%) and myeloculture (55%). They conclude that PCR may serve as an aid in diagnosing visceral leishmaniasis in immunocompromised patients. Rodriguez et al.,[212] using a PCR-hybridization technique, employing oligonucleotides directed against conserved regions of kinetoplast DNA, were able to detect the presence of *Leishmania*

cells in 98% of patients clinically diagnosed as having cutaneous leishmaniasis by a positive Montenegro skin test. They further believe that the technique is epidemiologically valuable in making the taxonomic discrimination between species.

Trypanosomiasis and *Trypanosoma* Species

African Trypanosomiasis: African Sleeping Sickness. Human trypanosomiasis is caused by a flagellate protozoan that inhabits the blood and tissues. The trypomastigote form, which measures 15 to 30 µm × 1.5 to 3.5 µm, has an elongated body with a posterior flagellum and a delicate undulating membrane that runs the length of the body. In Africa, wild game animals serve as the reservoir host. A central nucleus and a posterior kinetoplast are usually easily seen. These diagnostic forms can be seen in stained smears of peripheral blood (Image Plate 22-9F) and in the spinal fluid in certain stages of the infection. However, in chronic or light infections, Bailey and Smith,[11] in a study of blood samples obtained from 134 patients with *Trypanosoma brucei* subsp. *gambiense* infection, found the quantitative buffy coat test developed for malaria diagnosis to be the most sensitive diagnostic test for the detection of trypanosomal forms.

The life cycle involves animals and humans as the hosts, in which the mature trypanosomes circulate and divide in the peripheral blood and ultimately invade the CNS; an intermediate host and vector, the tsetse fly, is also involved. The immature forms develop in the salivary gland of the fly, ultimately forming infective metacyclic trypanomastigotes. The life cycle is illustrated in Figure 22-21.



■ **FIGURE 22-21** Life cycle of *Trypanosoma brucei* subsp. *gambiense* and subsp. *rhodesiense*. The life cycle shown here involves humans, and an insect, the tsetse fly, but in nature existed between the tsetse fly and animal hosts. On ingestion of trypomastigotes from an infected human through a blood meal, promastigotes are released in the stomach of the fly, and multiply in the midgut and hindgut, finally migrating to the salivary glands as epimastigotes. Humans are infected in turn through the bite of an epimastigote-infected fly. A chancre develops at the site of the insect bite, within which over a period of several weeks, the trypanosomes undergo multiplication and maturation. Ultimately, they invade the lymphatics and bloodstream, widely disseminating to lymph nodes and ultimately to the CNS. Organism multiplication continues to occur in the bloodstream despite humoral immunity of the infected host, a fundamental difference from infections caused by *T. cruzi*.

There has been substantial progress in controlling African trypanosomiasis. In a World Health Organization report issued in 1986, there were approximately 20,000 new cases diagnosed each year.[275] In 2009, the "10,000" threshold was broken (i.e., 9,878 cases were reported) due to sustained control efforts. In 2012, 7,216 cases were reported to the WHO. In the African form of trypanosomiasis, humans become infected through the bites of infected *Glossina* tsetse flies that harbor the infective procyclic trypanomastigotes in their salivary glands. Tsetse flies are found only in Africa. The capacity of these organisms to remain viable in a population of chronically infected people is due to the unique property of the organisms to periodically change the antigenic structure of the glycoproteins within their surface membranes, thus bypassing the effects of host antibodies.[63] The clinical manifestations of African trypanosomiasis are presented in Clinical Correlation Box 22-19.

Antigen-detection methods have been developed to diagnose African trypanosomiasis.[182,183] In a field trial in Zaire, Nantulya et al.[183] effectively used an antigen ELISA in evaluating 77 parasitologically proven cases of Gambian trypanosomiasis. Of these patients, 69 (89.6%) had antigens in serum and 35 (45.5%) had antigens in the CSF. Of the latter, 34 (97.1%) had elevated CSF white-cell counts, 29 (82.9%) had elevated protein levels, and 23 (65.7%) had trypanosomes microscopically identified in the CSF. The authors indicate that their ELISA test may be useful in staging disease and in follow-up to treatment. Kyambadde et al.[148] found that PCR demonstrated infection in 20 of 35 (57%) blood samples obtained from patients with suspected trypanosomiasis in Uganda, and in 21 of 34 CSF samples (61%). Of 21 blood samples negative for circulating diagnostic forms, 6 were positive by PCR (28.6%), and 8 of 21 CSF samples (38.0%) that were negative by double centrifugation were positive by PCR.

The particular subspecies causing the infection and the stage of disease are important when considering treatment. Pentamidine is useful for treating patients in the first stages of infections caused by *T. b. gambiense.* Other therapeutics, such as suramin, melarsoprol nifurtimox, etc., are available only through CDC.

South American Trypanosomiasis: Chagas' Disease. South American trypanosomiasis, also known as Chagas' disease, is caused by *Trypanosoma cruzi*. This parasite is found from the southern United States through Central and South America to Argentina. An estimated 10 to 12 million people are infected, approximately half of whom live in Brazil.[60] Although *T. cruzi* has been found in dogs, wild mammals, and insect vectors in the southern part of the United States, autochthonous human cases have been reported only very rarely.[106,244] The diagnosis may be made by observing circulating trypomastigotes in the peripheral blood, although the period of circulation is shorter than that observed in African trypanosomiasis. The trypomastigotes are similar in appearance to those of their African counterparts, except there is a distinct tendency for them to curl upon

themselves forming a letter "C" shape (Image Plate 22-9G).

An autochthonous infection reported in the United States was in rural Tennessee, when a mother found a triatomine bug in the crib of her 18-month-old boy.[118] The gut content of the bug was found to be infected with *Trypanosoma cruzi* both by light microscopy and PCR. A buffy coat prepared from a blood sample from the child was negative for organisms; however, it was positive by PCR, indicating low-level parasitemia. Thus, this autochthonous *T. cruzi* infection in the United States would have been missed without the mother's attentiveness and the highly sensitive PCR technique.

The life cycle of *T. cruzi* differs from the African species in that the triatomid (reduviid) bug serves as the arthropod vector. Both rodents and domestic animals, including dogs, cats, and pigs, serve as reservoir hosts for *T. cruzi*, and so eradication from endemic areas is not feasible. In the United States, opossums and raccoons have been found to be infected. In the incident reported above, three raccoons in the vicinity of the infected child had positive hemocultures for *T. cruzi*. Houses built of adobe, mud, or vegetative material, in which there are numerous cracks in the walls, provide the

CLINICAL CORRELATION BOX 22-19 African Trypanosomiasis

African trypanosomiasis, also known as African sleeping sickness, is caused by one of two subspecies of *Trypanosoma brucei*. Subspecies *rhodesiense* is endemic in the savannah and woodland regions of central and east Africa, whereas *Trypanosoma brucei* subsp. *gambiense* is endemic in the tropical rain forests of Central and Western Africa. The clinical disease caused by *T. brucei rhodesiense* tends to be of more rapid onset with a greater tendency to become rapidly progressive, even leading to death. Gambian trypanosomiasis is more chronic, characterized by neurologic deterioration, culminating in "sleeping sickness."

Patients infected with *T. brucei gambiense* first experience intermittent recurring fever associated with lymphadenopathy. Lymph nodes in the posterior cervical region of the neck are frequently involved, producing a lesion known as Winterbottom's sign. Ormerod[190] has demonstrated experimental evidence of a physiologic connection between cervical lymph glands and the ventricles of the brain, suggesting that Winterbottom's sign may be a marker for cerebral infection.

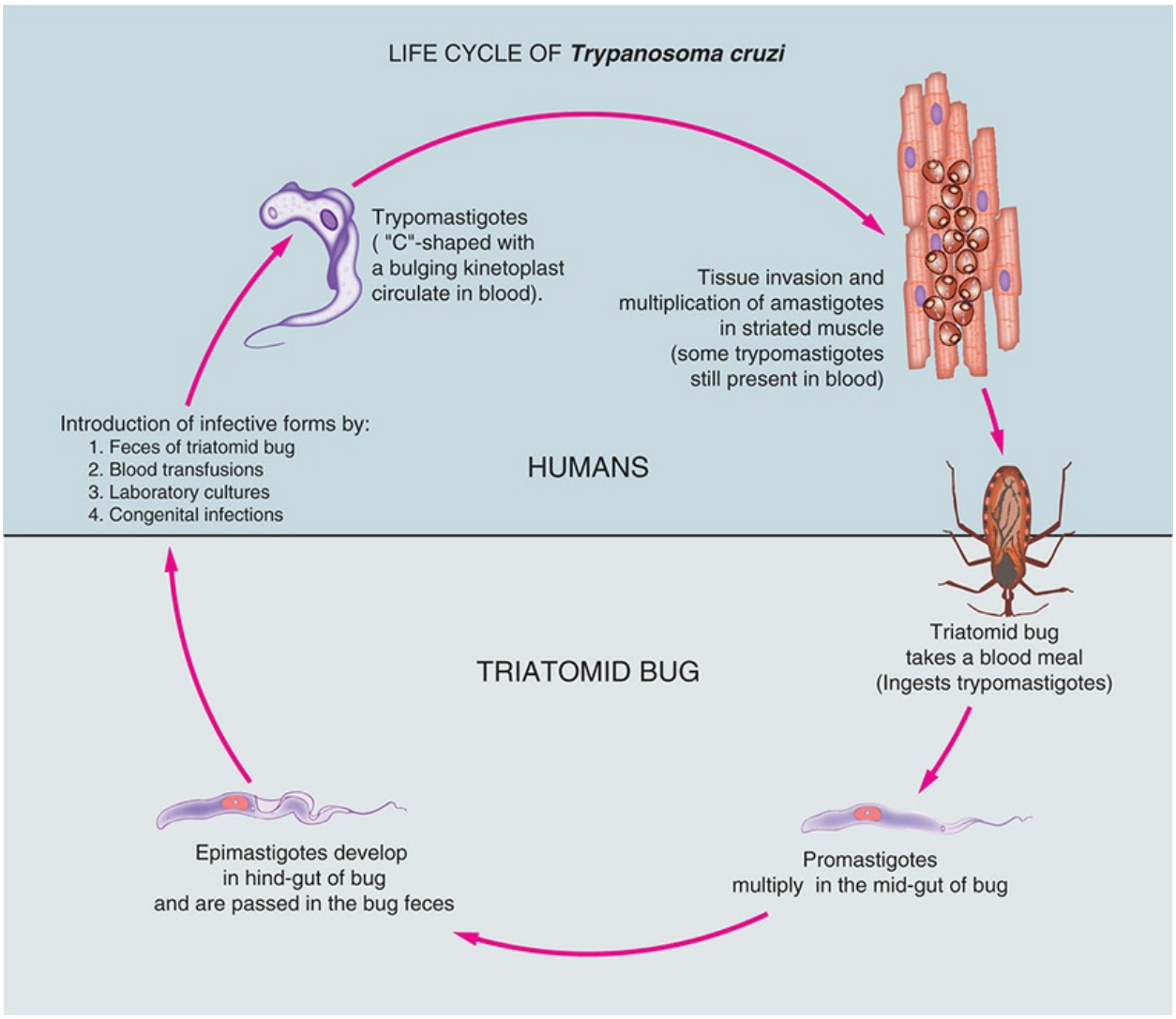
Hepatosplenomegaly may also be evident during this early stage of infection. As the disease becomes chronic, organism invasion of the CNS produces the sleeping sickness stage of the disease, characterized initially by behavioral and personality changes, leading to apathy, fatigue, confusion, and somnolence—signs of progressive meningoencephalitis. Emaciation and profound coma ultimately lead to death.

optimal breeding places for the reduviid bugs in endemic areas (Image Plate 22-9H). These insects are nocturnal and feed on sleeping victims. Prevention of the disease is largely aimed at improving housing conditions and decreasing the insect breeding environment. The life cycle of *T. cruzi* is illustrated in Figure 22-22.

Primary infection ranges from asymptomatic to fever, lymphadenopathy, and unilateral facial swelling. The great danger from South American trypanosomiasis is that visceral organs may be infected with the amastigotes of the parasite. This results in serious disease that may be fatal. The clinical presentations of South American trypanosomiasis are found in Clinical Correlation Box 22-20.

Trypanosoma rangeli is another trypanosomal organism transmitted to humans by reduviid bugs. Infections of humans and animals are asymptomatic. The trypanomastigotes can be identified in thick and thin smears of peripheral blood and morphologically resemble those seen in African trypanosomiasis. However, unless a careful clinical history is obtained, a misdiagnosis of *T. cruzi* infection may be made. Serologic testing may be of value in differentiating *T. rangeli* infections from Chagas' disease; however, duel infections may occur producing confusing results.

Serologic methods have been developed to detect *T. cruzi* serum antibodies to aid in the diagnosis of Chagas' disease.[93] Godsel et al.[92] identified in the serum of patients with Chagas'



■ **FIGURE 22-22** Life cycle of *Trypanosoma cruzi*. Humans become infected when the trypanosomal-infected fecal matter is discharged into the wound when the bug feeds. The bite is quite painful, and the infected feces are rubbed into the wound. In the human host, the C-shaped trypanosomes, with an enlarged kinetoplast compared with *T. brucei*, circulate in the bloodstream during the early acute phase of the disease (Image Plate 22-9G), and intermittent febrile episodes are experienced. The cycle is completed when a reduviid bug (Image Plate 22-9H) again bites an infected host.

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CLINICAL CORRELATION BOX 22-20 South American

Trypanosomiasis – Chaga's Disease

The etiologic agent of South American Trypanosomiasis or Chaga's Disease is *Trypanosoma cruzi*. Humans are infected through the bite of a triatomid bug (i.e. the reduviid bug) that is colloquially called a kissing bug. The bite of this insect vector does not transmit the parasite, but rather these are present in the feces of the insect. Patient who scratch at the bite site inadvertently inoculate the contaminated feces into the bite site. Acute infection is most commonly asymptomatic or produces only mild symptoms, but occasionally more severe reactions occur. Fever and swelling at the bite site are common in symptomatic patients. Unilateral periorbital swelling (Romaña's sign)occurs if the contaminated feces are rubbed into the eye. Trypomastigotes may be seen in the blood of the patient during the acute phase of disease.

The infection then may become chronic, with amastigotes replicating within somatic cells, such as the myocytes of the heart. In many instances, patients may never know that they have been infected, particularly if the acute infection was asymptomatic or produced subclinical disease with non-specific signs and symptoms. The damage done to the somatic cells of the patients continues for years, until organ systems fail. Infection of the heart may result in dysrhythmias or in dilated cardiomyopathy and congestive heart failure. Other common manifestations are the "mega-syndromes," which includes mega-esophagus and mega-colon, wherein these tubular organs are massively dilated and have lost the ability to effective produce peristalsis. These end-stage diseases can really only be effectively addressed through surgery, including cardiac transplantation.

disease a new recombinant antigen called FCaBP, a 24-kDa flagellar calcium-binding protein. This protein may be used as a component of a multiple-recombinant antigen preparation effective in screening for *T. cruzi* in donor blood supplies. Tantowitz et al.,[244] in a comprehensive review, discuss the importance of screening donor blood for Chagas' disease. The issue affects not only individuals living in countries with endemic disease, but also is of concern in the United States, where upward of 50,000 immigrants from endemic countries may be infected. Avila et al.[10] suggested a sensitive screening test for diagnosing chronic Chagas' disease in blood bank donors. They demonstrated 100% sensitivity compared with serologic tests in the detection of PCR-amplified products of *T. cruzi* kinetoplast minicircle DNA in 114 blood samples obtained from chronic patients with and without Chagas' disease, using a specific digoxigenin-labeled oligonucleotide probe. As mentioned, a chronic infection by *T. cruzi* may remain dormant for years. The organ damage that occurs throughout this silent period is irreversible and profound, often necessitating extensive surgery or even

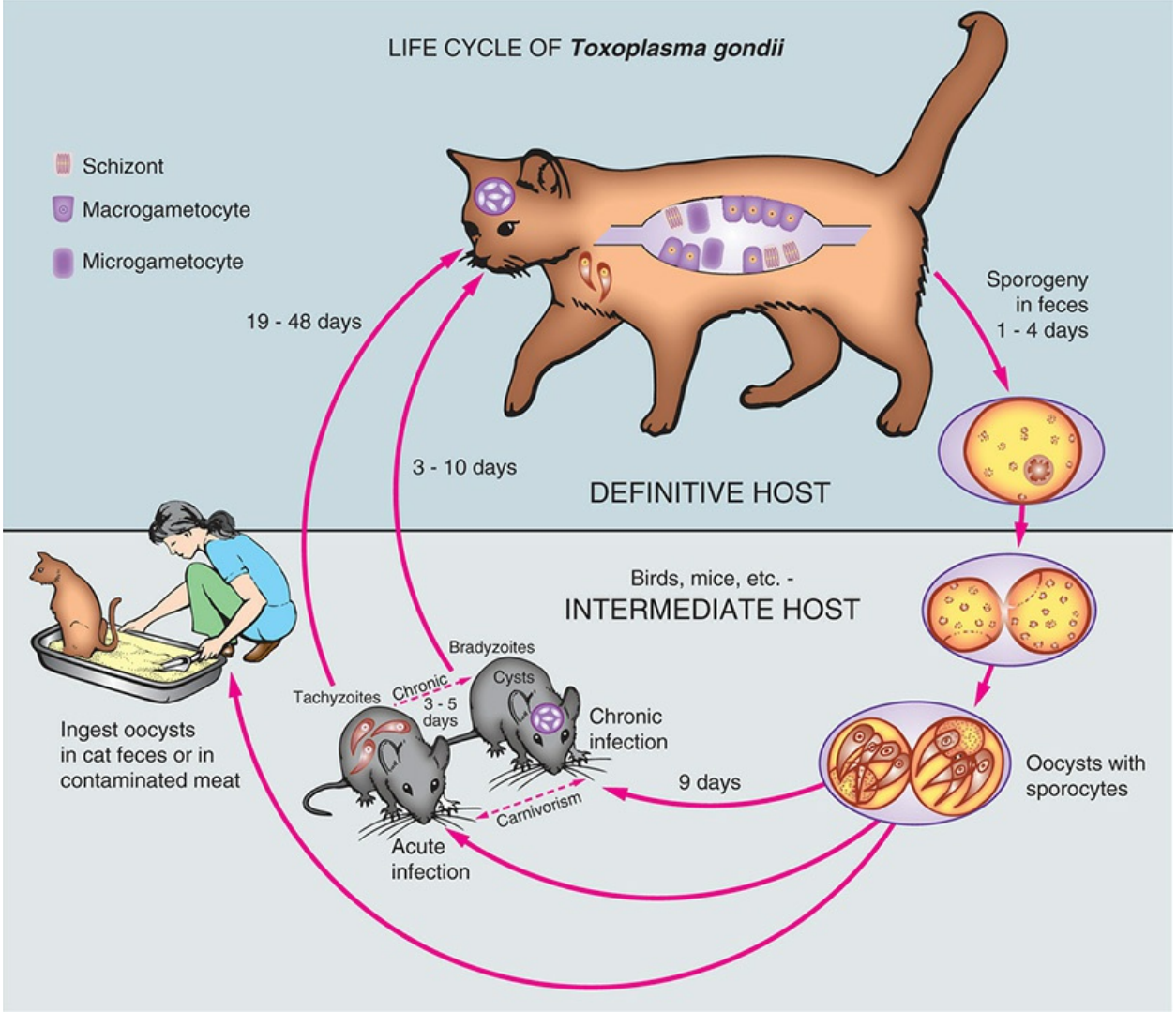
cardiac transplantation. Therefore, although there are few individuals in the United States with Chagas' disease, it has become the standard of practice to screen donated blood for evidence of infection.

Because the number of circulating trypomastigotes is very low in chronic Chagas' disease, PCR-based detection of *T. cruzi* kinetoplast DNA provides a highly sensitive method for establishing a diagnosis. Many studies have shown a high degree of sensitivity, comparable to xenodiagnosis, capable of detecting as few as one circulating trypomastigote in 20 mL of blood.[19,94] This method has also been found useful as a test for cure in the monitoring of patients who are undergoing therapy.

Toxoplasmosis and *Toxoplasma gondii*. *Toxoplasma gondii* is a protozoan parasite of felines, and infections of humans are largely accidental (i.e., in most instances, the human infection is a dead end without further propagation of the parasite in a feline, definitive host. The life cycle of *Toxoplasma gondii* is illustrated in Figure 22-23. Human infections are important because of a particular predilection for infection to involve CNS of the unborn child.

Three modes of transmission lead to most human infections: (1) directly from ingestion of infective oocysts in food (e.g., unwashed leafy vegetables) or water contaminated with cat feces; (2) indirectly, from ingestion of the raw or undercooked meat of animals that had ingested oocysts (it is estimated that 25% of lamb and pork meat sold at supermarkets contain viable tissue cysts); and (3) transplacental transfer to the fetus from a mother infected during pregnancy. Maternal infection rates during the reproductive years are estimated to be between 3% and 5%, but vary depending on the prevalence of disease.[78] Pregnant women should be strongly advised to avoid the handling of cats, cat feces (i.e., changing the litter box), and the ingestion of undercooked meat. Clinical symptoms may be minimal or absent in the acute stages of infection, and infants may not show some of the cardinal signs of infection—chorioretinitis, cerebral calcification,[215] hydrocephalus, or microcephaly—for several months or even years. Acquired infections in adults usually manifest as an acute mononucleosis-like illness, which includes lymphadenitis and lymphadenopathy. Most acute infections result in encystment of the organisms if the host is immunocompetent.

Two parasitic stages may be involved in human infections. After ingestion of oocysts or the encysted infective forms in uncooked meat, tachyzoites (Fig. 22-24 and Image Plate 22-10F) are released in the small intestine and first invade the mucosal epithelial cells, from which they enter the circulation, and then are widely distributed throughout the body. Considerable tissue damage may occur as the tachyzoites destroy the cells they parasitize; however, as the immune response develops, the tachyzoites become less active and ultimately aggregate within a membrane-enclosed cyst (Fig. 22-25, Image Plate 22-10G and H). Although inactive, the bradyzoites within these cysts may remain viable for weeks or for years. Most commonly, they ultimately



■ **FIGURE 22-23** Life cycle of *Toxoplasma gondii*. The life cycle of *T. gondii* has both sexual and asexual stages. The sexual stage occurs in the intestine of cats, where infective oocysts, measuring 10 to 12 μm in diameter, replicate within the intestinal mucosal epithelial cells and are excreted in the feces. The asexual stage commonly occurs in a variety of herbivorous and carnivorous animals that ingest the infective oocysts when foraging through contaminated soil and vegetative matter. Humans may also become infected by ingesting food or water contaminated with oocysts. Cockroaches, earthworms, snails, and slugs may also serve as transport hosts for oocysts.

disintegrate and become enmeshed in a hyaline scar and/or undergo calcification. The detection of intracerebral calcification in skull roentgenograms is one method for

establishing previous infection.

If the host is immunocompromised or becomes immunocompromised even years after the primary infection, the parasite may excyst; bradyzoites transform into tachyzoites and begin replicating causing a variety of diseases, including myocarditis, meningoencephalitis, atypical pneumonia, and rhinochoroiditis.

Laboratory Identification. The diagnosis of acute toxoplasmosis in an immunocompetent individual is usually made by serologic methods. In the immunocompromised with progressive disease, it can be made by demonstrating clusters of tachyzoites in stained smears using the PAS, Giemsa, and/or H & E stains. Fluorescent antibody staining and peroxidase–antiperoxidase techniques have been used to better demonstrate organisms in formalin-fixed and paraffin-embedded tissues. Tachyzoites are perhaps best seen in needle aspirates or impression smears stained with Wright–Giemsa.

- The tachyzoites are typically crescent-shaped, measure 3 to 4 × 6 to 7 μm, and have a dark-staining central nucleus (Fig. 22-26 and Image Plate 22-10F).
- One or more cysts, when observed in tissue sections of patients, usually indicate a chronic and usually inactive stage of infection, but serve as an important source of acute reactivation toxoplasmosis.

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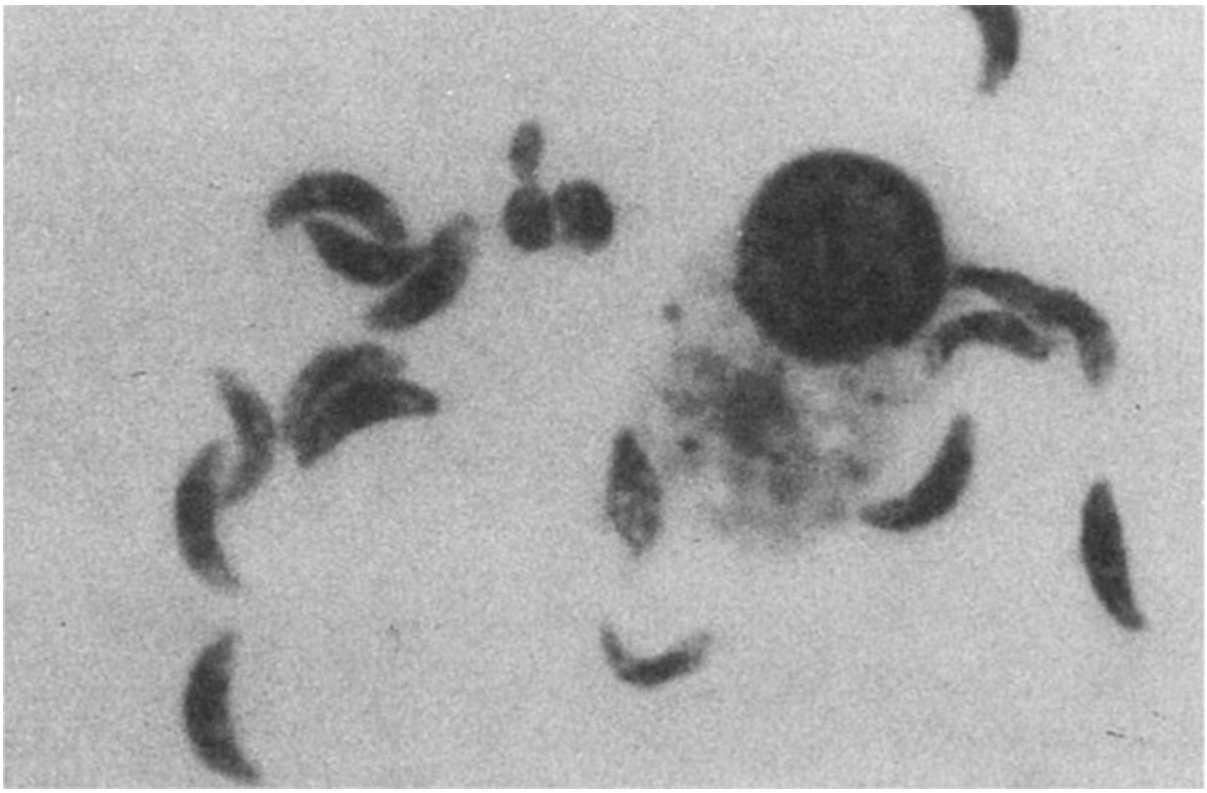
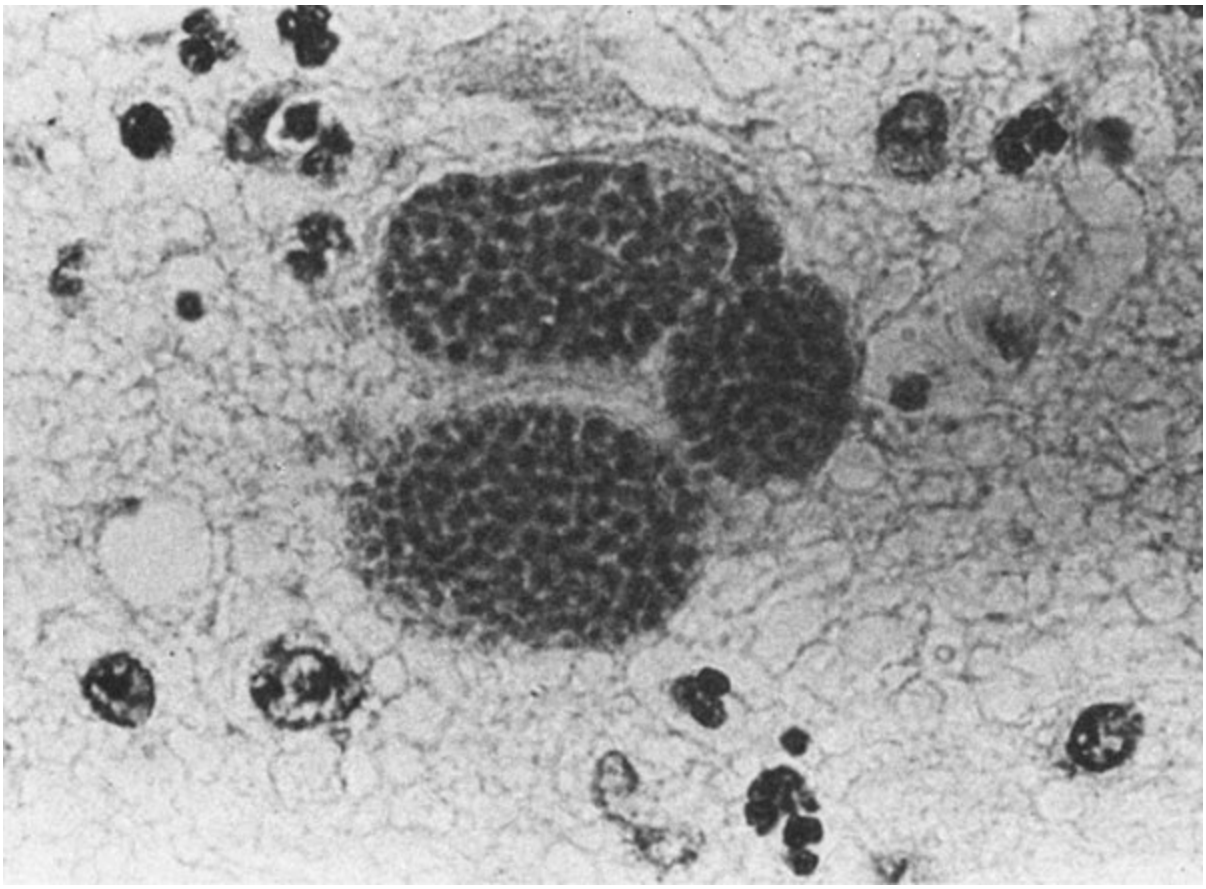


■ **FIGURE 22-24** High-power view of Giemsa-stained impression smear demonstrating *Toxoplasma* tachyzoites. Note characteristic bow-shaped or crescent-shaped, with central dark-staining nucleus (×1,000).



■ **FIGURE 22-25** High-power view of the toxoplasmal bradyzoite cysts. Each pseudocyst contains several scores of trophozoites. These bradyzoite cysts are most commonly seen in patients with latent disease. Note the absence of an inflammatory reaction adjacent to these pseudocysts (H & E ×1,000).

- Cysts measure up to 200 μm in diameter and contain several hundred bradyzoites 2 to 3 μm in diameter (Fig. 22-27 and Image Plate 22-10G and H).

The serologic diagnosis of toxoplasmosis is complex, and a full account is beyond the scope of this presentation. Pioneering work has been done by McCabe and Remington,[169] a summary of which is included in Box 22-5. Because of the high prevalence of increased titers of *Toxoplasma* antibody in the general population, test results must be carefully interpreted before a definitive diagnosis can be established. The time-honored Sabin–Feldman dye test, based on the principle that live *Toxoplasma gondii* tachyzoites lose their affinity for methylene blue dye in the presence of immune

BOX 22-5

Interpretation of Serologic Results in Clinical Syndromes of Toxoplasmosis[169]

A. Acute acquired toxoplasmosis: immunocompetent patient
   The diagnosis can be highly suspected if:
   1. Seroconversion from negative to positive is demonstrated.
   2. A twofold increase in titer between a baseline sample and a repeated test 3 weeks later.
   3. A high IgM titer with a single high IgG titer (>1:1,000).
   4. A low IgM titer indicates that the infection was acquired 4 months or more before. The IgM titer usually reverts to normal within 9 months.
      Note: A negative dye or immunofluorescence assay result in an immunocompetent person virtually precludes the diagnosis of acute toxoplasmosis.

B. Acute acquired toxoplasmosis: immunosuppressed patient
   The criteria cited for immunocompetent patients apply to immunoincompetent individuals as well. However, IgM antibody or twofold rises in IgG antibody often are not detected; therefore, a negative test does not rule out acute toxoplasmosis. Organisms must be demonstrated in tissue biopsies or impression smears of aspirates before a secure diagnosis can be made in this group of patients or by PCR.

C. Ocular toxoplasmosis
   The diagnosis can be suspected if:
   1. Low titers of IgG antibody in the presence of a typical retinal lesion.
   2. "C" is equal to or greater than 8 using the following formula when testing aqueous humor fluid:

      (Antibody titer in fluid×concentration of γ-globulin in serum)/(Antibody titer in serum×concentration of γ-globulin in body fluid)

D. Congenital toxoplasmosis
   The diagnosis of *Toxoplasma* infection in the neonate can be established if:
   1. There is a persistent or rising titer in serologic studies.
   2. A positive IgM test at any titer after birth in the absence of a placental leak, or the organism is demonstrated in tissue biopsies or impression smears of aspirates or by PCR.

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serum, being both sensitive and specific, may be used as the reference method, but is not used in clinical practice. Newer, sometimes automated, techniques are used in

most reference laboratories, which bypasses the necessity of having to work with live organisms. Current assays assess for both IgM and IgG, so that an assessment of the phase of infection (i.e., acute vs. remote) may be determined in many instances with a single serologic study, rather than having to compare acute and convalescent titers. A number of methods have been explored for the serologic diagnosis of toxoplasmosis.[46,153,273]

Nucleic acid amplification techniques for the detection of *Toxoplasma* have also improved significantly over the past decade, particularly with the introduction of real-time PCR technology. Several reports describe the *T. gondii* B1 gene as a valuable target for PCR.[71,123,143,193] The B1 gene-amplification procedure is sufficiently sensitive to detect a single isolated parasite and as few as 10 parasites in a background of 100,000 leukocytes.[193] Nucleic acid amplification techniques are more sensitive and have virtually replaced cell culture as the method of choice in diagnosing acute or active *Toxoplasma* parasitemia, particularly in the immunocompromised host.[123] This has been used to detect *Toxoplasma* parasitemia in patients with AIDS and unexplained fever or CNS abnormalities.[71] Examination of blood by PCR may also be useful in diagnosing extracerebral toxoplasmosis from the local reactivation of latent brain cysts.[66] Dupon et al.[65] also found the PCR examination of CSF and/or blood to be a valuable adjunct in detecting reactivation of latent cerebral toxoplasmosis. In addition to detecting the *T. gondii* B1 nucleic acid sequences, they cite other studies successfully using p30, TGR1 genes, and the 18S rDNA as targets. PCR of the CSF for *Toxoplasma* has largely replaced the need for brain biopsy to achieve the diagnosis, just as PCR for the herpes simplex virus (HSV) and JC virus has largely replaced brain biopsy for the diagnosis of HSV-associated meningoencephalitis and JC virus-associated progressive multifocal leukoencephalopathy. PCR when applied to amniotic fluid has also proven useful for the diagnosis of congenital toxoplasmosis.[104]

Trichomoniasis and *Trichomonas vaginalis*. Trichomoniasis, caused by *T. vaginalis*, is one of the most common sexually transmitted diseases, and importantly, it is curable. It has been estimated that 3.7 million individuals in the United States are infected, but only approximately 30% are symptomatic. Therefore, it is important to test individuals who have had unprotected sex.

Symptomatic trichomoniasis most commonly occurs in women, wherein there is a green frothy discharge, odor, and vaginal discomfort, such as itching and pain upon urination. Although surprisingly infections in men are often asymptomatic, if symptoms occur, they may include a urethral discharge and pain upon urination and ejaculation. *Trichomonas* does not disseminate. However, the treatment of infected patients is important, since infection with this parasite increases the likelihood of contracting another sexually transmitted disease, most importantly HIV.

Traditionally, trichomoniasis has been diagnosed using a "wet prep" often performed in the physician's office. The identification of *Trichomonas* is quite simple when the vaginal fluid from symptomatic women is examined microscopically, since the trichomonads are motile. Although this test is highly specific in this setting, the sensitivity of a microscopic examination diminishes in infected but asymptomatic patients. One study demonstrated that the direct examination was only 58% sensitive compared with culture. The sensitivity of the microscopic examination is also diminished if the specimen is not examined immediately, but rather transported to a distant laboratory. Nonmicroscopic assays for the detection of *Trichomonas* have become favored secondary to increased regulation of physician-performed microscopy and laboratory consolidation.

Laboratory Identification. The morphologic evaluation of *T. vaginalis* is usually straightforward when fluids from an infected, symptomatic woman are examined and motile trichomonads are seen. However, it is important that the parasitologists have the ability to differentiate *T. vaginalis,* which is sexually transmitted, from *Pentatrichomonas hominis,* which is part of the normal microbiota of the intestinal tract. The morphologic criteria used to identify *Trichomonas vaginalis* include the following:

- Trichomonads are pyriform, measuring 10 to 12 μm, with a single oval nucleus.
- There is no cyst form.
- A basal body is present at one end from which numerous flagella arise.
- There is an axostyle, which extends the length of the organism and extends from the distal end.
- An undulating membrane is present that extends from the basal body.
  - *Trichomonas vaginalis*: The undulating membrane 1/2 to 3/4 of the length of the organism.
  - *Pentatrichomonas hominis*: The undulating membrane runs the full length of the organism.

Trichomonas culture has been used to preserve the viability of the trichomonads using Diamond's medium. Although culture for *Trichomonas* is feasible, it requires a longer time to result and has not become the standard of practice. The OSOM *Trichomonas* Rapid Test is a CLIA-waived, lateral flow ICT assay that detects the antigens of *T. vaginalis.* These have a 95% agreement, according to the package insert, with the wet preparation, which represents a waived alternative for physician's offices for the assessment of symptomatic patients.

The BD Affirm VPIII system is a probe-based technology designed to detect the most common agents of vaginitis and includes *Trichomonas vaginalis.* According to the package insert, the sensitivity and specificity of this assay were 93% and 99.9%compared to direct examination and 90% and 99.9% compared to culture. This

product represents an option to assess for multiple causes of vaginitis simultaneously.

Nucleic acid amplifications are emerging as the gold standard for the detection of *T. vaginalis*. Importantly, this highly sensitive technology affords the opportunity to detect individuals who are infected, but who are not symptomatic. This assay utilizes transcription-mediated amplification, which has been used successfully for many years for the detection of *N. gonorrhoeae* and *C. trachomatis*. The APTIMA *Trichomonas vaginalis* is currently the only FDA-approved nucleic acid amplification assay. Chapin and Andrea found this assay

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to have a clinical sensitivity of 95% and a specificity of 98%.[33] Importantly, this technology affords detection in men, which is a cohort that has largely gone undetected and represent an important reservoir.[227]

Metronidazole is the most commonly used therapy. Tinidazole is an FDA-approved alternative that is equally effective.

Blood and Tissue Helminths

Filarial Nematodes and Filariasis

The filarial parasites discussed here are threadlike nematode worms.[194] The adults inhabit primarily the circulatory and lymphatic channels, but may also be found in muscles, connective tissues, and serous cavities. Some species form a subcutaneous nodule. Three important species of filariae worms that commonly cause disease in humans, *Wuchereria bancrofti*, *Brugia malayi*, and *Loa loa,* will be covered here.

There have been substantial efforts to combat filariasis in the past decades. Regardless, the WHO estimates that 120 million people are currently infected, with 40 million who are disfigured or will be incapacitated by the disease; although, similar numbers were reported in 1989 and 1999, this still represents progress, given the growing population in affected areas.[139] Ninety percent of patients have bancroftian filariasis, whereas brugian filariasis accounts for most of the remainder.[181] Bancroftian filariasis is seen primarily in urban areas due to poor sanitation and intense breeding of *Culex* mosquitos. Brugian filariasis is mainly a rural disease transmitted by *Anopheles* and *Aedes* mosquitos. The number of infected persons varies based on economic conditions associated with increased exposure to the insect vector and the formation of favorable mosquito breeding sites.[256]

Humans acquire the disease following a bite from an infected mosquito (*W. bancrofti* and *B. malayi*) or tabanid flies for *Loa loa*. Biting midges serve to transmit

the nonsheathed species, *M. perstans* and *M. ozzardi*. The life cycle of *Wuchereria bancrofti* as a prototype for the filarial worms is illustrated in Figure 22-26.

Laboratory Identification. The laboratory diagnosis is made by observing circulating microfilaria in stained peripheral blood smears.[102] Microfilariae circulate in the peripheral blood with a regular periodicity that is dependent on the species of worm. Those of *W. bancrofti* and *B. malayi* are nocturnal, whereas those of *Loa loa* are diurnal.[208] Therefore, to diagnose bancroftian filariasis, optimally blood should be obtained between midnight and 2:00 AM In patients with light infections or when samples are collected at suboptimal times, membrane filtration, centrifugation, sedimentation (Knott's concentration), and preparation of thick smears are techniques that may help to detect low numbers of circulating microfilaria.[211] The use of polycarbonate filters (Nuclepore) to trap microfilaria on a filter after the red blood cells have been lysed is an alternative concentration technique.[58] Because the 3 to 5 μm pore size filters are transparent when wet, they can be directly examined for the presence of microfilaria on a microscope slide.

Long et al.[157] describe an interesting approach to the laboratory diagnosis of filariasis using an acridine orange/microhematocrit tube technique. A microhematocrit tube incorporating heparin, EDTA, and acridine orange serves as the basis for this test. On centrifugation, parasites become concentrated in the buffy coat and can be visualized through the clear glass wall of the tube. The acridine orange stains the DNA of the parasites, and the morphologic characteristics, including the nuclear patterns in the tail sections, can be examined by fluorescence microscopy to achieve a species-level identification.

Mature female worms produce myriad larvae known of as microfilaria that circulate in the blood and serve as the mode of transmission when the mosquito vector takes a blood meal. An identification can be made by observing key characteristics unique to each filarial species.

Microfilaria:

- Measure between 240–300 × 7–10 μm.
- Are ribbonlike and can be seen in microscopic mounts of anticoagulated blood by their undulating motion, displacing the red blood cells from side to side as they move.
  - Note: This is rarely done. In most instances, they are observed in the thick and thin blood film preparations. As several of the species cause cyclic fevers, they are in the differential diagnosis with *Plasmodium* species. Therefore, the examination of every thick and thin blood film should begin with a low-power (i.e., 100× magnification; use the 10× objective) to search the film for the presence of microfilaria.

- A prominent sheath surrounds the microfilaria of the species discussed here. It is obvious in stained peripheral blood smears for some species, but for others appears as a nonstaining area around the microfilaria. The sheath extends beyond the tail section. The sheath is a close-fitting membrane that envelops the microfilaria of these filarial worms, representing the remnants of the ovum membrane from which it was derived.
- Species of sheathed microfilaria can be differentiated by observing the size and the pattern of extension of nuclei into the tail sections:
  - *W. bancrofti:* the nuclei do not extend to the tip of the tail (Image Plate 22-10A).
  - *B. malayi:* two nuclei extend into the tail, spaced about 10 μm apart (Image Plate 22-10B).
  - *Loa loa:* an uninterrupted column of nuclei extend into and to the tip of the tail (Image Plate 22-10C). Importantly, the sheath of *Loa loa* does not stain well, or even at all, with the common Giemsa's stain; it often appears as an unstained area around the microfilariae.

The microfilaria of two other microfilaria species, *Mansonella perstans* and *Mansonella ozzardi*, may also be seen in the peripheral blood; however, patients with these infections are often asymptomatic. Occasionally, eosinophilia, lymphadenitis, low-grade fever, maculopapular rash, and urticaria have been observed in infected individuals. The microfilariae of these species are much smaller than those discussed above and are devoid of a sheath.

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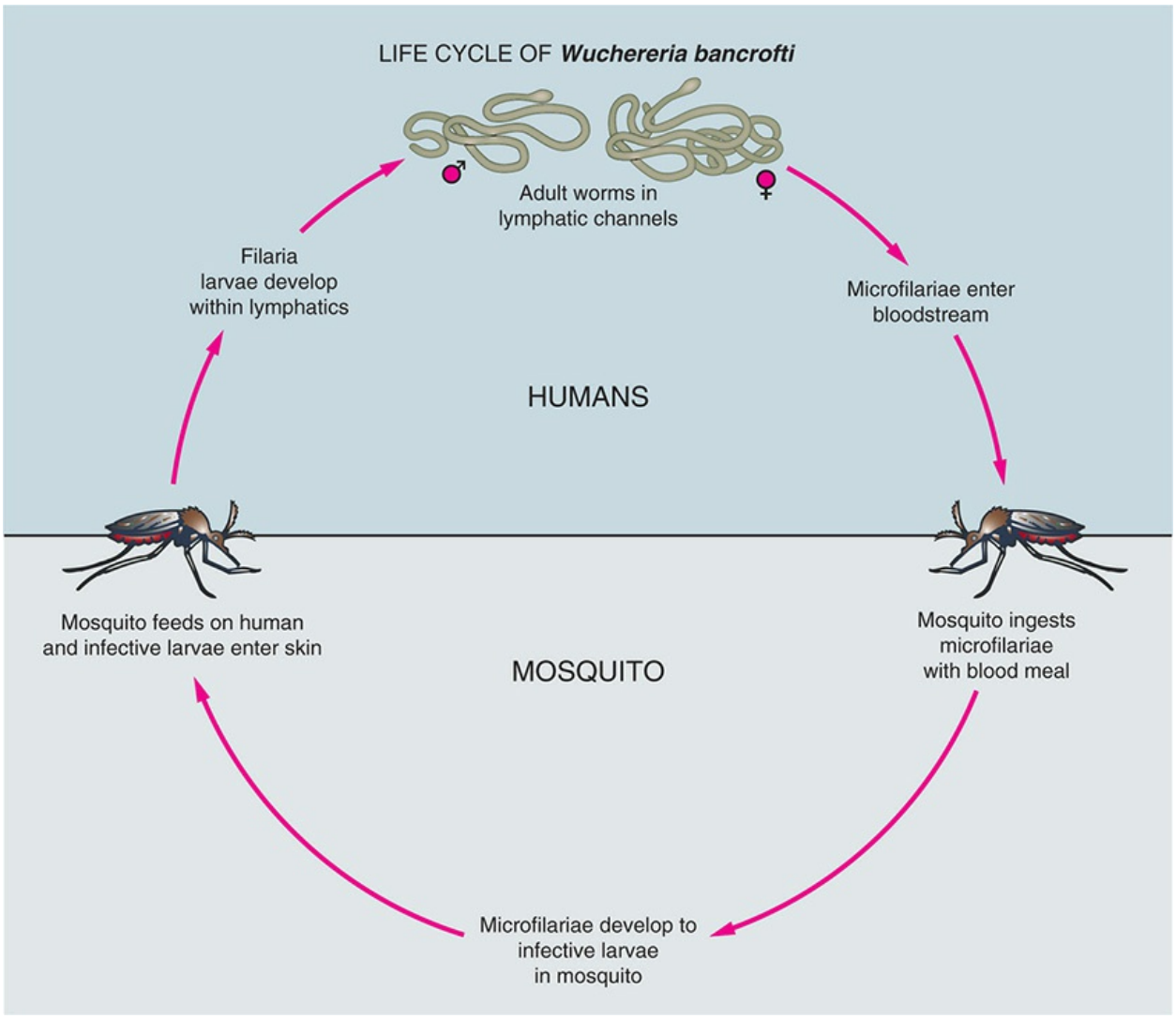

■ **FIGURE 22-26** Life cycle of *Wuchereria bancrofti*. Humans acquire the disease following a bite from an infected mosquito (*W. bancrofti* and *B. malayi*) or tabanid flies (*Loa loa*). Biting midges serve as the intermediate hosts for the nonsheathed species, *M. perstans* and *M. ozzardi*. When the vector bites an infected human host, the infective microfilariae are ingested and penetrate the stomach wall of the insect. An infective third larval stage develops in the stomach, which in turn migrates to the thoracic muscles of the insect and then to the proboscis. When the insect bites a new human host, these infective larvae move down the proboscis and are injected into the cutaneous wound while the mosquito takes a blood meal. These infective larvae reproduce locally in the subcutaneous tissue and ultimately enter the peripheral lymphatics, where they find their way to lymph nodes and lymphatic channels in various parts of the body. Over a period of several weeks, the larvae develop into white, threadlike adult worms. The lymphatics of the lower extremities and the epitrochlear and femoral lymph nodes are the sites most commonly involved. Mature female worms release microfilariae that circulate in the blood, and are ingested by the appropriate insect during the next blood meal, to complete the life cycle.

Because the detection of circulating microfilaria by direct examination of blood is relatively insensitive in establishing a diagnosis of filariasis, and is dependent on the collection of specimens at specific times in the day, several antigen-detection procedures have been introduced. Weil et al.[261] demonstrated filarial antigen in the sera of 56 of 57 patients with bancroftian microfilaremia living in an endemic area in South India. Antigen shedding may be irregular, particularly during times when circulating microfilaria may not be detected; thus, even these approaches lack sensitivity. Simonson and Dunyo[233] have also found that three recently introduced approaches for antigen detection also hold much promise—the ICT card test and the TropBio ELISA for serum specimens, and the TropBio ELISA for filter paper specimens.

The advent of PCR assays is proving to be useful not only in establishing a diagnosis in cases of light or amicrofilaremic infections, but also for use in monitoring therapy and

in discriminating between past and present infection.[72] Of much current interest is the application of PCR technology in the diagnosis of bancroftian filariasis by examining diurnally collected sputum specimens.[1] Preliminary data from this initial study are quite encouraging and portend great potential in the future.

Onchocerciasis and *Onchocerca volvulus*. Onchocerciasis is a filarial worm disease that involves primarily the subcutaneous portion of the skin wherein dense, fibrous nodules are formed that range in diameter from 5 to 25 mm.[189] These occur at the sites of bites of the vector, the *Simulium* black or buffalo fly. The nodules are more likely to be distributed in the trunk, thighs, and arms in African subjects, but more likely are localized in the neck and shoulders in inhabitants of Central America. Lesions of the skin and eyes occur secondary to the dead and dying microfilariae produced by the female worm in the subcutaneous nodule.[15] It is estimated that 25 million people worldwide are infected with *Onchocerca volvulus*, of whom 300,000 are blind and another 800,000 have visual impairment. Onchocerciasis is a leading cause of blindness in the world.[115] Although these numbers are staggering, substantial progress has been made. Two previously endemic countries have been declared onchocerciasis free—Colombia in 2013 and Ecuador in 2014. These successes are secondary to large-scale community-based treatment of the populations at risk with ivermectin (www.who.int).

The *Simulium* fly has its main habitat in the underbrush lining the banks of fast-moving streams. African onchocerciasis occurs mainly in the Congo basin, Zaire,

Angola, and the Sudan; in the Americas, it is found in the highlands of Guatemala, the states of Oaxaca and Chiapas in Mexico, in Colombia, and in northeastern Venezuela. The life cycle of *Onchocerca volvulus* is illustrated in Figure 22-27.

The diagnosis is clinically suspected when a patient from an endemic area presents with pruritic subcutaneous nodules with associated dermatitis, loss of elasticity, and hyperpigmentation. Pruritus is a major symptom in some patients, resulting in fatigue, weakness, and inability to sleep to the point of being physically and socially debilitating. Affected areas of skin may become hot and edematous, discolored, and prematurely wrinkled, a condition known as mal morado in Central America. In Africa, involvement of the hip region, particularly around the inguinal lymph nodes, results in a condition known as "hanging groin," often leading to femoral and inguinal hernias. Several excellent photographs illustrating these clinical presentations have been published by Garcia.[87]

Image Plate 22-10D is an H & E–stained section through a nodule, including the cross section of a gravid worm that contains many microfilariae. Although the microfilariae may remain localized to the site of infection, they may wander through the adjacent skin and reach other tissues, including the eye. Ocular involvement is the most serious complication, often leading to varying degrees of loss of sight, known as "river blindness." The eye serves as a trap for wandering microfilaria, which may be found either dead or alive in the anterior chamber, cornea, choroid, and vitreous humor. The mechanical action and/or effects from secretory toxins released by the adult worm in a hypersensitive patient and/or the inflammatory response to the microfilariae are thought to account for the development of bilateral blindness. The severity of blindness in various endemic regions is dependent on the *O. volvulus* strain involved, as determined by DNA sequence analyses.[188]

Zimmerman et al.[287] successfully used PCR to detect microfilariae in skin snips, providing significantly increased sensitivity in detecting active infection than that achieved by routine microscopic examination or serologic assays. Enzyme immunoassays have also been used to help establish the diagnosis in patients presenting primarily with river blindness. Ogunrinade et al.[188] evaluated an enzyme immunoassay using recombinant *Onchocerca volvulus* antigens OC 3.6 and OC 9.3 as an aid in diagnosing patients with suspected onchocerciasis. Forty of 42 (95%) serum samples they tested from patients with known onchocerciasis were reactive with the OC 3.6 antigen, while 81% were positive for the OC 9.3 antigen. Additional studies revealed that the OC 3.6 antigen was most helpful in detecting prepatent infections in humans; the OC 9.3 antigen was more sensitive in patients with mature, patent infections.

Treatment consists of the surgical removal of detectable nodules when possible. Ivermectin is currently considered the drug of choice in the treatment of onchocerciasis when surgical removal of lesions is not possible. This drug reduces the

numbers of microfilariae by blocking their release from the female worm. The drug is well tolerated, and side effects such as pruritus, arthralgia, and skin edema are usually minor. Diethylcarbamazine, beginning with small doses, plus suramin are alternative drugs that have been used successfully, although side effects from death of the microfilaria may require the addition of anti-inflammatory drugs. The administration of mebendazole or flubendazole (benzimidazole derivatives) has also been successful, with few side effects.

Dracunculiasis and *Dracunculus medinensis.* *Dracunculus medinensis* is a tissue roundworm that is taxonomically unique from other nematodes. It has been suggested that *D. medinensis,* also known of as the guinea worm, possibly represents the "fiery serpent" of biblical lore.[124] There has been an international campaign to eradicate *D. medinensis* with substantial success. There has been a steady reduction in dracunculiasis due to systematic intervention and eradication efforts. Dracunculiasis is now confined to only four countries in Africa, Chad, Ethiopia, Mali and South Sudan. Infections have diminished from an astounding 3.2 million in 1986 to 78,557 cases in 1998 (97% decrease), 148 cases in 2013, and 126 cases for 2014.[128] One important setback in the eradication efforts has been the discovery that this parasite, which was thought only to infect humans, also infects dogs, which can serve as a reservoir.

Humans acquire the infection through ingestion of infected Copepoda present in drinking water. The larvae develop into adult worms in the serous cavities and then the gravid females migrate to the subcutaneous tissue. These female worms can measure as long as 100 cm and cause a burning sensation and ulceration of the skin. The life cycle of the parasite is complete when the larvae produced by the female escape from the skin blister and are discharged into water in which the Copepoda live.

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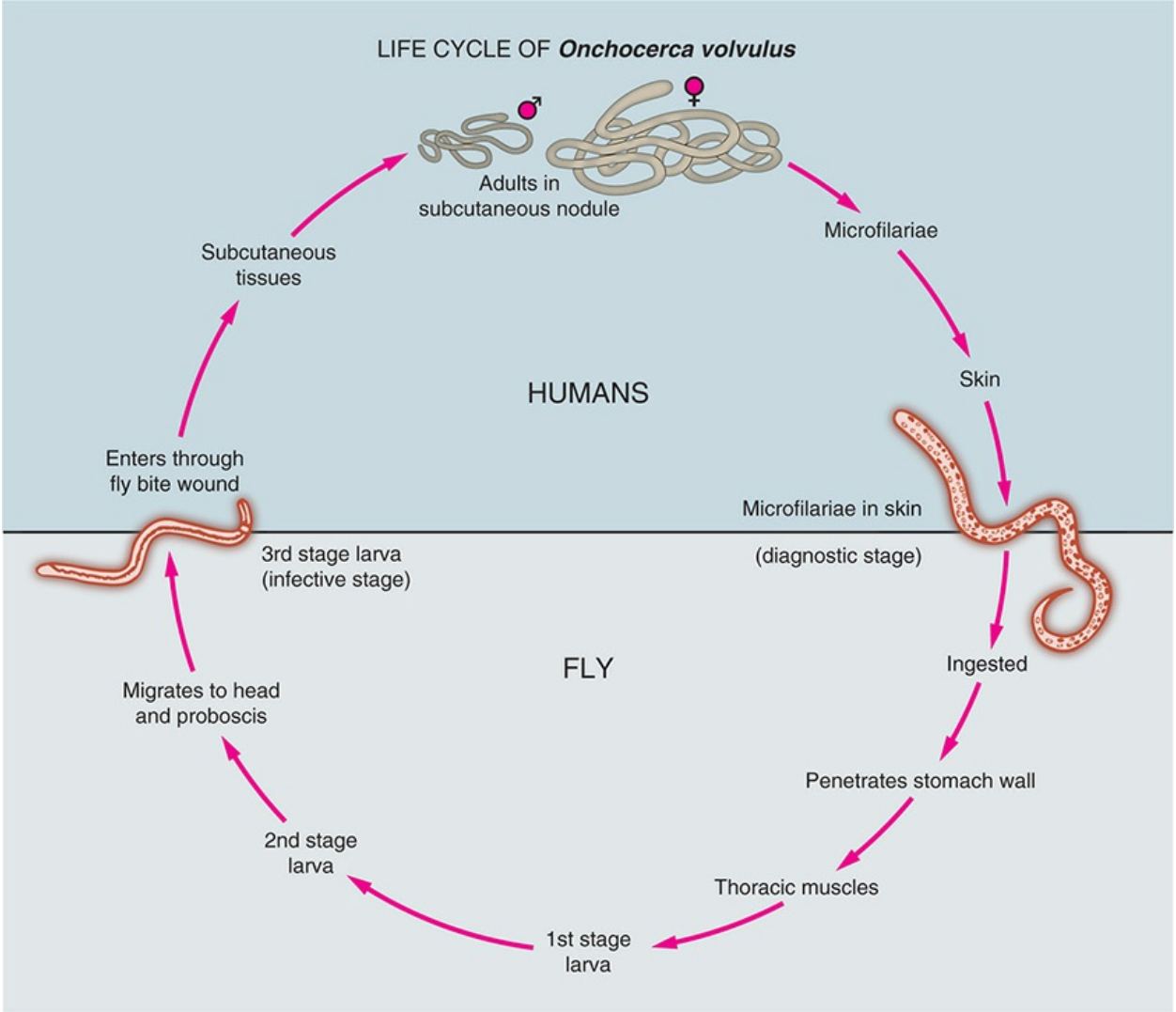

■ **FIGURE 22-27** Life cycle of *Onchocerca volvulus*. In the life cycle, humans become infected by bites from a *Simulium* fly, the mouth parts of which possess the infective third-stage larval forms of the parasite. The adult worms develop in tangled masses within nodules beneath the skin, and the females produce microfilariae (Image Plate 22-9D). The microfilariae remain localized to the infection sites and do not circulate in the peripheral blood except in rare instances. The life cycle is completed when a black fly again bites a diseased human at one of the infection sites, picking up the microfilaria in the mouth parts. The microfilariae then develop in the fly by penetrating the stomach wall, entering the thoracic muscles, and transforming through first- and second-stage larvae. They mature into the infective third-stage larval forms to complete the life cycle.

Interruption of this life cycle in endemic area, through both the treatment of infected persons and production of clean drinking water, promises to eventually eradicate dracunculiasis.

Traditionally, these have been from the subcutaneous tissue by winding them slowly on a stick. Modifications of this technique are still used, with an emphasis on sanitary condition and the prevention of secondary infections.

Dirofilariasis and *Dirofilaria* Species. *Dirofilaria* species are filarial parasites of animals that accidentally infect humans through the bite of the mosquito vector. In the definitive host, the infective larval forms injected into the subcutaneous tissue from the proboscis of the mosquito enter into the circulation and ultimately find their way to the heart, where they develop into adults (heart worms) (Image Plate 22-10E). The most well known of these is *D. immitis,* the dog heart worm, but there are other *Dirofilaria* species that infect other animals (e.g., *D. tenuis* and *D. repens*). The diagnosis is made in infected dogs by observing circulating microfilaria in the peripheral blood. However, when humans are bitten by infected mosquitos, the larvae, because they

are in the wrong host, are incapable of completing their life cycle. Rather, they lodge in and obstruct pulmonary arterioles, where they cause infarction and develop into local granulomatous nodules that may, on occasion, reach sufficient size to be diagnosed as "coin lesions" on chest roentgenogram examination. Microfilariae are never produced and circulate in human blood. The diagnosis is made by histologic observation of immature filarial larvae within the pulmonary granulomatous nodules. Flieder and Moran[74] recently reviewed 39 cases of histologically proven dirofilarial pulmonary infection. Approximately half of this group were asymptomatic, with only a pulmonary nodule detected on radiologic studies obtained for routine physical examinations; the remaining half presented with respiratory symptoms. Only 10%presented with peripheral blood eosinophilia.

Tissue Nematodes: Nonfilarial Worms

Humans may be inadvertent accidental hosts for several nematodes and cestodes that have life cycles in other animals. The adults of these species normally reside in the intestinal tract or select tissues of the definitive hosts; humans become infected either by ingesting the larvae in poorly cooked meat, through cutaneous penetration or by the ingesting fertile eggs. Of particular concern in the United States is the potential increased exposure to a variety of helminthic parasites through the ingestion of uncooked seafood in the form of dishes such as sushi, sashimi, lomi-lomi salmon, and pickled herring, among others. A brief discussion of some of these types of parasitic diseases will follow.

Trichinosis and *Trichinella spiralis*. Trichinosis is a disease of carnivores caused predominantly by infection with the nematode *Trichinella spiralis*. It results from the ingestion of raw or poorly cooked meat. Humans are accidental hosts and are most commonly infected through ingestion of pork or pork products that contain encysted larvae.[99] Infections have also been reported after ingestion of poorly cooked bear and walrus meat.[38] Smoking, salting, or drying the meat does not destroy the infective larval forms; however, prolonged freezing (20 days at −15°C) decontaminates the meat. The disease has a worldwide distribution. In resource-rich countries, the disease prevalence decreases with improved animal husbandry practices. For example, in the United States, 4% of human cadavers were found to be infected in 1968, but by 1985, less than 50 new cases were being reported each year in the United States,[38] a tribute to the meat inspection program and the stringent laws concerning improved animal husbandry (e.g., against feeding uncooked garbage to pigs). The life cycle is illustrated in Figure 22-28. The clinical manifestations of trichinosis are presented in Clinical Correlation Box 22-21.

Laboratory Identification. The laboratory diagnosis of trichinosis is most commonly made by detecting the spiral larvae in muscle tissue (Image Plate 22-11G and H). The deltoid muscle of the upper arm or the gastrocnemius muscle of the calf are usually selected for biopsy. The specimen may be examined microscopically by first digesting the muscle fibers with trypsin and then mounting some of the digested tissue on a microscope slide. Alternatively, a tease preparation may be prepared by squeezing a small fragment of muscle in a drop of saline solution between two glass slides, in each case looking microscopically for the coiled larval forms. Linear or spiral larval forms may also be observed in stained tissue sections, although their morphology is often not as well delineated.

Serologic tests are also helpful in establishing a diagnosis. Mahannop et al.,[164] using crude antigens obtained from the infective-stage larvae of *Trichinella spiralis* in an ELISA system for detecting serum IgG antibodies to *T. spiralis*, reported a 100%sensitivity in a group of patients with confirmed trichinelliasis. They found cross-reactions in sera collected from patients with capillariasis, gnathostomiasis, opisthorchiasis, and strongyloidiasis. Serologic tests for *Trichinella* are commercially available in reference laboratories.

Visceral Larva Migrans and *Toxocara* Species. *Toxocara canis* and *T. cati* are the intestinal roundworms of dogs and cats, respectively. These have a life cycle similar to

CLINICAL CORRELATION BOX 22-21 Trichinosis

Symptoms in most patients are mild, as the number of larvae is commonly small. Many individuals have flu-like symptoms. The minimal number of ingested larvae required to produce symptoms is about 100, and a fatal dose is estimated to be 300,000.[99] Upon ingestion of contaminated meat, gastrointestinal symptoms are the first to appear, including nausea, diarrhea, and abdominal cramps, suggesting acute food poisoning. These symptoms may persist with varying degrees of severity for as long as 10 weeks. Pain, swelling, and weakness of involved muscles develop during the larval invasion stage, accompanied by fever. Peripheral blood eosinophilia, with differential counts reaching as high as 50%, is seen during the muscle invasion stage. Periorbital edema is a diagnostic sign, as extraocular muscles are commonly involved. Damage to muscles may result in irregularities in chewing, swallowing, and breathing, depending on the muscles involved and the degree of involvement. In about 10%–20% of patients, the CNS may be involved to varying degrees, with symptoms suggesting polyneuritis, myasthenia gravis, or paresis of localized muscle groups. Acute myocarditis is the most serious complication, which may result in death in infections with heavy worm loads. A review of antiparasitic drugs effective for trichinosis myositis has been reviewed by Watt et al.[258] Mebendazole and albendazole are effective in killing the adult worms, but do not kill the encysted larvae.

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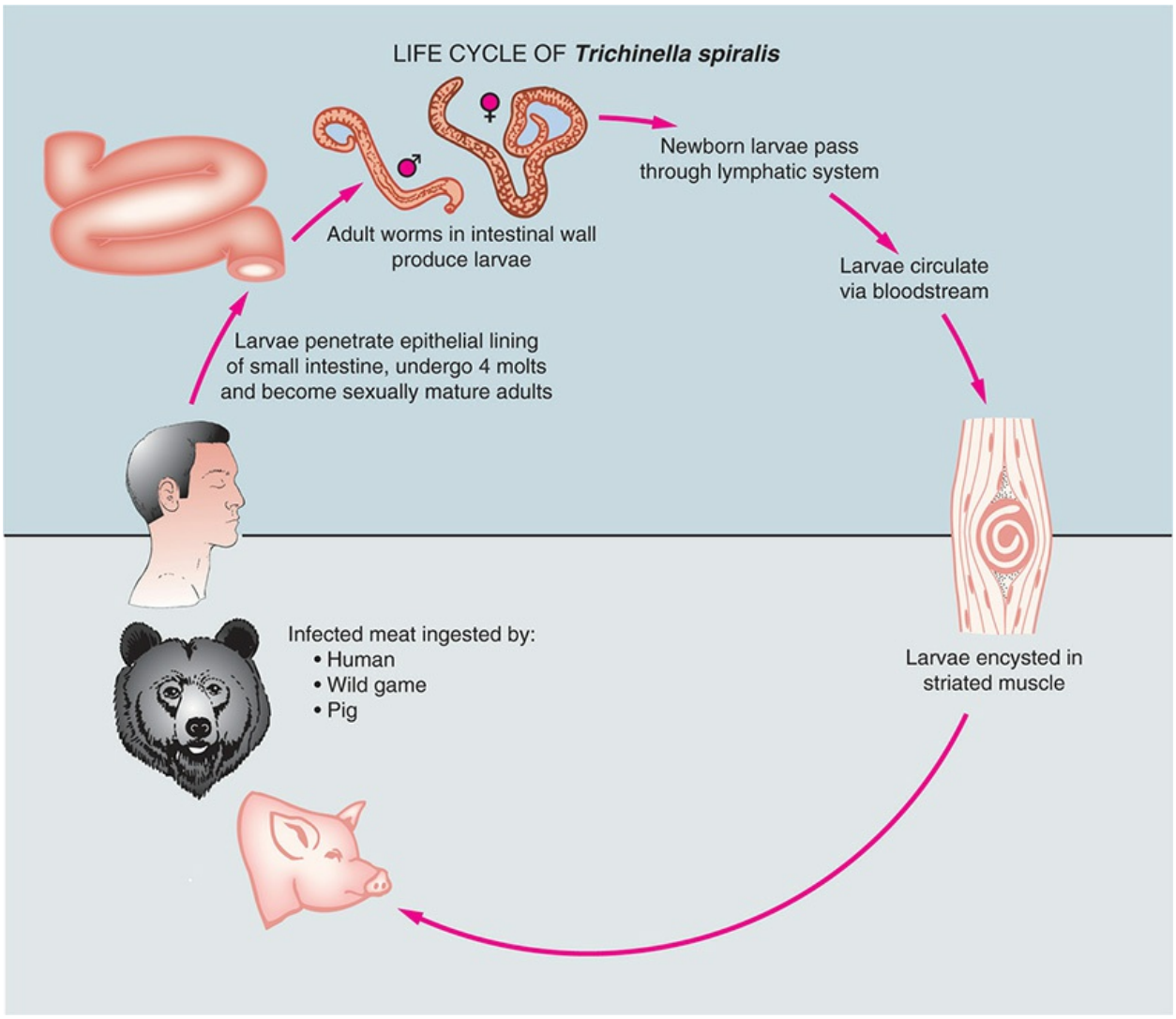

■ **FIGURE 22-28** Life cycle of *Trichinella spiralis*. When humans ingest trichinosis-contaminated meat, the encysted larvae are released in the intestine and burrow into the villi. The larvae mature and mate, and develop into adult male and female worms, measuring from 2 to 4 mm in length. The average life span of the adults in the human intestine is about 1 month. During this time, each female releases as many as 3,000 larval offspring. These larvae pass through the lymphatic system and enter the circulation and are deposited throughout the tissues of the body. Most of these larvae are killed; however, many of those that reach the skeletal muscle become encysted and survive. The heaviest period of muscle invasion is around day 10 after infection. By days 17 to 20 postinfection, these larval forms differentiate and become encapsulated, forming a coil two-and-a-half times on themselves, surrounded initially by an acute and later a chronic granulomatous inflammatory reaction (Image Plate 22-11 *G* and *H*). The adjacent muscle fibers undergo degeneration, leaving a cyst that measures about 0.25 to 0.50 mm. In approximately 3 months, the worms die and the cystic lesions undergo a process of calcification, which is completed in 6–12 months.

that of human roundworm, *Ascaris lumbricoides.* These are the most common causes of human larva migrans.

Visceral larva migrans is a condition in which the larvae of nematode parasites of lower animals infect humans, and since the parasite is in an inappropriate host, it migrates through the tissues without further development.

The infection begins in humans, an accidental host, through the ingestion of embryonated eggs in the soil. The disease is most common in children because of their inadvertent ingestion of soil and their close association with dogs. The embryonated eggs hatch in the intestine of the human host, liberating the larvae, which, in turn, penetrate the bowel wall and enter the circulation. However, because humans are abnormal hosts, the pulmonary cycle is not completed; rather, the larvae wander in various organs, chiefly the liver. Involvement of the eyes and CNS may also occur. The migrating larvae cause local tissue reactions or granulomas. In most instances, the larvae eventually die out with no sequelae. The infection may be suspected in a child with hepatomegaly, nonspecific pulmonary disease,

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and a high peripheral blood eosinophilia, reaching as high as 50% to 90%.

Although a definitive diagnosis is confirmed only by the identification of larvae in autopsy or biopsy specimens, serologic testing is recommended to direct management and therapy. Detecting elevated titers in ocular fluid specimens is confirmatory of eye involvement (i.e., ocular larval migrans).

Buijs et al.[23] report that the seroprevalence of *Toxocara* among young schoolchildren in the Netherlands as of 1994 was 11% in the Hague and 6% in Rotterdam. They found a significant correlation of recurrent asthma and bronchitis in *Toxocara*-seropositive children. They speculate that elevated allergen-specific IgE in these patients may account for the asthma. Jacquier et al.[134] evaluated a commercial ELISA kit marketed by Biokema-Affinity Products (Crissier-Lausanne, Switzerland), which is based on detection of excretory/secretory antigen derived from second-stage *T. canis* larvae. In a study of 1,000 serum samples randomly collected from healthy blood donors and children in Switzerland, the seroprevalence of toxocariasis was 2.7%. Of the positive samples, the Biokema kit had an overall diagnostic sensitivity of 91% and a specificity of 86%. The lower specificity was due to cross reactivity of serum from patients with filariasis, strongyloidiasis, and trichinosis. In the interpretation of serologic results, one must recall that a small percentage of the US population, estimated at 2%, may have residual low titers from previous infection. The CDC currently offers an enzyme immunoassay for *Toxocara canis*; acceptable specimens include serum and vitreous fluid.

Cutaneous Larva Migrans—Infections by Animal Hookworms. The filariform larvae of dog or cat hookworms, commonly of the species *Ancylostoma braziliense* and *A. caninum*, are capable of penetrating the exposed skin of humans, producing a pruritic, papular condition known as "creeping eruption." The larvae penetrate deeply into the subcutaneous tissue and produce linear tracts that extend for several millimeters each day. Impetigo, vesicular allergic reactions and intense pruritus are the major local clinical manifestations of cutaneous infections, which persist for long periods.[34] Deep tissue migration (visceral larva migrans) may occur, but is less common than with *Toxocara* species, as described above. The diagnosis is made by a history of exposure and the presence of subcutaneous linear tracts. Tremblay and associates[247] described an outbreak of cutaneous larva migrans in a group of 140 vacationers in Barbados. Risk factors were younger age, infrequent use of protective footwear, and walking barefoot to the beach.

Biopsies of suspicious lesions are often of little use in establishing a diagnosis, showing only necrosis and an eosinophilic cellular infiltrate. Yamasaki et al.[278] have developed a recombinant *Toxocara canis* antigen that was found to be highly specific for toxocariasis, providing for a reliable diagnostic approach in practice settings where this test is available. Camous[25] reports success in treating this condition with a single 400-mg oral dose of albendazole (cure rate, 46% to 100%), or a single 12-mg dose of ivermectin (cure rate, 81% to 100%). Mebendazole and albendazole are currently recommended therapies.

Anisakiasis and *Anisakis* and Related Species. Anisakiasis is a zoonotic disease in which humans, through the ingestion of raw, pickled, smoked, or poorly cooked seafood such as sushi, sashimi, and smoked or pickled fish, become an accidental host for the larval nematodes. The predominant causes are *Anisakis simplex* and *Pseudoterranova decipiens*. The number of infections in the United States has increased as raw fish such as lomi-lomi salmon, sushi, and sashimi have become popular. Deardorff and Kent[61] report that only wild-caught salmon were infected; those pen-reared were free of infection. Thus, knowing the origin of fresh fish may help in avoiding infections.

Upon entering the stomach, the larvae penetrate into the intestinal wall, forming small tunnels and burrows amid a dense granulomatous inflammatory reaction. In some instances, mucosal ulcers can be identified. Nausea and vomiting may be experienced within 24 hours after ingesting contaminated fish. Later manifestations include sharp, periodic, upper abdominal pain and diarrhea, at times simulating gastritis, duodenal ulcer, or on occasion, acute appendicitis. Daschner et al.[50] report hypersensitivity allergic manifestations in a series of 40 patients with anisakiasis. Urticaria, angioedema, erythema, bronchospasm, and varying degrees of anaphylaxis

were the common presenting symptoms. Lopez-Serrano et al.[158] report an additional 22 patients with gastroallergic anisakiasis. Two or more worms were detected by gastroscopy in three of these patients. Moneo et al.[174] demonstrated that these allergic reactions are in response to a potent allergen (Ani S 1) produced in the excretory gland of the *Anisakis simplex* adult worm. Because these symptoms are often delayed, the diagnosis of anisakiasis may be difficult, but must be considered, particularly if there is a clinical history of ingestion of raw or undercooked fish. Anisakis larvae may on occasion migrate beyond the stomach, producing metastatic infections in the omentum, liver, pancreas, and lungs.[218] Ikeda et al.[131] report on the successful treatment of nine patients with gastric anisakiasis by removing the causative larval worms through an endoscope, which immediately alleviated the acute abdominal pain. Surgical removal of the worm granuloma and/or albendazole therapy is recommended.

Yaquihashi et al.[280] report on the successful diagnosis of anisakiasis using a microenzyme-linked immunosorbent assay, with a monoclonal antibody directed against a specific *A. simplex* larvae. However, this approach is limited to a few research or reference laboratories, as commercial reagents are not currently available.

Gnathostomiasis and *Gnathostoma spinigerum*. *Gnathostoma spinigerum* is a nematode that normally infects the intestinal tract of cats and dogs; however, humans may become accidental hosts by ingesting poorly cooked or pickled seafoods containing immature larval forms. The disease is endemic in cats and dogs in Asia. After ingestion of contaminated fish by a dog or cat, the released larvae develop in the stomach or intestinal wall into adults that become encased in granulomatous inflammatory nodules. In humans, however, the larvae do not mature; rather, they penetrate the gastric wall and migrate throughout the tissues. Deep cutaneous or subcutaneous tunnels may develop, simulating cutaneous larva migrans, or hard, nonpitting

painful swellings may occur. Hira et al.[122] reported a Thai resident living in Kuwait, who contracted gnathostomiasis and presented with acute pain in the right iliac fossa. A mass of the terminal ileum and cecum was removed, revealing an immature male *G. spinigerum* worm. Grobusch and associates[103] reported the removal of this parasite from a cutaneous granuloma of the arm in a woman from Bangladesh living in Germany. Marked eosinophilia and a grossly elevated IgE were also observed in this patient. The diagnosis was made by demonstrating *Gnathostoma*-specific serum antibodies using an enzyme immunoassay.

Parastrongyliasis (Angiostrongyliasis) and *Parastrongylus* Species. Human

parastrongyliasis, previously and often still referred to as angiostrongyliasis, is caused by the larvae of nematodes, the adults of which live in rats as the definitive host. The disease presents in humans in two clinical forms, depending on the species: *P. cantonensis*, endemic in Thailand, Tahiti, and Taiwan, among other South Pacific locales, causes a syndrome of meningitis, eosinophilic pleocytosis in the CSF, and peripheral blood eosinophilia (known as eosinophilic meningitis). *P. costaricensis*, the rat lungworm, endemic primarily in Costa Rica, with infections also reported in Mexico and in Central and South America, causes abdominal disease primarily of the distal small intestine and ascending colon, the sites of penetration by the developing larvae.

The adult female produces eggs at the site of infection in the rat lung, which are then swallowed and passed in the feces. Slugs, land snails, and other mollusks (less commonly, also freshwater prawns, land crabs, and frogs) ingest these eggs and serve as an intermediate host within which the infective third-stage larvae develop. Humans acquire the infection by ingesting foods, usually leafy vegetables, contaminated with infected snails and slugs or the slime layer they produce. After ingestion by humans, the larvae of *P. cantonensis* migrate to the brain, causing eosinophilic meningitis. Symptoms vary from mild headache, stiff neck, and weakness to more severe symptoms, including nausea, vomiting, pruritic skin rash, and a variety of neurologic symptoms, including paresthesias, fourth and sixth cranial nerve palsies, and in heavy infections, coma and death.

Witoonpanich et al.[271] report two instances of fatal infections that occurred among three infected members of family. Two days after ingestion of Pila snails, a generalized itchy maculopapular rash developed in all three patients, followed by myalgia, marked paresthesia, fever, and headache. Weakness of the extremities, urinary retention, and cloudiness of consciousness progressing to coma were experienced by the two patients with a fatal outcome. Autopsy revealed multiple tracks and cavities with the presence of *P. cantonensis* in the brain and various levels of the spinal cord.

In the normal host, the rat, *P. costaricensis* adults occupy arteries and arterioles in the ileocecal part of the intestine. Eggs deposited in the rat tissue hatch and escape in the feces. Slugs also serve as intermediate hosts, and humans become infected by ingesting slug-contaminated foods. The larvae penetrate the tissues in the ileocecal portion of the human intestine, including the appendix, where a combination of the adult worms and the deposited eggs cause a severe granulomatous inflammatory reaction, resulting in the formation of a tumorlike mass. Hulbert et al.[130] have reported on the infection of two children who acquired *P. costaricensis* infections within the United States. One patient presented with symptoms suggestive of acute appendicitis, whereas the other was thought to possibly have a Meckel's diverticulum.

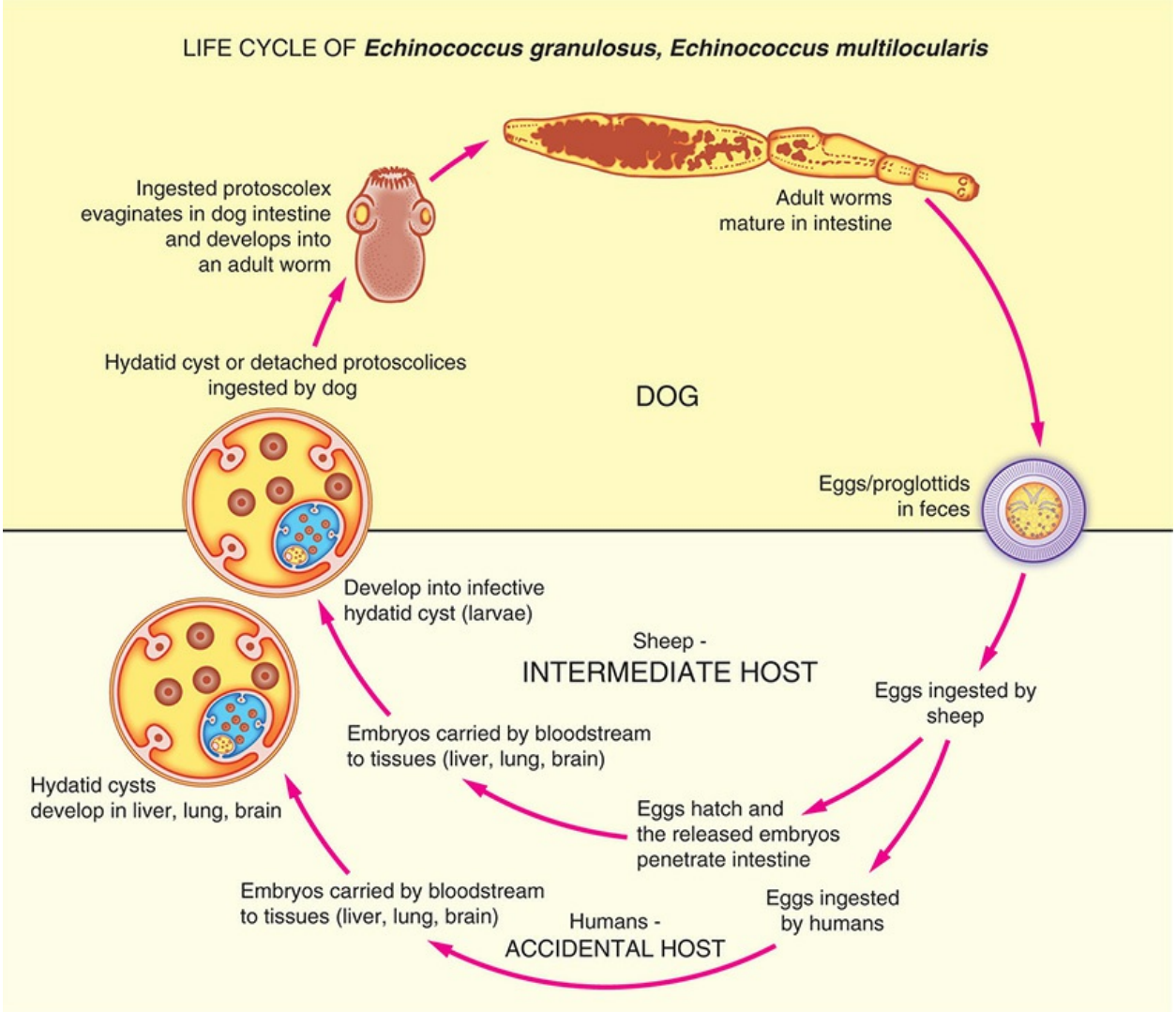
The diagnosis is usually made in patients in endemic areas on the basis of the symptoms described together with increased eosinophils in the spinal fluid and in the

peripheral blood. Larvae or young adult worms may be recovered in the CSF. A positive serologic ELISA titer, particularly when found in both serum and in the CSF, can also aid in the diagnosis.[258,260]

Echinococcosis (Hydatid Disease) and *Echinococcus* Species. Echinococcosis, or hydatid disease, is possibly one of the more difficult parasitic diseases to understand because of the peculiar cystic lesions that form when the larval stages of the parasite invade the viscera.[246] There are two main species that may infect humans, and these have a somewhat different morphology and patterns of behavior. These are *Echinococcus granulosus* and *Echinococcus multilocularis*. Humans are accidental hosts for both species. The normal life cycle of *E. granulosus* involves dogs or foxes as the definitive hosts, within the intestines of which the adult tapeworms reside. These tapeworms are very small, measuring only about 3 to 6 mm in length; they possess three proglottids and a scolex armed with a double row of hooklets (Image Plate 22-11F). Sheep, cattle, or swine serve as the intermediate hosts in which cystic larval disease develops, to be described below. *Echinococcus multilocularis* differs slightly in this regard as the definitive hosts are dogs, foxes, wolves, and cats, while the intermediate hosts are small rodents, including squirrels, field mice, and voles. If humans are infected, they are also intermediate hosts in whom the larval form of the parasite is harbored. The life cycle of *Echinococcus* is illustrated in Figure 22-29.

The cysts of *E. granulosus* grow slowly and usually remain quiescent for many years. Only rarely do cysts rupture, at times into the biliary tract and at other times through the liver capsule into the peritoneal cavity. If the cyst should rupture, either spontaneously in the body or during surgery, the danger from death from anaphylactic shock is high. Metastatic cystic lesions can also develop in virtually any of the visceral organs following the rupture of the primary cyst into the peritoneal cavity. If cyst material seeds the peritoneal lining, massive proliferation can occur with vascular invasion and spread to other organs. Brain cysts may also occasionally be found. The cysts of *E. multilocularis* grow more rapidly, with invasion of hepatic parenchyma simulating a carcinoma. Examples of hydatid cysts are illustrated in Image Plate 22-11B and C.

Laboratory Identification. The laboratory diagnosis may be made by demonstrating the daughter cysts or brood capsules with protoscolices in surgically removed tissue. The inner lining of the cyst is a germinal membrane from which numerous daughter embryos develop. These form as tiny polypoid structures (brood capsules) that line the inner reproductive membrane from which large numbers of daughter cysts are produced (Image Plate 22-11D). When the immature forms (protoscoleces) break free from the membrane and float in the fluid within the cyst, they are known as



■ **FIGURE 22-29** Life cycle of *Echinococcus granulosus*. Hexacanth eggs, either singly or within proglottids, closely resembling those of *Taenia* species, are passed in dog feces, and become embryonated in the soil. Under normal circumstances, these eggs are ingested by the natural intermediate hosts—sheep, cattle, or swine—or by the small rodents mentioned earlier in the case of *E. multilocularis*. The larvae are released from the eggs in the intestines of the intermediate hosts and, by means of their hooklets, penetrate the bowel wall and enter the circulation.

The circulating embryos are filtered out in the capillaries of various organs, usually the liver because it is the first organ to drain the mesenteric blood. *Echinococcus granulosus* produces a single multilayered cyst from which small cysts called bladder worms may form (Image Plate 22-11B). The cysts of *E. multilocularis*, as the species name implies, have multiple locules. Multiple cysts up to 5 cm in diameter, resembling what Aristotle called

hailstones, may be seen in some cases (Image Plate 22-11C). The life cycle is complete when infected viscera of the definitive host are eaten by a dog, fox, or other related carnivore. Humans may also become the intermediate host by ingesting the *Echinococcus* eggs, the larvae of which also may migrate to various organs, particularly the liver and lungs.

hydatid sand (Image Plate 22-11E). If examined under a microscope, each "grain of sand" is, in fact, a tiny embryonic beginning of a new tapeworm complete with an inverted scolex and a rostellum armed with hooklets (Image Plate 22-11E).

Several immunodiagnostic techniques are available by which a clinical diagnosis may be confirmed. Gottstein[98] provides a comprehensive review of the molecular and immunologic approaches to the diagnosis of hydatid disease. Most immunodiagnostic techniques involve the detection of *Echinococcus*-specific antibody in the serum of suspected patients, using a variety of crude antigens. The problem with methods using crude antigens is the serologic cross reactivity of echinococcal antibody tests with other parasite diseases, liver cirrhosis, and collagen diseases. Problems with cross reactivity have been substantially improved with the development of a selected antigen called arc-5. In a study conducted by Schantz

and McAuley,[223] a high percentage of the arc-5-positive persons subsequently were shown to harbor hydatid cysts. Cross reactivity of the arc-5 antigen has been demonstrated in the sera of patients with cysticercosis; however, the differential diagnosis should be possible clinically in such cases.

Verastegui et al.[252] offer an enzyme-linked immunoelectrotransfer blot assay for the diagnosis of hydatid disease that circumvents the cross reactivity in patients with cysticercosis. The antigen, prepared from lyophilized bovine hydatid fluid, was found to contain three 8-, 16-, and 21-kDa bands, that cross-reacted in only 12%, 4%, and 4%, respectively, of sera from patients with cysticercosis, a significant reduction from most ELISA assays. Newer EIAs provide greater than 90% sensitivity and specificity in detecting *Echinococcus*-specific antibodies. Using an ELISA procedure, Poretti et al.[206] demonstrated 91% sensitivity and 82% specificity for detecting *E. granulosus*-specific antigen in direct assay of cyst fluids, providing a 99% discrimination between seropositive cystic hydatid cases and noncestode parasitic infections or malignancies. Helbig et al.[116] were able to differentiate between cystic and alveolar disease with an assay using recombinant larval antigens, the results of which proved to be more specific than radiologic imaging studies. The CDC currently offers EIA and blot assays on serum to assist with the diagnosis of echinococcosis.

Although nucleic acid amplification tests have been devised for the detection of

Echinococcus, they have not come into practice. Namely, because if the cyst is aspirated or excised, the diagnosis is usually morphologically evident, and if the cyst is intact, serologic studies are superior.[98]

Coenurosis and *Taenia multiceps* and *T.* serialis. Coenurosis is another human disease related to tapeworms of dogs and foxes, *Taenia multiceps* and *T. serialis.* The normal intermediate hosts are rabbits, sheep, cattle, horses, and other herbivorous animals. Humans become infected by ingesting food or water contaminated with dog feces containing the eggs of these *Taenia* species. The disease in humans primarily involves the CNS, where the migrating metacestode larvae (i.e., a coenurus) develop into cysticercus-like cyst. These cysts differ from echinococcal cysts by having multiple protoscoleces, but these are without hooklets. No brood capsules or daughter cysts are seen. Symptoms are often those produced by a space-occupying lesion, including headache, vomiting, and localizing neurologic symptoms such as hemiplegia, paraplegia, aphasia, and seizures. Basal arachnoiditis leading to the posterior fossa syndrome and internal hydrocephalus is also a presenting symptom. The diagnosis is usually made following surgical removal of the cyst and the histologic recognition of the coenurus.

Sparganosis and *Spirometra* and *Sparganum* Species. Dogs and cats serve as the definitive host for several species of diphyllobothroid tapeworms belonging to the genus *Spirometra*. *Spirometra* eggs are passed in dog or cat feces into freshwater hatch and are ingested by minute *Cyclops* crustaceans, within which they develop into procercoid larvae. These larvae in turn develop into plerocercoid larvae, individually known as sparganum, in the flesh of a second intermediate host (fish, snakes, frogs) that feed on the *Cyclops.* Humans become infected by ingesting an infected *Cyclops,* by eating the raw, infected flesh of the second intermediate host, or from the practice in certain cultures of applying raw flesh of an intermediate host to the skin, eyes, or vagina as a poultice. On ingestion, sparganum larvae penetrate the bowel wall and enter the circulation, or penetrate through the mucous membranes.

Most lesions in humans are subcutaneous, where slow-growing, painful, red, edematous nodules develop. The definitive diagnosis is made only after surgical removal of a sparganum and identifying the slender, delicate white worm that measures 60 to 80 × 1 to 2 mm wide. Aberrant spargana have been observed in the external eye, where edematous swelling of the eyelid simulating Romaña's sign of Chagas' disease has been reported, in lymph channels producing elephantiasis-like swellings, and in the brain in the form of cerebral abscesses.

Serologic and Molecular Diagnosis of Parasitic

Infections

The serologic approach to the evaluation of parasitic diseases is most applicable when invasive techniques other than the routine examination of blood, feces, or other body fluids are required to establish a diagnosis. For example, the infective parasitic forms in toxoplasmosis, extraintestinal amebiasis, trichinosis, and cysticercosis are often lodged deep within tissues and organs, and either deep needle or open surgical biopsies are needed to confirm the diagnosis. In such cases, serologic diagnosis may be possible if several potential problems as outlined by Garcia[87] are considered:

1. Certain parasites that pass through several developmental phases may not provide sufficiently constant or continuous antigenic stimuli to elicit antibody formation.
2. Antibody responses in specific individuals may be lacking, either because of a limited antigenic stimulus, the individual is too immunocompromised to generate an antibody response, or because a relevant antigen is not present in the test system.
3. The antigens used in assays are a poorly defined heterogeneous mixtures or extracts of parasitic forms. Such antigen preparations may show cross reactivity that affects specificity, making interpretation difficult.
4. Patients living in endemic areas may have higher baseline antibody titers than those in nonendemic areas; thus, if possible, changes in titer must be ascertained. Serologic tests are usually more valuable in travelers to endemic areas. These individuals would not be expected to have any preexisting antibodies, and so a serologic response would be strong evidence of a newly acquired infection.
5. Reliable test kits for general diagnostic use often are not available from commercial sources. Even when

available, the incidence of parasitic disease is usually so low in most nonendemic locales. The low prevalence of disease has a negative influence on the positive predictive value of any test.

Several of the applications of antibody and antigen detection provided in Garcia's review are reflected in short sections included within the discussion of each animal parasite in this textbook. We have elected to include such discussions within each section rather than having a stand-alone presentation here. Many of the details of the various methods currently being used in research laboratories are beyond the scope of

this book. In a practical sense, the volume of parasitic diseases encountered in most practice settings in the United States is low, making serologic testing impractical in most diagnostic microbiology laboratories. In most instances, specimens from patients suspected of having a particular parasitic disease are referred to local and regional reference laboratories, including the various state public health laboratories. Each reference laboratory will have in operation a mixture of time-honored and newer technologies that is unique to that laboratory, and consultation is necessary to learn which application and the types of specimens required may be most applicable. Figure 22-30 is the list of serologic tests performed at the CDC for various parasitic diseases, along with the titers considered to be diagnostically significant, as originally published by Walls and Smith.[255]

Most of the advances in diagnostic clinical parasitology over the past 5 years involve the introduction of new molecular techniques for the detection of the nucleic acids of select parasites. Two FDA-approved commercially available molecular diagnostic products are available. These take a syndromic approach to the diagnosis of infectious gastroenteritis and include a panel of bacterial, viral, and parasitic pathogens. *Cryptosporidium, Entamoeba histolytica,* and *Giardia* are included in the Luminex Gastrointestinal Panel. The FilmArray includes *Cyclospora cayetanensis* in addition to the three parasites included in the Luminex panel. There are similar products in development by other companies.

Serum/Plasma Specimens

Antibody Detection Tests Offered at CDC

Disease	Organism	Test	Acceptable Specimens
Amebiasis	*Entamoeba histolytica*	Enzyme immunoassay (EIA)	Serum
Babesiosis	*Babesia microti* *Babesia* sp. WA1	Immunofluorescence (IFA)	Serum
Baylisascaris	*Baylisascaris procyonis*	Immunoblot	Serum or CSF
Chagas disease	*Trypanosoma cruzi*	IFA	Serum
Cyticercosis	Larval *Taenia solium*	Immunoblot (Blot)	Serum, CSF
Echinococcosis	*Echinococcus granulous*	EIA, Blot	Serum
Filariasis	*Wuchereria bancrofti* and *Brugia malayi*	EIA	Serum
Leishmaniasis	*Leishmania braziliensis* *L. donovani* *L. tropica*	IFA	Serum
Malaria	*Plasmodium falciparum* *P. malariae* *P. ovale* *P. vivax*	IFA	Serum
Paragonimiasis	*Paragonimus westermani*	Blot	Serum
Schistosomiasis	*Schistosoma sp.* *S. mansoni* *S. haematobium* *S. japonicum*	FAST-ELISA Blot	Serum
Strongyloidiasis	*Strongyloides stercoralis*	EIA	Serum
Toxocariasis	*Toxocara canis*	EIA	Serum, vitreous fluid
Trichinellosis (Trichinosis)	*Trichinella spiralis*	EIA	Serum

■ **FIGURE 22-30** Serologic assays available from the CDC.

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Application of molecular techniques has finally penetrated into diagnostic parasitology. It is hopeful that these very sensitive methods will afford the increased detection of patients with parasitic diseases.[196,263]

Drugs Commonly Used in the Treatment of Parasitic Diseases

Drug therapy of parasitic diseases is directed to interrupting the invasive capabilities of

parasites. Infections that occur primarily within the gastrointestinal tract are best treated with compounds that are poorly absorbed, resulting in high concentrations of active drug within the intestinal lumen. Agents that are well absorbed and achieve high serum and tissue levels are required for the treatment of invasive parasitic diseases, particularly the larval forms. In patients with intestinal and extraintestinal amebiasis, a combination of an absorbable and nonabsorbable drug to treat residual organisms in the intestine may be required. Many parasites exist in a cyst form, which may be poorly penetrated by many of the drugs, leading to potential reactivation of infection at a later date. For example, the inactive, encysted forms of *Toxoplasma gondii* are drug-resistant and may reactivate when immunity is lost.

Table 22-1 summarizes the mode of action and other attributes of select commonly used antiparasitic drugs. For a full review of all current drugs, including recommended drugs and dosages to be used for most human parasitic diseases, we refer the reader to select publications and infectious disease texts.[87,175]

TABLE 22-1 Drugs Commonly Used in the Treatment of Parasitic Infections

Drug	Type of Agent	Mode of Action	Comments
Amphotericin B	Polyene macrolide	Increases cell membrane permeability, leading to a loss by leakage, first of ions and then of other cellular contents. Binding is effected by sterols in membrane.	Mammalian cells may also be affected with severe side effects. Used in treatment of infections with *Acanthamoeba* species and in advanced cases of mucocutaneous leishmaniasis.
Chloroquine	4 aminoquinoline (quinine)	The concentration of drug achieved in malarial parasitized erythrocytes is several hundred times greater than in normal erythrocytes. The drug binds to ferriprotoporphyrin IX (FP), a degradation product of the hemoglobin present in the parasite food vacuole. The complex of chloroquine FP is lytic for the parasite.	Mainstay of malarial therapy for over two decades. *P. falciparum* drug resistance occurs in strains that can somehow sequester FP so that it does not bind to chloroquine. Chloroquine is not curative for *P. vivax*, because of the presence of hepatic hypnozoites.
Emetine	Alkaloid (cycloheximide)	Directly lethal to trophozoites of *E. histolytica* by causing degenerative changes in the trophozoites. The drug causes interruption of cell division by irreversibly inhibiting protein synthesis by preventing movement of mRNA along the 60s ribosomal subunit.	Used in the therapy for severe intestinal amebiasis or amebic liver abscess as an alternative to metronidazole. Mammalian cells are also affected, resulting in severe side effects and limiting its therapeutic use.
Diethylcarbamazine	Piperazine derivative (two nitrogens in benzene ring)	The drug is thought to alter surface properties and mobilization of microfilariae, causing them to leave the circulation through a specific neuromuscular effect. The microfilariae become trapped in the liver and are subject to phagocytosis.	Used primarily in the treatment of filarial infections. The drug is well absorbed, reaching peak serum concentrations within 3 hours, and is widely distributed throughout the body.
Ivermectin	A 22,23 dihydro derivative of avermectin B, a complex macrocyclic lactone produced by *Streptomyces avermitilis*	The drug acts on γ-aminobutyric acid (GABA) receptors on the parasite musculature. Inhibitory neurons that cause release of GABA from presynaptic terminals are stimulated, in essence blocking signal transmission from interneurons to excitatory neurons. The effect is the loss of locomotor activity by the parasite, making normally motile parasites highly susceptible to various host defenses.	The drug has a broad spectrum of antinematodal activity but has no effects on flukes and tapeworms. It has been effectively used in onchocerciasis, cutaneous visceral larval migrans, *Angiostrongylus meningitis*, and various gastrointestinal nematode infections.

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Mebendazole	Benzimidazole—benzine + five ring structure, including three carbon and two nitrogen molecules	Selective inhibition of glucose uptake in nematodes and cestodes, leading to increased utilization of parasite glycogen; parasites are thereby deprived of their main energy sources. Under action of the drug, the parasite is immobilized and in vitro larval development is interrupted.	Mebendazole is active against nematodes and used primarily in the treatment of trichuriasis, ascariasis, hookworm, and *Strongyloides* infections. The drug is minimally absorbed from the gastrointestinal tract, thus being most effective in the treatment of intraintestinal worms.
Melarsoprol	Arsenical (a dimercaprol derivative of melarsen oxide)	Arsenoxides attach to trypanosomes by covalent bonding—sulfur groups in arsenoxide exert lethal effect by blocking essential biologically active glycolytic enzymatic groups. Arsenicals react with sulfhydryl groups, leading to inactivation of various enzymes synthesized by the parasite for glycolysis.	Used in the treatment of trypanosomiasis. The drug enters parasite cells more rapidly than human cells, and thus is more toxic to the parasite. The drug crosses the blood–brain barrier and enters the cerebrospinal fluid, making it highly effective in the treatment of CNS infection.
Metronidazole (Flagyl)	5-nitroimidazole	Metronidazole is metabolized into derivatives, including superoxide radicals, that interfere with DNA metabolism in parasites, causing extensive breaks in the DNA strands and interrupting the helical structure. Protein synthesis in the parasite is thus disrupted.	Used in the treatment of invasive amebiasis and also for infections with *Trichomonas vaginalis* and *Giardia intestinalis*. Because only 10% of the drug is bound to serum proteins, it reaches high concentrations in the tissues, including lung, bile, bone, liver, and brain, exceeding levels required to inhibit the organisms against which it is active.
Niclosamide	Heterocyclic pyrazinoisoquinoline derivative	The oxidative phosphorylation of mitochondria of cestodes is interrupted. The effect on mature adult worms is lethal, inducing complete muscular paralysis in certain species. The drug induces the scoleces to detach and the worms to disintegrate before they are eliminated in the feces.	Niclosamide is an alternative drug for the treatment of tapeworms. Treatment for *Hymenolepis* worms is increased to 5 days because onchospheres develop in the jejunal villi, and cysticercoids emerge into the intestinal lumen about 4 days later.
Praziquantel: (pyrazinoquinoline)	8-aminoquinoline derivative	The drug acts by increasing the membrane permeability to calcium, causing contractions and paralysis of the musculature of worms. Neuromuscular effects lead to increased motility and spastic paralysis, causing the worms to detach and disintegrate in the intestine.	Used for the treatment of schistosomiasis, cysticercosis, tapeworm infections, and liver, lung, and intestinal flukes. The drug achieves excellent therapeutic levels in the liver, bile, and muscle tissue, and crosses the blood–brain barrier to reach the brain and spinal fluid as well.
Primaquine	Tetrahydropyrimidine	The drug is gametocidal and sporonticidal for all species of human malaria. Its mechanism of action is unknown, although inhibition of DNA synthesis is most likely.	Primaquine is a poor blood schizonticide but is effective against exoerythrocytic hypnozoites. Therefore, it is effective in preventing relapses due to *P. vivax* and *P. ovale*. Radical cure of malaria can be effected by a combination of chloroquine and primaquine therapy.
Pyrantel pamoate	Naphthylamine sulfonic acid, a polyanion dye	Pyrantel (and its analogs) acts as a cholinergic antagonist, causing the depolarization of muscle cells within the parasite, leading to irreversible contractures.	Used primarily in the treatment of infections with ascariasis, hookworms, pinworms, and *Trichostrongylus*. The drug is insoluble in water and poorly absorbed from the gastrointestinal tract, leading to little toxicity.

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Suramin		The drug inhibits glycerol 3-phosphate oxidase and glycerol 3-phosphate dehydrogenase, preventing reoxidation of nicotinamide adenine dinucleotide and decreasing adenosine triphosphate synthesis. This interruption in metabolism is lethal to the parasite.	The drug is widely distributed in the body but, because of avid protein binding in the serum, does not pass the blood–brain barrier. The drug is used in the treatment of early trypanosomiasis; however, as it does not penetrate into the CNS, it is not effective in the treatment of progressive CNS disease.
Thiabendazole	Benzimidazole	The precise mechanism of action of thiabendazole is unknown, but it is thought that is likely inhibits the fumarate reductase of the helminth. Inhibition of fumarate reductase interrupts the citric acid cycle and subsequently energy production.	The drug is well suited for the treatment of disseminated *Strongyloides* infection and any larval forms causing visceral and cutaneous larval migrans. The bulk of absorbed drug is metabolized in the liver and excreted as metabolites in the urine.
Stibogluconate sodium	An antimony-containing compound	The drug is thought to act on sulfhydryl-containing enzymes within the parasite, decreasing the flow of glucose into the tricarboxylic acid cycle, resulting in the accumulation of glycolytic byproducts that are toxic to the developing amastigotes.	Used primarily in the treatment of leishmaniasis. After intramuscular or intravenous administration, a serum peak is achieved in 2 hours, and over 90% of the drug is excreted within 8 hours. However, the drug does gradually accumulate in the tissues, explaining why long courses of therapy are necessary.

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CHAPTER 23

Diagnosis of Infections Caused by Viruses, *Chlamydia/Chlamydophila*, *Rickettsia*, and Related Organisms

thePoint For additional ancillary materials related to this chapter, please visit the point.

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- Evolution of Cell Culture Techniques
- Evolution of Diagnostic Virology Services
- Levels of Service

Taxonomy and Nomenclature

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- Paramyxoviruses
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Respiratory Syncytial Virus
Other Paramyxoviruses
Picornaviruses
Rhabdoviruses
Arenaviruses
Filoviruses
Togaviruses and Flaviviruses
Bunyaviruses
California Encephalitis Viruses
Hantaviruses
Human Gastroenteritis Viruses
Rotaviruses
Caliciviruses
Astroviruses
Enteric Adenoviruses
Coronaviruses
Coltiviruses
Retroviruses
Herpesviruses
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Introduction

Diagnostic virology has evolved rapidly in recent years from a predominantly cell culture–based service to one that is dominated by molecular diagnostics. In this transition period, we have chosen to retain descriptions of the traditional techniques, as some of these are still used in laboratories without molecular capabilities. The emphasis, however, will be on the new molecular diagnostic assays that have transformed the clinical virology laboratory in a way that provides clinicians with the rapid and accurate detection of viral pathogens in a timely manner.

Historical Review

The first stage of historical interest spanned the years during which the existence of infection by submicroscopic particles was established. For many decades after Carlos Finlay hypothesized that yellow fever was transmitted by the bite of a mosquito, the only means available for isolation of viral agents was the inoculation of animals or embryonated eggs. Agents of disease continue to be uncovered by the traditional use of subhuman primates (e.g., kuru and Creutzfeldt–Jakob viruses),[158] electron microscopy (e.g., severe acute respiratory syndrome [SARS]),[178] and even human volunteers (e.g., Norwalk agent of infectious gastroenteritis).[259] The next phase utilized cell cultures as a means to propagate viruses. Today, these previously used methods are complemented by sophisticated molecular diagnostic methods, including

next-generation sequencing (NGS).[19]

Evolution of Cell Culture Techniques

One of the major historic breakthroughs in diagnostic virology was achieved by Enders and colleagues,[129] who demonstrated that the virus that caused poliomyelitis could be isolated in nonneural cells in vitro. In the 1950s and 1960s, there was a rapid expansion of knowledge about clinical features, epidemiology, and diagnosis of common viral infections, largely provided by the expanding use of cell culture. Diagnostic efforts, which were an integral part of the clinical studies, were incorporated into university and government research laboratories. The high cost of the definitive identification of isolates, the absence of effective antiviral chemotherapy, and the paucity of cell culture facilities all combined to limit the importation of these tests into the diagnostic microbiology laboratory. A more detailed review of the historical development of virology is provided by Levine.[292]

Evolution of Diagnostic Virology Services

The first step was the incorporation of diagnostic services into clinical microbiology laboratories, many of which had previously limited their scope to bacteriology (including mycobacteriology), mycology, and parasitology. Box 23-1 lists some of the factors that facilitated and encouraged the transfer of technology and techniques from the research laboratory setting to a clinical diagnostic laboratory, usually in a pathology or microbiology department. A more detailed discussion of this transition can be found in previous editions of this book.

The complexity and sophistication of traditional viral diagnosis limited the introduction of these methods to clinical laboratories located in large medical centers, many of which were associated with academic centers. In the previous decade, the pace of technologic development, particularly in the field of molecular diagnostics, has accelerated so that some viral diagnostic testing for multiple pathogens (e.g., respiratory viral panel) can be introduced into community hospitals.

In addition to the scientific issues, an important determinant was the promulgation and enforcement of the CLIA ‘88 (Clinical Laboratory Improvement Amendments, 1988), the first major development in oversight of laboratories since the Clinical Laboratory Improvement Act of 1967 (CLIA ‘67). The numerous requirements for personnel and performance of tests were familiar to most clinical laboratories, but foreign (and extremely unwelcome) to most research laboratories, and essentially out of the reach of physician office laboratories. Although many of the molecular assays that target viruses are Food and Drug Administration (FDA)-approved, there are still many laboratory-developed tests (LDTs) in use throughout the country. The FDA has cited oversight for these assays, but the form of the oversight remains undetermined.

Many of these assays, for which there are not currently available FDA-approved options (e.g., quantitative Epstein–Barr virus PCR), are critical for patient care. It is a concern that if the oversight rules imposed by the FDA are too stringent, then local testing for these pathogens, which provides superior turnaround time, may be discontinued.

1502

BOX 23-1

Some Factors That Facilitate the Transfer From a Research Laboratory to a Diagnostic Laboratory

Factors That Encouraged Transfer of Testing to the Clinical Laboratory

- Appearance of clinical scenarios in which viral diagnosis is essential; e.g., herpes simplex in pregnant women.
- Development of antiviral chemotherapy.
- Availability of reliable commercial sources of mammalian cells for culture.
- Application of monoclonal antibody technology to diagnosis, with commercial availability of reliable reagents.
- Facilitation of more rapid diagnosis by application of molecular assays that target viruses of clinical relevance.
- Application of enzyme immunoassays to direct diagnosis.
- Application of more simple-to-use molecular diagnostic methods.
- Support for public health epidemiology of transmissible disease.

Developments in Diagnostic Virology

- Increasing frequency of clinical scenarios that depend on viral diagnosis; e.g., monitoring of viral loads in transplant recipients and the increased sensitivity of HPV assays.
- Increased number of diseases for which antiviral chemotherapy, including combination therapy, is possible.
- Commercial sources of cell culture materials are available, which include cells on coverslips for shell vial cultures, the development of mixed cell lines (e.g., R-Mix) and the availability of genetically modified to enhance detection (e.g., ELVIS system).
- Commercial availability of high-quality immunofluorescent reagents; some of these are pooled (e.g., respiratory viruses) or improve the efficiency of screening.
- Movement of nucleic acid amplification technology from the research laboratory into the clinic in the form of laboratory-developed tests (LDTs); commercial availability of systems or validated reagents for multiplex, monoplex, and

quantitative assays, many of which are FDA-approved, and available on simple-to-use platforms.
- Although most immunoassays are quick and inexpensive, they suffer from poor sensitivity; improved immunoassays have been developed to increase the sensitivity, which employ reader devices. These assays are being threatened by waived, point-of-care molecular assays.
- Support for outbreak investigation and tracking; some of the newer immunoassay devices have wireless capability to report influenza results to public health laboratories in an HIPPA-compliant manner.

Levels of Service

Each laboratory director or supervisor must determine the level of service that is appropriate for local clinical needs. This should be done after consultation with medical staff. It is far better to limit the scope of effort to the tests that can be performed with high proficiency than to attempt broad coverage when resources are insufficient and proficiency may be lost owing to infrequent performance. Several likely levels of service are listed in Box 23-2.

Most small hospital laboratories will probably fit into level 1 or 2,[428] whereas larger community hospital and university laboratories may offer services at level 3, 4, or 5. These hospitals are most likely to serve the patients who need comprehensive virology services. It is feasible to use such laboratories as regional diagnostic resources. The service levels that are possible have changed dramatically in recent years with the introduction of simple-to-use molecular diagnostic tests, some of which are CLIA-waived.

Taxonomy and Nomenclature

Taxonomy is the science of systematic classification using general rules and principles; the term is most commonly used for the study of living things. Nomenclature is the activity of ascribing names to the taxonomic subjects.

Many viral infections, such as influenza, chickenpox, measles, and hepatitis, were well characterized clinically long before the etiologic agent was recognized. Once isolated, the virus was given the name of the corresponding clinical condition. In an attempt to bring order to the scheme of classification, taxonomists have used modern knowledge of viral structure and antigenic composition to construct a systematic classification.[93] Orders, families, and genera have Latin stems, but the concept of species has been more difficult for virologists.[48,463] Often, the classical vernacular

names are used as species designations.

The International Committee on Taxonomy of Viruses (ICTV) has proposed the ICTV Universal System of Virus Taxonomy. A summary of the conventions of viral nomenclature is provided in Box 23-3. The hierarchical names are italicized and may be preceded by the taxon, which is not italicized. In the case of the species names, we are left with the peculiar situation that the same words may be italicized or not, depending on whether they are part of a taxonomic designation or are being used as the vernacular name. The taxonomic designation needs to be used only once in a scientific publication. For instance, a discussion might refer to measles virus (*Measles virus*, genus *Morbillivirus*, family *Paramyxoviridae*). At the moment, this approach to taxonomy and nomenclature appears to have preference, but the subject has not been settled definitively.

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BOX 23-2

Levels of Diagnostic Service

Level 1: No virology services performed. Specimens are collected for transfer to a reference laboratory.
Level 2: Limited virology procedures performed. Such laboratories may or may not also have the capability to detect viral antigens in body secretions and fluids. These laboratories also may or may not have the capability to inoculate specimens to tissue culture for transfer to reference laboratories. These laboratories may use fully automated (i.e., specimen-to-result) platforms for molecular testing.
Level 3: These laboratories are able to recover selected viral groups that grow in commonly used cell cultures. They may also perform limited identification of selected agents for which transportation, cell isolation, and simple immunologic confirmation systems are commercially available, with referral of other isolates to reference laboratories. These laboratories may use more complex, FDA-approved molecular diagnostic tests in an unmodified manner.
Level 4: These laboratories are expected to have personnel with a high degree of expertise in virology who are able to isolate all of the common viral groups and to perform clinically relevant identifications. These laboratories are able to perform complex laboratory-developed tests (LDTs), in addition to FDA-approved assays, and possibly traditional DNA sequencing.
Level 5: In addition to a high degree of expertise in virology, these laboratories may perform antiviral susceptibility testing. In addition to LDTs, these laboratories perform next-generation sequencing.

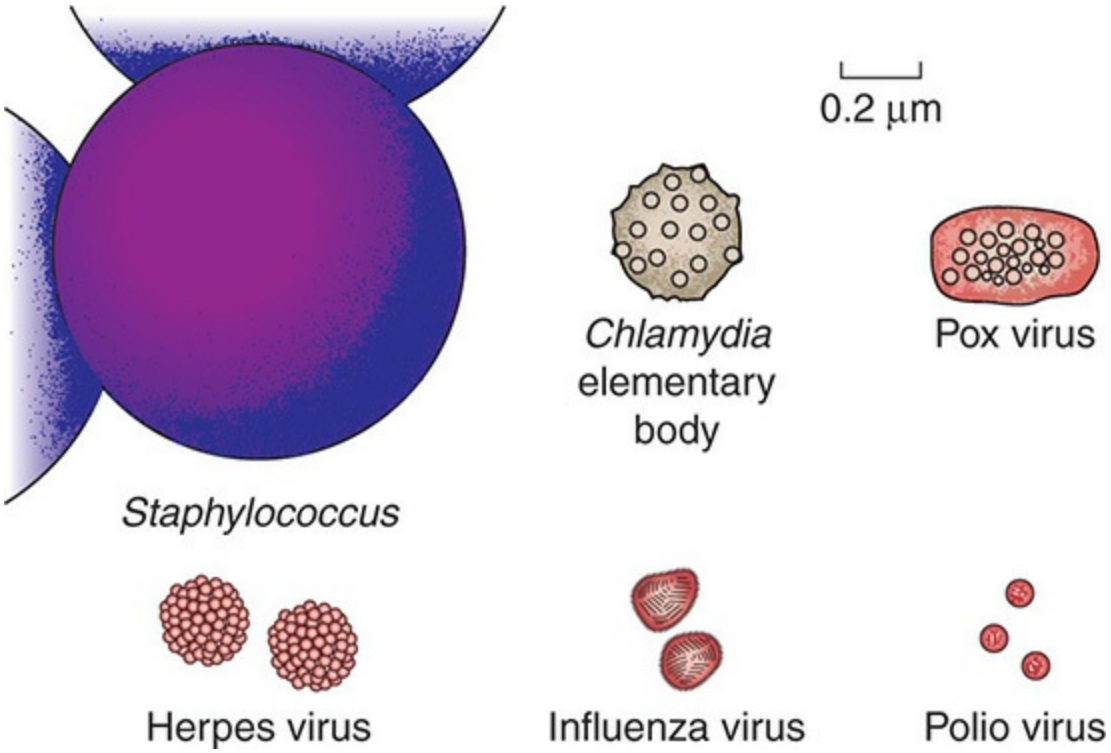
BOX 23-3

Conventions for Naming Viruses

Taxonomic Hierarchy	Ending	Example
Order	-virales	Mononegavirales
Family	-viridae	Paramyxoviridae
Subfamily	-virinae	Pneumovirinae
Genus	-virus	*Pneumovirus*
Species	None	*Human respiratory syncytial virus*

Much of traditional viral taxonomy derives from detailed knowledge of viral structure, but is now supplemented by nucleic acid studies. Viruses are among the smallest of infectious agents (Fig. 23-1). The poxviruses, which are the largest members of the virus family and similar in size to the smallest bacteria, are close to the resolution of the light microscope; an electron microscope is necessary to visualize all other viruses.

Almost without exception, viruses contain either DNA or RNA, but not both. Most of the medically important DNA viruses are double-stranded, an exception being parvovirus; most of the medically important RNA viruses are single-stranded, an exception being rotavirus (i.e., reoviruses). The center of any viral particle, the ribonucleoprotein core, contains coiled nucleic acid and protein.[204] Around the core is a protective shell composed of repeating protein units called capsomeres, which, in the aggregate, make up the capsid shell. With the nucleoprotein core, the nucleocapsid unit is complete. The architecture of most viruses has a symmetry that is either helical, similar to nucleic acid, or icosahedral, the architecture of a geodesic dome. These two symmetries, illustrated in Figure 23-2, provide the most efficient means for assembling the structural shells, an economy that is essential for these very small particles with limited genetic resources.

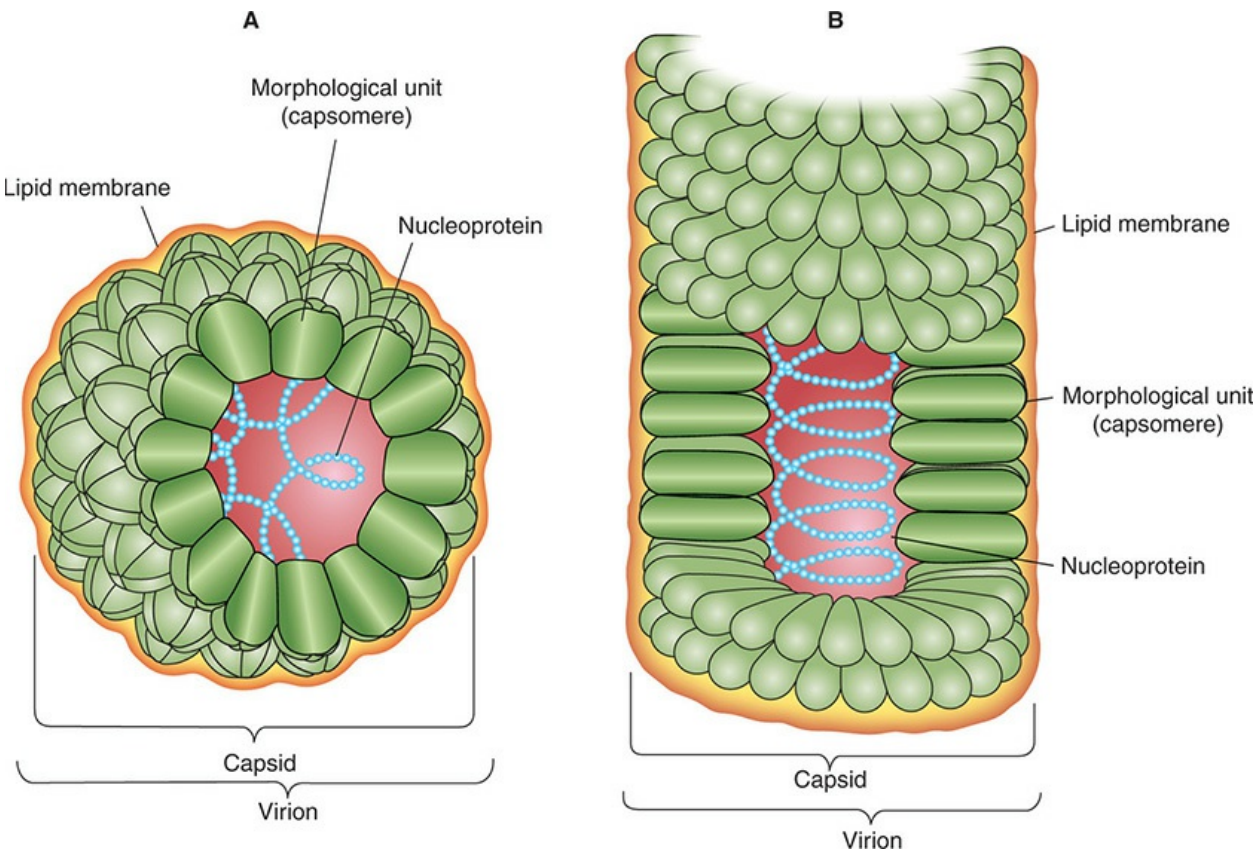


■ **FIGURE 23-1** Viruses are the smallest of infectious agents. The relative size of a bacterium, *Staphylococcus*, is compared with *Chlamydia*, with the largest virus group (poxvirus), and with one of the smallest viruses (poliovirus, a member of the enterovirus group).

As a general rule, DNA viruses replicate and are assembled in the nucleus; RNA viruses are assembled in the cytoplasm. The major exceptions to the site of replication are influenza viruses (RNA), which begin replication in the nucleus, and poxviruses (DNA), which replicate in the cytoplasm. The complex events that occur after a virus infects a cell are reflected only incompletely in the morphologic cellular changes. In some instances, however, one can see clearly how the virus has appropriated the genetic resources of the cell (Fig. 23-3). The final assembly of some viruses occurs at the nuclear or cytoplasmic membrane. The formation of the "envelope," for enveloped viruses, occurs as the virion moves from the nucleus to the cytoplasm or passes from the cytoplasm to the extracellular space; it is during these transitions that an external lipid-containing envelope is added to the nucleocapsid (Figs. 23-2 and 23-3 A-F). Lipid solvents and detergents readily inactivate such enveloped viruses, whereas their nonenveloped counterparts are not affected. The replication of some viruses results in death of the cell, often through cell lysis, with release of the assembled nucleocapsids. The enveloped viruses that mature at the cytoplasmic membrane are released from the cell by "budding" through the membrane (Figs. 23-3F and 23-4). In this process, the envelope accumulates portions of the lipid membrane from the cell,

as well as virus-specific glycoproteins that have been inserted into that membrane.

1504



■ **FIGURE 23-2** Virus morphology. Viruses are constructed with great economy. A central nucleoprotein core is surrounded by a protein capsid, which is made up of individual capsomeres. Some viruses also have a host-derived lipoprotein membrane around the nucleocapsid. The two most common organizational patterns of symmetry are icosahedral **(A)** and helical **(B)**. Ultrastructurally, the icosahedral viruses appear round, although the facets may occasionally be visualized (see the illustration of adenovirus in Table 23-2).

The major groups of viruses are summarized in Tables 23-1 and 23-2. Illustrative electron micrographs of negatively stained viral particles (provided by Frederick A. Murphy) are juxtaposed with a brief description of the families and genera. The list is not exhaustive, but includes the most important human pathogens. The taxonomic characteristics are summarized in the tables, but the vernacular names are used subsequently in the text.

Structural and biochemical features that are used for classification include the

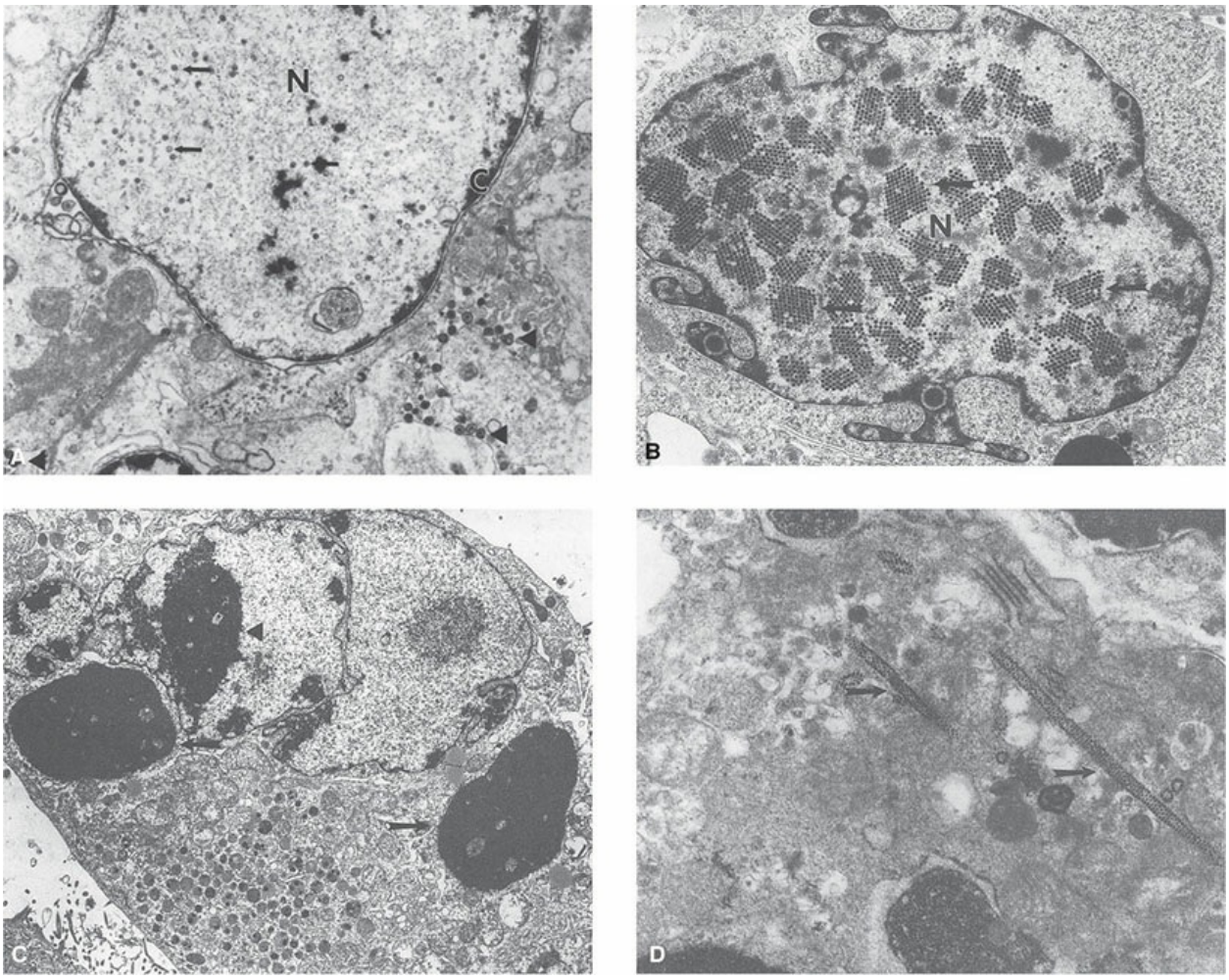
characteristics of the nucleic acid, the configuration or symmetry of the nucleocapsid (helical, icosahedral, or complex), the presence or absence of a lipid envelope, the size of the virion, the number of capsomeres, the stability of the virion to chemical treatment, and the antigenic composition.

The polarity of RNA viruses is considered positive if it can function as mRNA (i.e., proteins needed for construction of the virion can be synthesized directly from the viral nucleic acid once it is released into the cell by disruption of the virion capsid). An RNA virus with negative polarity must use an RNA-dependent RNA polymerase (i.e., an enzyme that must be packaged with the nucleic acid in the infecting virion) to make a complementary RNA molecule (i.e., an RNA molecule with a positive polarity), after which the necessary proteins can be synthesized. The genome of most RNA viruses is contained in one linear segment, but some viruses have a segmented genome. The segmented genome allows enhanced potential for genetic reassortments when two strains infect the same cell. One of the explanations for the amazing ability of influenza viruses to change dramatically (i.e., the cause of antigenic shift) and adapt is its segmented genome that facilitates recombination of segments when two different virions infect the same cell.

Clinical Manifestations of Viral Infections

The clinical manifestations of viral infections are legion. General symptoms include fever with or without chills, malaise, and myalgias. Specific symptoms derive primarily from the propensity of the virus to infect various cells and tissues (tropism), but may also depend on the route of infection, the virulence of the strain, the immunologic status of the patient,

1505



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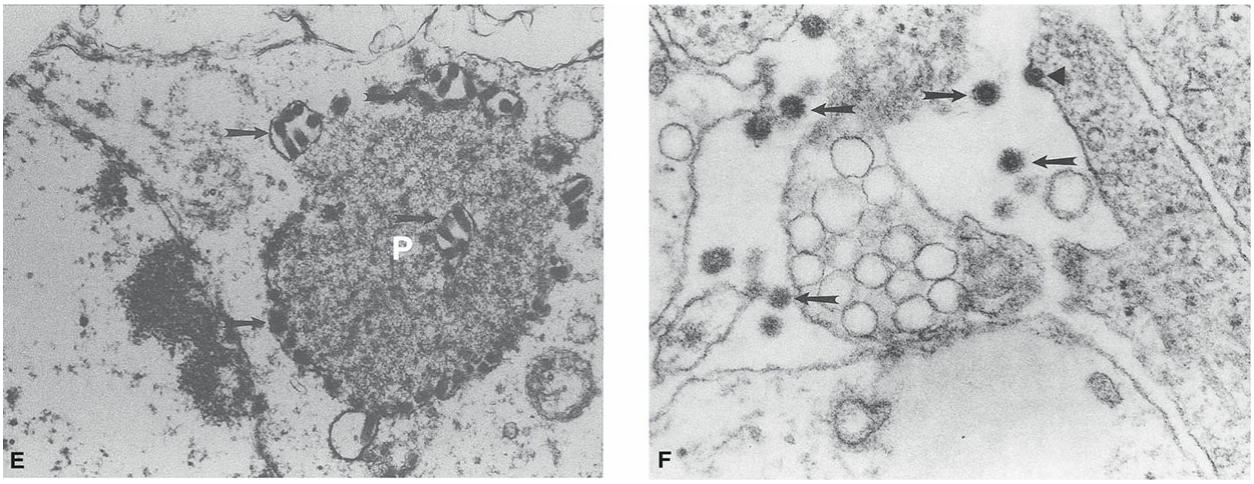
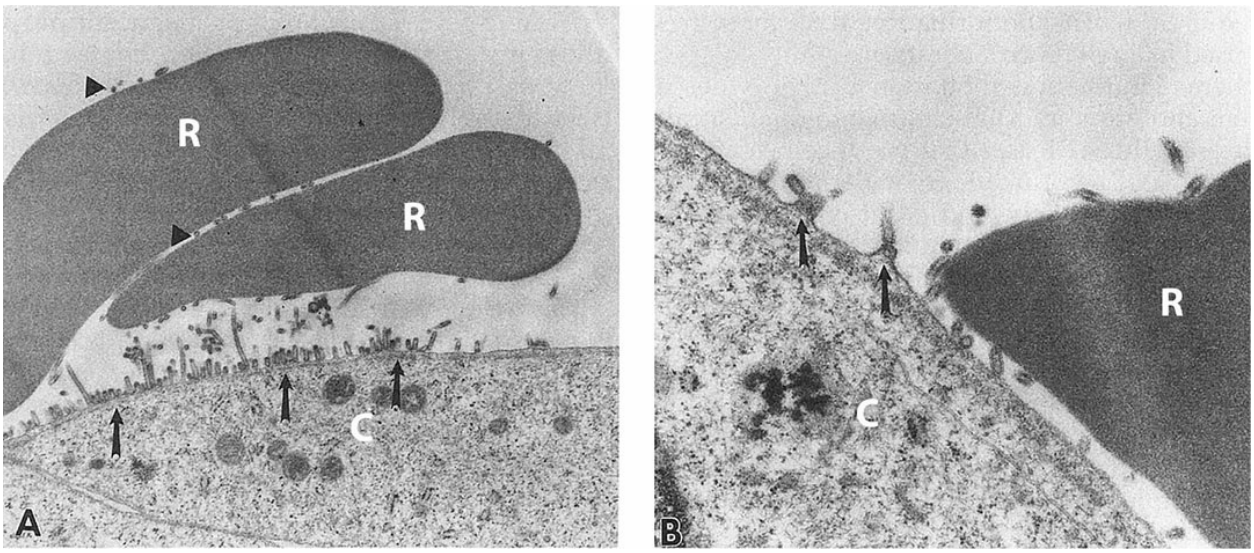


■ **FIGURE 23-3** Ultrastructure of viruses. **A.** Herpes simplex virus. The herpesviruses are assembled in the nucleus of the cell (N). The cellular chromatin (C) is pushed to the edge

of the nucleus by the viral deoxyribonucleoprotein, which stains lightly in the micrograph. This material corresponds to the eosinophilic intranuclear inclusion seen with the light microscope (Image Plate 23-1). Nonenveloped virions (arrows) are assembled in the nucleus. Outside the nucleus, some mature virions that have acquired a lipid envelope from the host cell (arrowheads) are visible (MRC-5 cells, infected with herpes simplex virus, type 1, ×22,400). **B.** Adenoviruses are assembled in the nucleus (N). They have icosahedral symmetry and are not enveloped. They are often closely packed in paracrystalline arrays (arrows). As the viruses accumulate, the whole nucleus may become filled with virions, producing the inclusions seen with the light microscope (Image Plate 23-1E) (Infected HEp-2 cells ×22,400). **C.** Respiratory syncytial virus is assembled in the cytoplasm and matures at the cell membrane. Inclusions may be seen in the cytoplasm of infected cells, both in vivo and in vitro (Image Plate 23-1G). The inclusions (arrow) are intracytoplasmic and are composed of fibrillar ribonucleoprotein. Contrast the density of the nucleolus (arrowhead) with that of the inclusions (Infected HEp-2 cells, ×3,150). **D**. Coxsackievirus B. The enteroviruses are small RNA-containing viruses that have icosahedral symmetry and are often packed in a paracrystalline array (arrows). They are assembled in the cytoplasm. Inclusions are not seen with the light microscope (Infected rhesus monkey kidney cells, ×56,000). **E.** Rabies virus. This bullet-shaped rhabdovirus is assembled in the cytoplasm. The fibrillar nucleoprotein (P) and the elongated viruses (arrows) are found together. The mass of viral material corresponds to the Negri body, which is seen with the light microscope (Image Plate 23-1H). The internal structure that may be seen in classical Negri bodies comes from the incorporation of cytoplasmic material into the body of the inclusion (Infected human brain, ×25,000). **F.** Eastern equine encephalitis virus. This togavirus illustrates the maturation of virus particles at the surface of the cell. Mature virions (arrows) are present in the extracellular space. A maturing (budding) virion is denoted by the dense nucleoprotein core and a condensation of the cellular membrane where the envelope is forming (arrowhead). Some viruses, such as the myxoviruses, insert their hemagglutinins into the membrane at this stage; in classical virology, this afforded their detection by the adherence of red blood cells to the viral hemagglutinin in the cell membrane (hemadsorption) (Infected mouse brain, ×60,000). (E, courtesy of Daniel Perl; F, courtesy of Frederick Murphy).



■ **FIGURE 23-4** Electron microscopy of hemadsorption and hemagglutination of influenza A virus. A. Two red cells (R) have adhered to a monolayer of rhesus monkey kidney cells (C). The red cell-to-red cell adherence is caused by the large number of influenza virions that are "budding" from the surface of the kidney cells (arrows) or adhering to the red blood cells (arrowheads) (×35,000). B. At higher magnification, the bulges in the cell membrane at the point of budding can be seen clearly (arrows). The hemagglutinin and neuraminidase spikes on the virus particles appear as a "fringe" (×80,000).

and other predisposing factors such as underlying pulmonary or cardiac disease. The reader is referred to several excellent textbooks for additional details.[285,395]

Orthomyxoviruses

The orthomyxoviruses, which include influenza A, B, and C viruses, produce a wide variety of respiratory infections.[50,368] The influenza or "flu" syndrome begins with the abrupt onset of headache, fever, chills, and dry cough; later, high fever, myalgias, malaise, and anorexia appear. In young children, gastrointestinal symptoms may be prominent. Almost all influenza infections occur during the winter. Influenza A and B viruses account for most epidemic respiratory diseases in which there is excess mortality. They are rarely isolated in the absence of infection. Influenza A virus infects a variety of mammalian and avian species. Transmission of swine and avian influenza from pigs/chickens to humans has been described occasionally.[481] Influenza C virus is an uncommon cause of human infection, but may produce bronchitis and pneumonia.[335] Influenza viruses may directly produce serious lower respiratory tract disease or, more commonly, may set up the patient for a secondary bacterial

infection.[510] A fortunately uncommon twist to the secondary infection scenario has been the production of toxic-shock syndrome by the invading *Staphylococcus aureus*.[300] More commonly, *S. aureus* would cause a necrotizing pneumonia in the postinfluenza setting. Other complications of influenza include myocarditis[130] and encephalopathy,[222,443] although the virus is not usually detected in these situations.

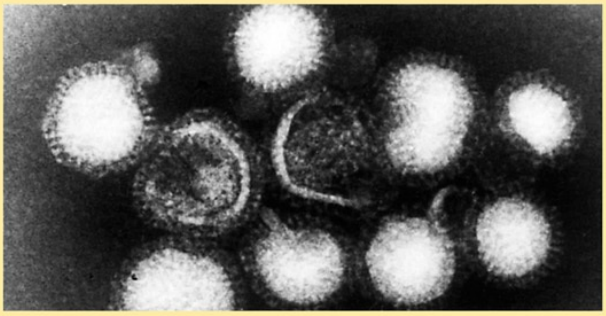
The structure and function of influenza virus are intimately related both to pathogenesis and to laboratory diagnosis.[279] The nucleoprotein antigen in the viral core defines the type of virus; for example, influenza A or B. This antigen is stable and is recognized by many of the diagnostic antisera, which are able to detect all circulating strains of a particular type. Two glycoprotein antigens on the surface of the influenza virion are important for pathogenesis, epidemiology,

TABLE 23-1 Classification of RNA Viruses (Selected Genera and Species That Infect Humans)

Family: *Orthomyxoviridae*
Species: *Influenza A virus*
Species: *Influenza B virus*
Species: *Influenza C virus*

Size: 80–120 nm, spherical or pleomorphic
Nucleic acid: Segmented, negative polarity, single-stranded RNA
Symmetry: Helical
Lipid envelope: Present
Antigens: Hemagglutinin and neuraminidase
Natural habitat: Humans and animals
Distribution: Worldwide

Mode of transmission: Aerosol droplet spread from person to person; rarely animal to person
Route of infection: Respiratory
Diseases: Respiratory infections, including coryza (common cold), tracheobronchitis, and pneumonia. Extrapulmonary symptoms are prominent in the influenza syndrome; rarely, extra-pulmonary disease such as myocarditis may be present.



Influenza A virus. The particles are roughly spherical but irregular. The internal helical structure is difficult to appreciate, but the fringe-like projections of hemagglutinin and neuraminidase are shown well. × 135,000.

Family: *Paramyxoviridae*
Subfamily: *Paramyxovirinae*
Genus: *Respirovirus*
Species: *Human parainfluenza virus 1*
Species: *Human parainfluenza virus 3*
Genus: *Rubulavirus*
Species: *Human parainfluenza virus 2*
Species: *Human parainfluenza virus 4*
Species: *Mumps virus*
Genus: *Morbillivirus*
Species: *Measles virus*
Subfamily: *Pneumovirinae*
Genus: *Pneumovirus*
Species: *Human respiratory syncytial virus*
Genus: *Metapneumovirus*
Species: *Human metapneumovirus*

Size: 150–300 nm, pleomorphic
Nucleic acid: Nonsegmented, negative polarity, single-stranded RNA
Symmetry: Helical
Lipid envelope: Present
Antigens: Hemagglutinin, neuraminidase, fusion protein, nucleocapsid (depending on virus)
Natural habitat: Humans and animals
Distribution: Worldwide

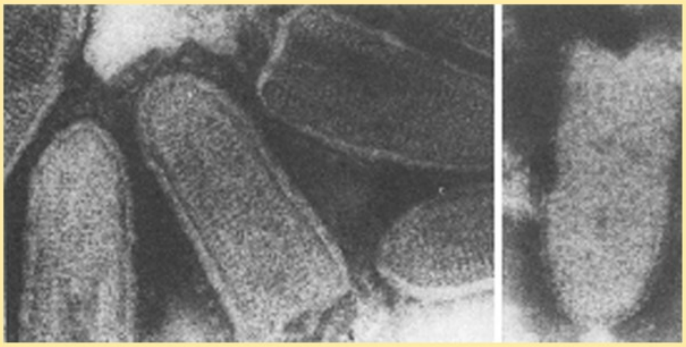
Mode of transmission: Droplet aerosol or direct inoculation
Route of infection: Respiratory
Diseases: Common cold, tracheobronchitis, rarely pneumonia (parainfluenza); tracheobronchitis, bronchiolitis, and pneumonia (respiratory syncytial virus); parotitis, pancreatitis, orchitis, meningitis (mumps); rash, pneumonia, encephalitis, subacute sclerosing panencephalitis (measles)

Human parainfluenza virus 1. The irregular virion has been disrupted, releasing a long strand of uncoiled RNA. The hemagglutinin fringes on the virion are evident.× 72,000

Family: *Rhabdoviridae*
Genus: *Lyssavirus*
Species: *Rabies virus*

Size: 50–95 nm by 130–389 nm, bullet shaped
Nucleic acid: Nonsegmented, negative polarity, single-stranded RNA
Symmetry: Helical
Lipid envelope: Present
Natural habitat: Wild and domestic animals
Distribution: Worldwide

Mode of transmission: Infected secretions, rabies usually transmitted by bites
Route of transmission: Cutaneous or respiratory
Disease: Encephalitis (rabies)

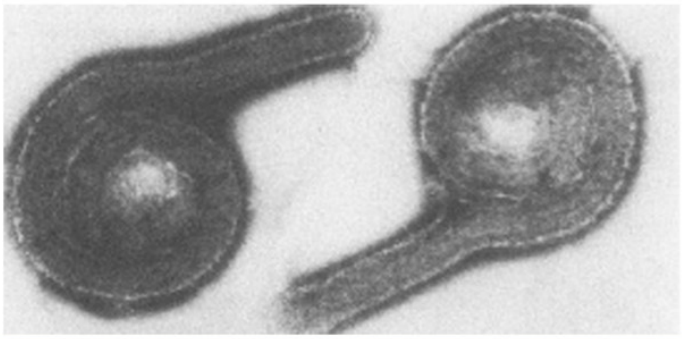


Vesicular stomatitis virus. The bullet shape of the virion is evident. The coiled helical nature of the internal ribonucleoprotein is reflected in the striations.× 216,000

Family: *Filoviridae*
Genus: *Marburgvirus*
Species: *Marburg marburgvirus*
Genus: *Ebolavirus*
Species: *Zaire ebolavirus*
Species: *Bundibugyo ebolavirus*
Species: *Tai Forest ebolavirus*
Species: *Sudan ebolavirus*

Size: 80 nm by 800–1,000 nm, filamentous
Nucleic acid: Nonsegmented, negative polarity, single-stranded RNA
Symmetry: Helical
Lipid envelope: Present
Natural habitat: Nonhuman primates
Distribution: Africa, Philippines

Mode of transmission: Unknown, but close contact required
Diseases: Hemorrhagic fever with fatality rates as high as 90%

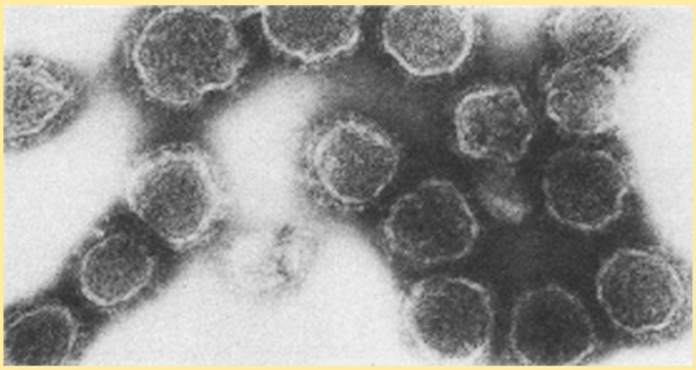


Ebola virus. Long, filamentous form has assumed a "crook" shape. × 59,000

Family: *Bunyaviridae*
Genus: *Orthobunyavirus*
Species: *California encephalitis virus*
Species: *La Crosse virus*
Genus: *Hantavirus*
Species: *Hantaan virus*
Species: *Sin Nombre virus*
Genus: *Nairovirus*
Species: *Crimean Congo hemorrhagic fever virus*
Species: *Rift Valley fever virus*
Species: *Sandfly fever Naples virus*

Size: 80–120 nm
Nucleic acid: Segmented, single-stranded RNA. May be negative polarity (*Bunyavirus, Hantavirus,* and *Nairovirus*) or contain examples of each polarity (*Phlebovirus*)
Symmetry: Helical
Lipid envelope: Present
Antigens: Surface hemagglutinin
Natural habitat: Small animals

Distribution: Worldwide
Mode of transmission: Bite of infected mosquitoes (*Bunyavirus, Nairovirus, Phlebovirus*); exposure to rodent excreta (*Hantavirus*)
Diseases: Febrile illness, aseptic meningitis (*Bunyavirus*); febrile illness, hemorrhagic fever, respiratory failure (*Hantavirus*); febrile illness, hemorrhagic fever (*Nairovirus*); febrile illness, encephalitis, hemorrhagic fever (*Phlebovirus*)

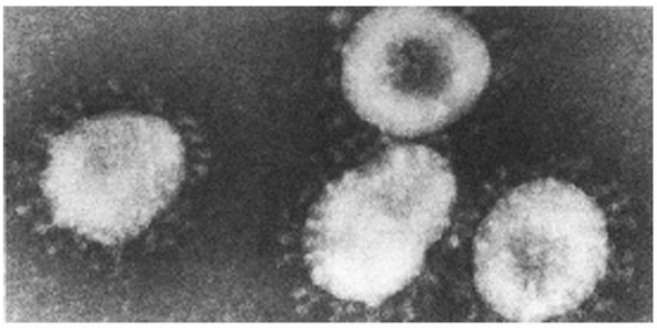


LaCrosse virus. Irregular virus particles; the lipid envelope and surface projections are evident.× 117,000

Family: *Coronaviridae*
Genus: *Alphacoronavirus*
Species: *Human coronavirus 229E*
Species: *Human coronavirus OC43*
Genus: *Betacoronavirus*
Species: *Severe acute respiratory syndrome-related virus*

Size: 120–160 nm
Nucleic acid: Nonsegmented, positive polarity, single-stranded RNA
Symmetry: Helical
Lipid envelope: Present
Natural habitat: Humans or animals (Coronaviruses)

Distribution: Worldwide
Transmission: Person to person, presumably by aerosol
Route of infection: Respiratory
Diseases: Common cold; rarely lower respiratory disease; severe acute respiratory syndrome

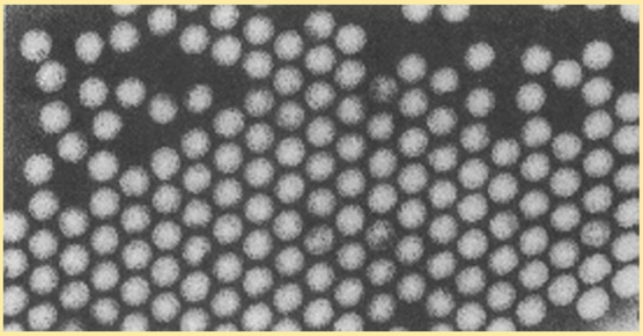


Human coronavirus OC43. The club-shaped projections are the most distinctive features of these pleomorphic particles. × 180,000

Family: *Picornaviridae*
Genus: *Enterovirus*
Species: *Enterovirus C (human poliovirus 1–3)*
Species: *Enterovirus A (human coxsackievirus A2)*
Species: *Enterovirus B (human echovirus)*
Species: *Rhinovirus A-C*
Genus: *Hepatovirus*
Species: *Hepatitis A virus*
Genus: *Parechovirus (Rhinovirus)*
Species: *Human parechovirus*

Size: 22–30 nm
Nucleic acid: Nonsegmented, positive polarity, single-stranded RNA
Symmetry: Icosahedral
Lipid envelope: Absent
Natural habitat: Human gastrointestinal tract; survives in environment (*Enterovirus, Hepatovirus, Parechovirus*); human upper respiratory tract (*Rhinovirus*)

Distribution: Worldwide
Transmission: Fecal–oral from human to human or contaminated water (*Enterovirus, Hepatovirus, Parechovirus*); human respiratory tract by aerosol or on hands (*Rhinovirus*)
Route of infection: Gastrointestinal (*Enterovirus, Hepatovirus, Parechovirus*); respiratory (*Rhinovirus*)
Diseases: Febrile illness, meningitis, encephalitis, myocarditis (*Enterovirus, Parechovirus*); hepatitis (*Hepatovirus*); common cold (*Rhinovirus*)

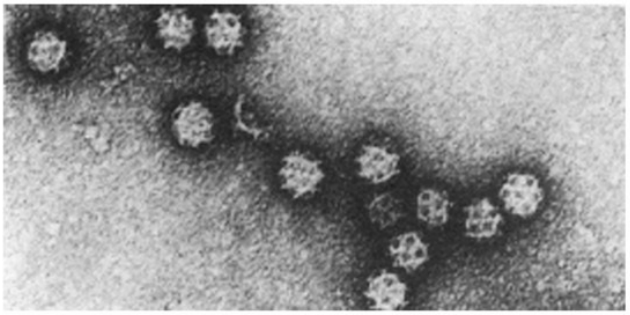


Poliovirus 1. The particles are small, regular, and nonenveloped. × 180,000

Family: *Caliciviridae*
Genus: *Norovirus*
Species: *Norwalk virus*
Genus: *Sapovirus*
Species: *Sapporo virus*

Size: 35–40 nm
Nucleic acid: Nonsegmented, positive polarity, single-stranded RNA
Symmetry: Icosahedral
Lipid envelope: Absent
Natural habitat: Human gastrointestinal tract

Distribution: Worldwide
Transmission: Fecal–oral; contaminated water
Portal of entry: Gastrointestinal
Diseases: Gastroenteritis

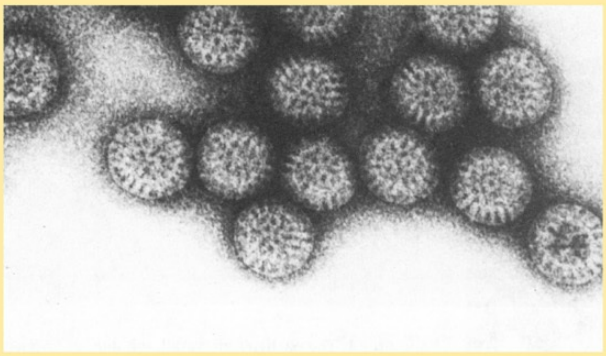


Swine exanthemata virus. Very small, regular particles with prominent cuplike depressions. The surface depressions of the human Norwalk-like viruses are not as pronounced.

Family: *Reoviridae*
Genus: *Orthoreovirus*
Species: *Mammalian orthoreovirus*
Genus: *Rotavirus*
Species: *Rotavirus A-C*
Genus: *Coltivirus*
Species: *Colorado tick fever virus*

Size: 60–85 nm, variable morphology
Nucleic acid: Segmented, double-stranded RNA
Symmetry: Icosahedral
Lipid envelope: Absent
Natural habitat: Gastrointestinal tract of humans (*Rotavirus*) and animals (*Rotavirus*)

Distribution: Worldwide
Transmission: Fecal–oral by direct contact; animal to human
Portal of entry: Gastrointestinal tract
Diseases: Gastroenteritis (*Rotavirus*); None (*Rotavirus*)



Rotavirus. The distinctive feature of the *Reoviridae* is the presence of a double shell, each with icosahedral symmetry. × 135,000

Family: *Astroviridae*
Genus: *Mamastrovirus*
Species: *Mamastrovirus 1, 6, 8, and 9*

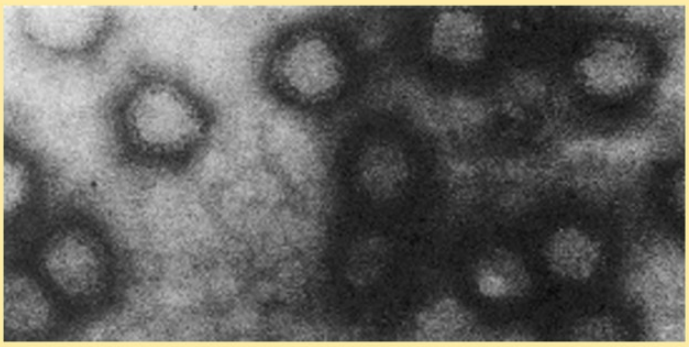
Size: 28–30 nm
Nucleic acid: Nonsegmented, positive polarity, single-stranded RNA
Symmetry: Icosahedral
Lipid envelope: Absent
Natural habitat: Gastrointestinal tract of human

Distribution: Worldwide
Transmission: Fecal–oral; contaminated water
Portal of entry: Gastrointestinal tract
Diseases: Gastroenteritis

Family: *Togaviridae*
Genus: *Alphavirus*
Species: *Eastern equine encephalitis virus*
Species: *Venezuelan equine encephalitis virus*
(Other arthropod-borne species)
Species: *Chikungunya virus*
Genus: *Rubivirus*
Species: *Rubella virus*

Size: 60–70 nm
Nucleic acid: Nonsegmented, positive polarity, single-stranded RNA
Symmetry: Icosahedral
Lipid envelope: Present
Natural habitat: Small mammals and insects (*Alphavirus*); humans (*Rubivirus*)

Distribution: Worldwide
Transmission: By mosquito bites (*Alphavirus*); aerosols or transplacental (*Rubivirus*)
Portal of entry: Cutaneous (*Alphavirus*); respiratory or vascular (*Rubivirus*)
Diseases: Hepatitis, meningitis, encephalitis, arthritis, febrile illness (*Alphavirus*); rash illness, congenital infection (*Rubivirus*)



Rubella virus. The spherical virions have a lipid membrane and surface hemagglutinin projections.× 135,000

Family: *Flaviviridae*
Genus: *Flavivirus*
Species: *Yellow fever virus*
Species: *St. Louis encephalitis virus*
Species: *Japanese encephalitis virus*
Species: *West Nile virus*
Species: *Dengue virus*

Size: 40–60 nm
Nucleic acid: Nonsegmented, positive polarity, single-stranded RNA
Symmetry: Icosahedral
Lipid envelope: Present
Natural habitat: Small mammals and insects (*Flavivirus*); humans (*Hepacivirus*)

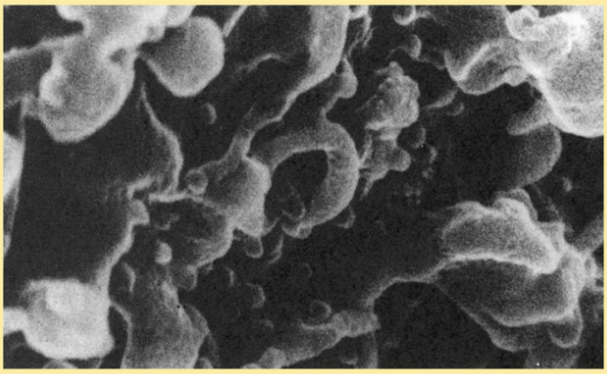
Distribution: Worldwide
Transmission: By bite of mosquito or tick (*Flavivirus*); by blood (*Hepacivirus*)
Portal of entry: Cutaneous (*Flavivirus*); vascular; sexual (*Hepacivirus*)
Diseases: Hepatitis, meningitis, encephalitis febrile illness, hemorrhagic fever (*Flavivirus*); hepatitis, cirrhosis, hepatocellular carcinoma (*Hepacivirus*)

(Numerous other species, many arthropod-borne)
Genus: *Hepacivirus*
Species: *Hepatitis C virus*
Genus: *Hepevirus*
Species: *Hepatitis E virus*

Family: *Retroviridae*
Genus: *Deltaretrovirus*
Species: *Primate T-cell lymphotropic virus,* Types 1 and 2 (HTLV)
Genus: *Lentivirus*
Species: *Human immunodeficiency virus,* Types 1 and 2 (HIV)

Size 80–100 nm
Nucleic acid: Nonsegmented, positive polarity, single-stranded RNA dimer (packaged with RNA-dependent DNA polymerase)
Symmetry: Variable
Lipid envelope: Present
Natural habitat: Humans

Distribution: Worldwide
Transmission: Sexual, contaminated needles, transfusions, maternal-fetal/neonate
Portal of entry: Mucous membranes, vascular
Diseases: T-cell leukemia and lymphoma (HTLV); acquired immunodeficiency syndrome (HIV)

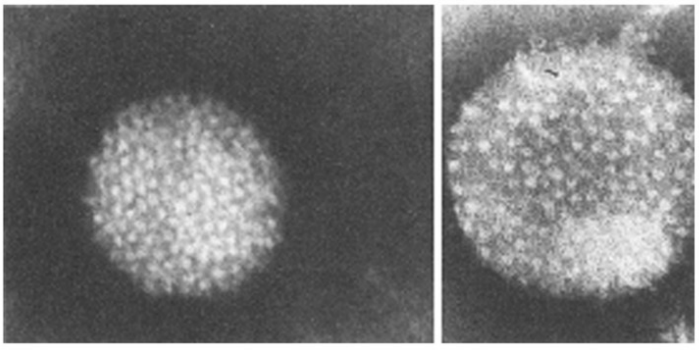


Human immunodeficiency virus. Scanning electron micrograph of human T lymphocyte with virus particles budding from surface

Family: *Arenaviridae*
Genus: *Arenavirus*
Old World Group
Species: *Lymphocytic choriomeningitis virus (LCM)*
Species: *Lassa virus*
New World Group
Species: *Junin virus*
Species: *Machupo virus*
Species: *Sabia virus*
Species: *Guanarito virus*

Size: 50–300 nm
Nucleic acid: Bisegmented, ambipolarity, single-stranded
Symmetry: Pleomorphic
Lipid envelope: Present
Naturally habitat: Rodents

Distribution: Worldwide (LCM); South America (Junin, Machupo, Sabia, Guanarito); Africa (Lassa)
Transmission: Contact with rodent or human secretions
Portal of entry: Respiratory, cutaneous (lacerations)
Diseases: Febrile illness, meningitis (LCM); hemorrhagic fever (Lassa, Junin, Machupo, Sabia, Guanarito viruses)

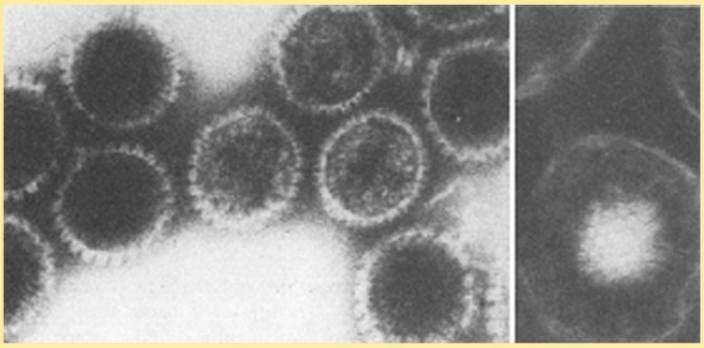


Lymphocytic choriomeningitis virus. The spherical virus has large surface projections. The name is derived from the Latin word *arenosus* (sandy), which was suggested by the internal ribosome-like particles that are seen by transmission electron microscopy. × 225,000

TABLE 23-2 Classification of DNA Viruses (Selected Genera and Species That Infect Humans)

Family: *Herpesviridae*
Subfamily: *Alphaherpesvirinae*
Genus: *Simplexvirus*
Species: *Human herpesvirus 1*
(Herpes Simplex virus Type 1; HSV1)
Species: *Human herpesvirus 2*
(Herpes Simplex virus Type 2; HSV2)
Genus: *Varicellovirus*
Species: *Human herpesvirus 3* (Varicella-Zoster virus; VZV)
Subfamily: *Betaherpesvirinae*
Genus: *Cytomegalovirus*
Species: *Human herpesvirus 5* (Cytomegalovirus; CMV)
Genus: *Roseolovirus*
Species: *Human herpesvirus 6A* (HHV6A)
Species: *Human herpesvirus 6B* (HHV6B)
Species: *Human herpesvirus 7* (HHV7)
Subfamily: *Gammaherpesvirinae*
Genus: *Lymphocryptovirus*
Species: *Human herpesvirus 4* (Epstein–Barr virus; EBV)
Genus: *Rhadinovirus*
Species: *Human herpesvirus 8* (Kaposi's sarcoma-associated herpesvirus; HHV8)

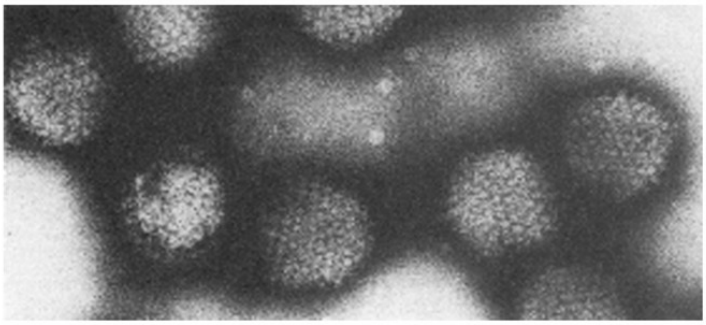
Size: 120–300 nm
Nucleic acid: Double-stranded DNA, nonsegmented
Symmetry: Icosahedral
Lipid envelope: Present
Natural habitat: Humans
Distribution: Worldwide
Transmission: By infected oral or genital secretions, by blood, or transplacentally
Portal of entry: Respiratory, cutaneous, intravascular, transplacental
Diseases: Pharyngitis, cervicitis, local skin lesions, pneumonia, esophagitis, encephalitis, disseminated infection (HSV1), skin lesions, meningitis, neonatal sepsis (HSV2); congenital infection, hepatitis, mononucleosis, pneumonia (CMV), chickenpox, disseminated infection, zoster/shingles (VZV), pharyngitis, mononucleosis, hepatitis (EBV); exanthema subitum (HHV6, HHV7), Kaposi's sarcoma, lymphoma (HHV8)



Herpes simplex virus. (Left) Naked nucleocapsids demonstrate the regular structure of the virion. Clearly defined structural units appear to have a central depression. (Right) Enveloped particle has, in addition, a lipid membrane derived from the infected cell. ×189,000.

Family: *Adenoviridae*
Genus: *Mastadenovirus*
Species: *Human mastadenovirus A-G*

Size: 70–90 nm
Nucleic acid: Double-stranded DNA, nonsegmented
Distribution: Worldwide
Symmetry: Icosahedral
Transmission: Fecal–oral, possibly aerosol droplet
Lipid envelope: Absent
Portal of entry: Respiratory, Gastrointestinal
Natural habitat: Humans
Diseases: Conjunctivitis, keratitis, pharyngitis, tracheobronchitis, pneumonia, cystitis; gastroenteritis (types 40 and 41)



Adenovirus, type 5. The regular arrangement of the structural units of the virion is clearly evident. ×234,000.

1513

Family: *Papillomaviridae*
Genus: *Alphapapillomavirus*
Species: *Alphapapillomavirus 1 (Human papillomavirus 32; HPV)*
Genus: *Betapapillomavirus*
Species: *Betapapillomavirus 1 (Human papillomavirus 5; HPV)*

Size: 55 nm
Nucleic acid: Double-stranded DNA, nonsegmented.
Lipid envelope: Absent
Symmetry: Icosahedral
Distribution: Worldwide
Transmission: Contact with infected secretions, sexual; the virus remains dormant in tissues and/or integrates into the host genome
Portal of entry: Cutaneous, mucocutaneous (oral and/or genital), respiratory
Diseases: Cutaneous, genital and respiratory papillomas (i.e., warts), and epithelial neoplasia, predominantly squamous cell carcinoma of the uterine cervix and of the head and neck

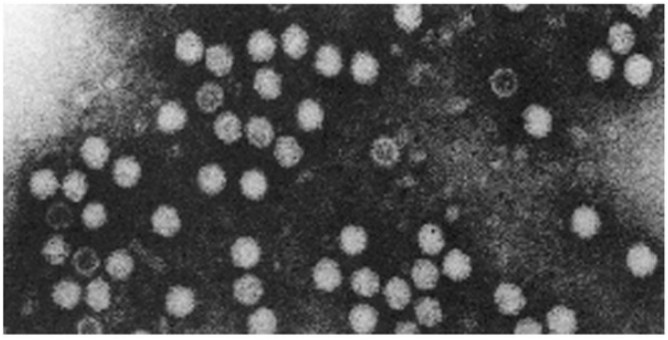
Human papillomavirus. These viruses have never been propagated successfully in cell cultures. The diseases have been associated by demonstration of virions, viral antigens, or nucleic acid in lesions. ×135,000.

Family: *Polyomaviridae*
Genus: *Polyomavirus*
Species: *BK polyomavirus*
Species: *JC polyomavirus*

Size: 40–45 nm
Nucleic acid: Double-stranded DNA, nonsegmented.
Lipid envelope: Absent
Symmetry: Icosahedral
Distribution: Worldwide
Transmission: Unknown; respiratory and urine/oral mechanisms are postulated. Virus remains latent and may reactivate in the presence of a suppressed immune system.
Portal for entry: Likely respiratory or oral.
Diseases: Hemorrhagic cystitis and renal transplant nephropathy (BK virus); progressive multifocal leukoencephalopathy (JC virus).

Family: *Parvoviridae*
Subfamily: *Parvovirinae*
Genus: *Erythroparvovirus*
Species: *Primate erythroparvovirus* (Human parvovirus B19)

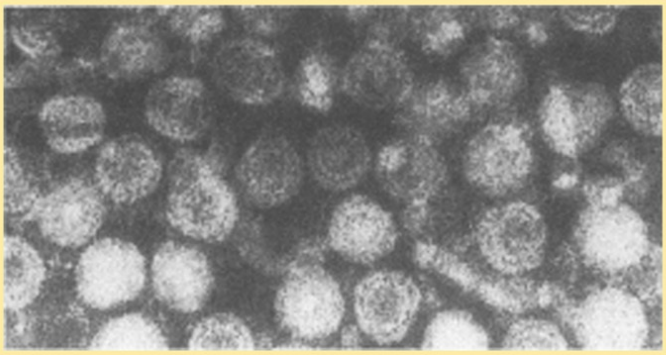
Size: 21–26 nm
Distribution: Worldwide
Nucleic acid: Single-stranded DNA; nonsegmented
Symmetry: Icosahedral
Lipid envelope: Absent
Natural habitat: Humans
Transmission: Person-to-person (route unknown); transfusion
Portal of entry: unknown; vascular
Diseases: Erythema infectiosum (fifth disease), hemolytic disease of newborn, anemia



H-1 virus. The virions are very small and regular in size and shape. Parvoviruses are unusual in that they contain single-stranded DNA.

Family: *Hepadnaviridae*
Genus: *Orthohepadnavirus*
Species: *Hepatitis B virus*

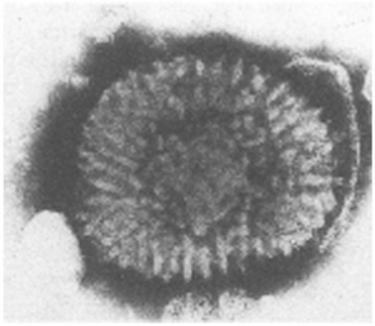
Size: 42–50 nm
Distribution: Worldwide
Nucleic acid: Double-stranded, circular DNA; nonsegmented
Transmission: Infected secretions and blood, sexual, transfusion
Symmetry: Complex
Portal of entry: Skin, vascular, genital
Lipid envelope: Present
Diseases: Hepatitis, cirrhosis, hepatocellular, carcinoma
Natural habitat: Humans
Antigens:
Surface antigen (HBsAg)
Core antigen (HBcAg)
Early antigen (HBeAg)



Hepatitis B virus. These 42-nm particles, which are the infectious agent, were originally known as Dane particles. ×225,000.

Family: *Poxviridae*
Subfamily: *Chordopoxvirinae*
Genus: *Orthopoxvirus*
Species: *Variola virus* (smallpox)
Species: *Vaccinia virus*
Species: *Cowpox virus*
Species: *Monkeypox virus*
Genus: *Parapoxvirus*
Species: *Orf virus*
Genus: *Molluscipoxvirus*
Species: *Molluscum contagiosum virus*
Genus: *Yatapoxvirus*
Species: *Yaba monkey tumor virus*

Size: 140–260 × 220–450 nm
Nucleic acid: Double-stranded DNA; nonsegmented
Symmetry: Complex
Lipid envelope: Usually present
Natural habitat: Humans and animals
Distribution: Worldwide; variola "eradicated"
Transmission: Respiratory, cutaneous
Portal of entry: Respiratory, cutaneous
Diseases: Cutaneous ulcerative lesions, disseminated disease (*Orthopoxvirus*), cutaneous ulcerative lesions (*parapoxvirus*, *Yatapoxvirus*), molluscum contagiosum (*Molluscipoxvirus*)



Vaccinia virus. The convoluted outer coat of these large, brick-shaped particles is evident. When smallpox existed, negative stain electron microscopy was a useful tool for differentiation of the small pox virus from those of varicella. ×49,500.

and therapy. The dominant antigen is the hemagglutinin, which is responsible for attachment to respiratory epithelial cells and is therefore an important target for influenza vaccines. The surface neuraminidase enzyme participates in entry of the virus into the cell. These surface antigens are constantly changing, so that they are a moving target for therapeutic and diagnostic antibodies. It is the constant, incremental change (antigenic drift) and periodic, quantum change (antigenic shift) that allow

recurring epidemics and pandemics, respectively, of influenza.

The viruses are named by their structure and geography. For instance, an isolate might be characterized influenza A/Leningrad/360/86 (H3N2). The name means that this strain was the 360th isolate of influenza A in Leningrad in 1986, and it contains the third antigenic type of hemagglutinin and the second type of neuraminidase. The hemagglutinin and

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neuraminidase are not useful antigens for diagnostic purposes, but they are essential for the epidemiologist to study so that the next year's vaccine can contain antibodies to currently circulating antigens. Although it has not yet been accomplished, attempts continue to predict the future drift of influenza A virus strains, so that the vaccine for the next flu season can be formulated more rationally.[44]

To date, a number of different hemagglutinin types have been recognized. The initial antigen (H1) was hypothesized to be a constituent of the strain that caused pandemic influenza in 1918, based on serologic analysis. Independent evidence to this end has been developed using modern molecular techniques on tissue from influenza victims.[390] Two samples came from formalin-fixed lung tissue in England, but the sample that yielded the most complete sequence was taken from the body of an Inuit woman who had been buried in Alaskan permafrost. There was a remarkable homogeneity among the three samples from diverse geographic locations.

Subsequent major epidemics have been associated with major shifts in the hemagglutinin: H2 during the Asian influenza epidemic in 1957 and H3 during the Hong Kong influenza epidemic of 1968. Subsequently, strains with either the H1 or H3 hemagglutinin circulated until a new strain of avian influenza (H5 hemagglutinin) killed 6 of 18 individuals infected in Hong Kong. This virus, which was spread directly from chickens to humans without another intermediary, had particularly virulent characteristics.[205] Fortunately, spread from human to human appeared limited. Subsequently, another strain (H9 hemagglutinin) appeared in Hong Kong with similar epidemiologic characteristics, but fortunately less mortality. H5 virus has reappeared in Southeast Asia, once again with high mortality rates in patients who were exposed to poultry, but without significant person-to-person transmission.[214,269]

Outbreaks of, what has been termed, Highly Pathogenic Asian Avian Influenza A (HPAI) (i.e., H5N1) have occurred in 15 countries, with documented infections in more than 700 people. The majority of infections have occurred in Indonesia, Vietnam, and Egypt, but there have been infected individuals who have traveled to Europe and Canada. Most of the infected patients have an epidemiologic exposure to infected poultry. Most infections have occurred in adults <40 years old and in children. This strain may cause pneumonia, respiratory failure, and death, but in some,

particulary children, milder disease has been reported.[72] Most importantly, the HPAI Asian H5N1 virus is not efficiently transmitted from person to person.

The fact that avian influenza viruses have crossed the species barrier to infect humans is cause for vigilant monitoring. Devastating pandemic disease awaits only the emergence of a strain with the combination of high virulence and efficient human-to-human transmission.

The most recent influenza pandemic was the H1N1 pandemic of 2009.[71] This particular strain, which was epidemiologically linked with swine, was highly contagious, but, fortunately, no more virulent than the influenza A strains typically encountered each year. Disease, therefore, ranged from mild to severe. The new virus originated in Mexico, but quickly spread to California. This virus, fortunately, was initially susceptible to oseltamivir, which was widely used for treatment. There were two interesting observations from the pandemic and the years that followed. First, the pandemic H1N1 strain did not disappear, but rather became an endemic strain of influenza (i.e., very similar to the current endemicity of West Nile virus in North America). The other interesting finding was the speed at which this virus developed resistance to oseltamivir.

Paramyxoviruses

The paramyxoviruses are a varied group that include parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus (RSV), and human metapneumovirus (hMPV).

Parainfluenza Viruses. The parainfluenza viruses, of which there are four serotypes, are important causes of respiratory disease, which is usually mild in adults but may be severe in young children or immunocompromised patients.[208]Parainfluenza viruses 1 and 2 cause croup (laryngotracheobronchitis) in children between the ages of 2 and 6 during the winter respiratory virus season in the Northern Hemisphere. Parainfluenza virus 3 is an important cause of bronchiolitis in children less than 2 years of age; infections occur throughout the year, with peaks in the fall and spring. Parainfluenza virus 4 causes less severe infections, which is likely the reason that it is less commonly recovered in the clinical virology laboratory (i.e., patients with subclinical disease do not present for testing). Many assays, including direct fluorescent antibody (DFA) reagents and molecular diagnostic tests, do not test for the presence of parainfluenza virus 4.

Mumps Virus. Mumps virus produces infectious parotitis. It also causes aseptic meningitis, which is usually mild, and may also affect the pancreas, the gonads, and more rarely the joints. The introduction of an effective vaccine has greatly reduced the incidence of mumps virus infection, although the vaccine is not 100% effective.

Interestingly, after the introduction of mumps vaccine, the circulating strains, which had been homogeneous, became more diverse.[247] There are small outbreaks of mumps every year. These often occur in the fall or winter, when there is crowding, or among individuals with close associations, such as sport teams. According to the Centers for Disease Control and Prevention (CDC), there were 1,151 individuals who contracted mumps in 2014 (http://www.cdc.gov/mumps/outbreaks.html).

Measles Virus. Measles virus (rubeola virus) is unlikely to be encountered in the clinical laboratory,[427] but unfortunately, this virus continues to cause focal outbreaks, the most recent of which was in California. Laxity in monitoring and enforcing immunization practices has led to explosive outbreaks in both young and adults, who may have waning immunity. Other at-risk populations include children, especially in inner-city areas where levels of immunization are low, and individuals who decline vaccination for a variety of reasons.[54] Molecular analysis suggests the existence of multiple imported strains, rather than a single endemic strain.[400]

The public health implications of exposure in our mobile society were illustrated by a classical epidemic of 247 individuals that began in a ski area in Breckenridge, Colorado.[58] After dispersal of infected individuals, patients with measles were detected in 10 states.

Three factors minimize the likelihood of isolating measles virus: the diagnosis is usually made on clinical grounds, and specimens are often not submitted to the laboratory.

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In addition, virus is isolated from respiratory secretions and urine only early in the course of the infection. Finally, many laboratories are moving from cell culture, which would support the growth of the virus, to specific molecular diagnostic assays. Therefore, the measles virus would not be detected by routinely used molecular respiratory virus assays, unless specifically sought (i.e., a specific reverse transcription polymerase chain reaction [RT-PCR] assay directed to the measles virus would need to be ordered).

The diagnostic rash is produced by complexes of viral antigen and antibody. Thus, at the time the diagnosis is made by a physician, the virus is being neutralized and cultures are likely to be negative. Atypical clinical symptoms were seen formerly in patients who had received early inactivated measles vaccines,[10] but this phenomenon is no longer prevalent. Pneumonia and encephalitis are the most serious complications of measles.[128,253] In addition, the measles virus rarely produces a chronic encephalitis known as subacute sclerosing panencephalitis.[365]

Respiratory Syncytial Virus. RSV causes recurrent respiratory infections from infancy through adult life. Epidemics are common and may be hospital-associated. There are two distinct serologic groups of RSV (A and B). A single group predominates in most epidemics,[343] but the viral type does not correlate with severity of infection.[313] Disease is concentrated in the winter months, although summertime infections may occur in warm climates.[478]

Infection is spread by large particle droplets and by fomites (e.g., contaminated environmental particles, such as dust), rather than by small particle aerosols. This viral infection is a major cause of nosocomial infection; careful attention to the principles of infection control is essential.[177] Good laboratory diagnostic support, which is most effectively accomplished using rapid molecular diagnostics, is needed not only for treating the individual patient, but also for limiting nosocomial spread of the infection.

RSV may produce croup, bronchitis, bronchiolitis, or interstitial pneumonia.[196] It is the most common cause of respiratory disease in hospitalized infants less than 1 year of age, rivaled only by the hMPV (see below). RSV infections are often severe in young children. If congenital heart disease or immunologic deficiency is present, the disease may be especially lethal. In one study, 8 (73%) of 11 infants with congenital heart disease and pulmonary hypertension died of the infection.[301] Many previously normal children, especially infants, are sufficiently ill that ventilatory support is required. Apnea is a prominent part of the syndrome.

Immunity to RSV is short-lived and incomplete. RSV was long thought to be a disease of infants and young children, but adult infection, including serious disease, is now recognized to be an important clinical problem.[138,197] Epidemics in nursing homes have been reported.[139]

The serious nature of RSV infection in immunocompromised adults has been recognized.[314] Serious RSV infection occurs also in adults with chronic diseases, such as diabetes, cirrhosis, and chronic obstructive pulmonary disease.[193]

Other Paramyxoviruses. Several other paramyxoviruses have been recognized as important causes of human infections. Most notable among these is the hMPV. This virus was initially recognized in Hong Kong, but has now been documented worldwide. It is a member of the subfamily *Pneumovirinae*, which includes RSV[325]; the human virus is closely related to two avian viruses. The disease produced by hMPV resembles RSV both clinically and epidemiologically.[31] It has been isolated using LLC-MK2 cells (i.e., a continuous rhesus monkey kidney cell line), a method not commonly used in diagnostic laboratories. The virus was recognized by cytopathic effect, but currently identification is most effectively made using molecular techniques. There are now molecular assays commercially available for the detection of hMPV, but widespread use is not commonplace.

Two other more recently recognized paramyxoviruses have been associated with severe neurologic disease, as well as respiratory infection. Both of these viruses are transmitted to humans from animals (i.e., zoonotic infections). Nipah virus has caused encephalitis in pig farmers in Malaysia and Singapore.[176] The *Pteropus* (i.e., large fruit) bats are the natural reservoir for the Nipah virus. Disease produced by this virus ranges from asymptomatic disease to fatal respiratory infections or encephalitis. The Nipah virus is closely related to Hendra virus, which shares the same natural reservoir. The Hendra virus produces disease in horses and a few human infections have occurred in Australia. The range of clinical presentations is like that of the Nipah virus described above. Laboratory-acquired infections have also been noted.

Picornaviruses

The picornaviruses, which include the rhinoviruses and the enteroviruses, constitute the other large and important group of RNA viruses. The rhinoviruses are frequent causes of the common cold. They are acid-labile and do not infect the gastrointestinal tract. Transmission of rhinoviruses occurs by generation of respiratory aerosols[96] and through contact with contaminated inanimate surfaces (i.e., fomites).

There are so many rhinovirus serotypes that immunologic diagnosis is impractical, as well as not being of clinical value. In the past, culture has only infrequently been requested because rhinovirus infections are uncomfortable, but almost never life-threatening, and there is no treatment. Some of the newer multiplex respiratory viral assays include rhinovirus or a combined rhinovirus/enterovirus combination result. These may be useful in immunocompromised hosts, wherein infections with even the usual "nuisance" viruses could contribute to increased overall morbidity.

The classical enteroviruses are polioviruses 1 through 3, coxsackieviruses A and B, and echoviruses.[359] The Coxsackie group received its name from a small town in Upstate New York where the first isolates were recovered. "Echo" stands for *e*nteric *c*ytopathic *h*uman *o*rphan, a name applied before the pathogenic potential of the viruses was appreciated. Subsequently, other members of the genus have been recognized and designated enteroviruses 68 through 71. A thorough molecular assessment, however, has changed this taxonomy.[290] The enteroviruses are now separated into four species, Enterovirus A through D, each of which contains numerous serotypes. This group taxonomically also contains the rhinoviruses. Enteroviruses can be isolated from 40% to 80% of patients with "aseptic" meningitis.[84]

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Many more instances of sporadic and epidemic aseptic meningitis are probably caused

by enteroviruses, based on the finding that the epidemic curve for all cases of aseptic meningitis parallels exactly the curve for cases from which enteroviruses have been isolated. This is becoming clearer, as molecular diagnostic methods are more commonly being employed. The peak incidence of disease is in the summer and early fall.

The enteroviruses, especially coxsackievirus B, also produce myocarditis, pericarditis, and epidemic pleurodynia. An association of these viruses with cardiomyopathy, a noninflammatory disorder of cardiac muscle, also exists.[456] Coxsackievirus A causes herpangina and hand-foot-and-mouth disease. The enteroviruses also produce febrile disease with rash, which is usually maculopapular but rarely can be vesicular. Enterovirus 70 causes epidemic hemorrhagic conjunctivitis and enterovirus 71 produces central nervous system (CNS) infection, including a polio-like paralysis. Most recently, Enterovirus D68 caused significant epidemics in the 2014 to 15 respiratory viral season. This virus produces respiratory infections that range from mild to severe and life-threatening (www.cdc.gov/non-polio-enterovirus/about/ev-d68.html).

Enteroviral infection is most common in infants and young children. Paradoxically, paralytic poliovirus disease is most prevalent in young adults. The spread of enteroviruses is facilitated by poor hygiene and sanitation, and by crowding. In populations among which virus spreads actively (e.g., low socioeconomic groups), infection occurs at an early age and paralytic disease is infrequent. Persons in high socioeconomic groups escape infection as infants and are more likely to experience paralysis after infection as a teenager. A similar phenomenon has been documented with varicella-zoster virus, Epstein–Barr virus (EBV), and rubella virus. Fortunately, paralytic disease was almost entirely caused by virulent poliovirus serotypes and has been virtually eliminated by immunization in most countries. Wild-type poliovirus has now been eradicated from much of the world; the World Health Organization (WHO) reports that the world has never been better positioned to eradicate polio.[65,497] Key areas wherein diseases transmission remains include Nigeria, Central Africa, and the Horn of Africa, wherein the outbreaks have occurred, as well as Pakistan and Afghanistan. Paralytic disease may be caused by traditional coxsackieviruses and enteroviruses, but only very rarely. The production of paralytic disease by enterovirus 71 has been noted.

The pathogenesis of most enterovirus infections includes primary infection of the respiratory or gastrointestinal tracts followed by viremia and infection of a distant target organ, such as skin, heart, or meninges. The inactivated (Salk) poliovirus vaccine elicits neutralizing serum antibodies, but not mucosal immunity. The vaccine works because the serum antibodies block the viremic spread to the meninges. The inability of the Salk vaccine to prevent mild gastrointestinal infection is not a serious problem for the individual patient, although it does allow continued circulation of the

virulent strain.

In recent years, most infections of paralytic poliomyelitis in developed countries have been caused by the attenuated (Sabin) vaccine strains. Consequently, inactivated (Salk) poliovirus vaccines are once again recommended for use in the United States. Up-to-date information concerning polio vaccination can be found on the CDC website (http://www.cdc.gov/vaccines/vpd-vac/polio/vacc-in-short.htm).

The symptoms of most enterovirus infections are produced by direct viral cytopathic effect on the target organ. Meningoencephalitis is accompanied by productive infection of the CNS by the actively replicating virus. In contrast, the clinical syndrome of myocarditis is caused by an immunologic reaction to enterovirus-infected cardiac tissue. By the time the patient becomes symptomatic, the virus has usually been eliminated from the heart.

It is clear from the pathogenesis of enteroviral infection that the culture or molecular detection of virus from patients with aseptic meningitis (i.e., productive infection) makes sense, but the similar testing of patients with myocarditis (immunopathologic disease) is unlikely to yield a viral isolate or positive result.

The human *parechovirus* is another member of the *Picornaviridae* that has been discovered and the pathogenicity of which continues to be described.[441] This virus has been associated with diarrhea, sepsis, hepatitis, and most importantly meningitis/encephalitis, which is similar to that caused by enteroviruses.[30,142] Molecular methods, which are necessary to detect this virus in a timely manner, are available from major reference laboratories.

Rhabdoviruses

Many other important RNA viruses have been described, some of which cause highly lethal infections. These viruses are primarily tropical and are therefore less likely to be encountered by clinical laboratories than are the picornaviruses and the myxoviruses. Among these are the rhabdoviruses, including the agent of rabies, which is important worldwide.

The diagnosis of rabies is made by detection of viral antigen or viral nucleic acids in clinical specimens from animals or humans. The culture of rabies virus is infrequently performed because of the simplicity, accuracy, and speed of direct immunofluorescence. This historical scourge is endemic in wild animals in the United States but is extremely uncommon in humans. Human exposure continues, however, from hunting dogs exposed to infected wild animals, from infected bats, and from domestic animals.[353] Although bats may be insectivorous and the contact may be only casual, these animals remain an important reservoir for the virus. In some instances, the only recognized contact was finding a bat in a bedroom in the absence of a recognized bite. In other instances, it may be impossible to identify any source for human rabies. Not uncommonly, the diagnosis is not suspected until autopsy.

Great anxiety is caused by possible exposures because of the lethality of the infection. The cost of preventing human disease can be enormous, as was shown in New Hampshire when a large number of individuals were potentially exposed to a rabid kitten in a pet store.[59] The estimated cost for epidemiologic investigation and prophylaxis was $1.5 million. Ongoing epizootic disease in raccoons in the eastern United States is of particular concern because of the frequency with which these aggressive animals invade human habitations. The molecular structure of rabies strains reflects their animal host. It is now possible to identify the likely source of a viral isolate from the nucleic acid composition of the virus.

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Unfortunately, rabies has been added to the list of transplant-transmitted infections.[438,439] In 2004, encephalitis, which was eventually determined to be caused by the rabies virus, occurred in three organ recipients (i.e., one liver and two kidney recipients). The donor, who died of complications from a subarachnoid hemorrhage, was not suspected of having an infectious disease, and rabies is not among the viruses for which routine screening is performed prior to organ donation. It was only during the subsequent investigation that friends of the donor recalled that he had been bitten by a bat. The subsequent investigation and an autopsy review revealed a fourth patient, also a recipient of the infected liver tissue, who also died of the disease. In addition to this unfortunate incident, there was also a case of inadvertent surgical transmission of rabies as a result of a corneal transplantation reported.[234]

Arenaviruses

The arenaviruses are a family of RNA viruses that share fascinating biologic characteristics.[42] Their natural host is a variety of rodents, which are persistently infected without an effective host response or symptomatic disease. Infectious virus is excreted in the urine, which is the source of human infection. Those at risk of infection are, therefore, individuals who come in contact with infected rodent excreta, either in their homes or at work in the fields. Person-to-person transmission occurs regularly only with Lassa virus, usually in healthcare workers who have been exposed to infected body fluids, especially at autopsy. Several of the hemorrhagic fever viruses appear to have been transmitted to sexual partners by convalescent men. The infected patient does not pose a significant risk to individuals with casual contact. Laboratory infections have been uncommon, but may occur (e.g., a laboratory worker was infected with Sabia virus).[56] The sources of infection have been aerosolization of culture fluids and probably rodent urine. Epidemics of lymphocytic choriomeningitis (LCM) virus have occurred among laboratory workers who worked with hamsters and

among individuals who owned hamsters as pets.[186] The major human pathogens, the geographic distribution, rodent reservoir, clinical disease, and date of discovery are summarized in Table 23-3.

LCM virus was discovered during investigation of the St. Louis encephalitis virus epidemic in 1933. It was soon recognized that the virus caused aseptic meningitis in humans. The frequency of infection is probably underestimated because clinicians do not suspect the infection and diagnostic tools are not readily available. Investigators found antibody to LCM virus in 5.1% of healthy women in Birmingham, Alabama.[445] Junin virus was the first recognized arenavirus cause of hemorrhagic fever, Argentinian hemorrhagic fever, in the pampas region. The mysteries of the epidemiology were unraveled a few years later by Karl Johnson et al. during investigation of an outbreak of Bolivian hemorrhagic fever in the Beni province, caused by Machupo virus.[249] Further notoriety for the viruses followed the recognition of severe hemorrhagic disease in several West African countries, caused by Lassa virus. Other additions to this group come from South America: Guanarito virus in Venezuela and Sabia virus in Brazil. The old viruses remain an important cause of disease, as demonstrated by the outbreak of Bolivian hemorrhagic fever in 1994 in the provinces of El Beni and Santa Cruz.[57]

The arenaviruses have a distinctive morphology (Table 23-1) that led to the recognition of the group.[345] They are roughly spherical, enveloped particles with prominent ribosome-like structures in the center. The name of the group derives from the Latin word *arenosus* (sandy), which was suggested by the internal particles. The original proposal, arenoviruses, was subsequently modified to arenaviruses to avoid confusion with adenoviruses.

Filoviruses

For many years, the only known member of this group was Marburg virus, which caused a mysterious, fatal illness among monkey handlers in a laboratory in Marburg, Germany in 1967.[405] The source of the virus was African green monkeys, but attempts to identify the agent in freshly caught monkeys

TABLE 23-3 Characteristics of Arenaviruses Pathogenic for Humans

Virus	Geographic Distribution	Rodent Host	Human Disease	Fatality Rate	Year of Discovery
Lymphocytic choromeningitis virus (LCM)	Europe, North and South America	*Mus domesticus; Mus musculus*	Aseptic meningitis	<1%	1939
Junin virus	Argentina	*Calomys musculinus*	Hemorrhagic fever	15%–30%	1958
Machupo virus	Bolivia	*Calomys callosus*	Hemorrhagic fever	25%	1963
Lassa virus	West Africa	*Mastomys* spp.	Hemorrhagic fever	15%	1969
Guanarito virus	Venezuela	*Zygodontomys brevicauda; Sigmodon alsoni*	Hemorrhagic fever	25%	1990
Sabia virus	Brazil	Unknown	Hemorrhagic fever	Too few cases to determine	1990
White water arroyo virus	Southwestern United States	*Neotoma albicula*	Hemorrhagic Fever	Several cases presumptively identified	1999
Lujo virus	Southern Africa	Unknown	Hemorrhagic Fever	80%	2008

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in Africa were unsuccessful. Over the next decade, there were few isolations of the virus, which remained a curiosity, albeit a lethal one. In 1977, a new virus, designated Ebola virus, was isolated from simultaneous outbreaks of disease in Zaire and Sudan. The outbreaks shared epidemiologic and clinical characteristics, and the viruses were found to be closely related, although antigenically distinct. The disease was characterized by high fever, shock, involvement of multiple organ systems, and diffuse hemorrhage. The mortality rate for Marburg virus was 25%; for Ebola–Sudan, 50%, and for Ebola–Zaire, 90%. As with Marburg, the source of the infection was unknown, but transmission was clearly associated with exposure to blood and body fluids of infected patients. The medical staffs of some of the small mission hospitals were decimated by the mysterious disease.

The two epidemics in remote areas of Sudan and Zaire caused alarm in public health and infectious disease circles, but did not make an impact on the general public. That situation changed dramatically when a large outbreak occurred in 1995 in Kikwit, Nigeria. More than 300 cases occurred over a short period, approximately one-third of which were in healthcare workers, and the fatality rate was approximately 80%. The epidemic was eventually controlled by introducing strict barrier precautions to prevent families and healthcare workers from having contact with secretions of infected patients. The source of this extensive epidemic was not clear.

A third strain of Ebola virus was identified in the Ivory Coast in 1994. Conjecture about the source in nature of Ebola virus had focused on monkeys because of the simian origins of Marburg virus, but no hard evidence had been found to support the hypothesis. A strain of Ebola virus most closely related to the Zaire type was isolated from the blood of a researcher, who probably acquired the infection while performing a necropsy on an infected chimpanzee. The highly lethal epidemic among chimpanzees was the first demonstration of natural infection in wild animals. Grooming and other

close contact was not a risk factor for acquiring infection by a chimpanzee, but consumption of meat was a risk.[150] The high mortality rate among the chimpanzees makes it unlikely that these primates are the natural reservoir of infection.

Additional evidence for a simian source of Ebola virus comes from an epidemic of lethal infection among cynomolgus monkeys in two animal holding facilities—the first in Reston, Virginia, and the second in Texas. In both instances, the animals had been imported from the Philippines; the virus was antigenically distinct from the strains isolated in Zaire, Sudan, and the Ivory Coast. Only five cases of human infection with the Reston strain of Ebola virus have been documented, all of which were asymptomatic. There was evidence of transmission of infection from animal to animal by aerosols, a terrifying epidemiologic characteristic that has fortunately been missing from the human epidemics. Data indicating epidemic disease among captive cynomolgus monkeys in the Philippines before export have also been reported. The fatality rate among infected monkeys was 82%, and infection was more likely to develop in monkeys held in a group cage than those held in individual cages.

The Ebola epidemic of 2014 to 2016 continues to rage in West Africa at the time of writing of this chapter, but fortunately due to extensive control measures and heroic efforts by many appears to be coming under control. As of June 09, 2015, 27,273 cases of Ebola virus disease were documented, with 11,173 deaths.[70] This equates to a death rate of 41%. This epidemic has been centered in the West African countries of Sierra Leone, Guinea, and Liberia. Transmission to countries outside the endemic areas has occurred largely via air travel; infected patients have been reported from the United States, the United Kingdom, Mali, and Italy.[70]

The filoviruses are RNA viruses with a characteristic ultrastructure (Table 23-1) and a distinctive filamentous morphology that gives the family its name. The two viruses, Marburg and Ebola, can be distinguished by ultrastructural morphology, and the types of Ebola virus are antigenically distinct.

These Biosafety Level 4 agents are unlikely to be encountered by clinical diagnostic laboratories. However, infection and specimen control measures have undergone review, given the importation of a few patients infected with or exposed to the Ebola virus in the current epidemic.[70] Interested readers can consult several excellent reviews of the biology of these fascinating viruses and the diseases they produce.[369,405]

Togaviruses and Flaviviruses

The *Togaviridae* and *Flaviviridae* now include many of the viruses that were previously known as arboviruses (arthropod-borne viruses), as well as some other, non–arthropod-borne viruses.[70] These groups are single-stranded RNA viruses with a lipid envelope.[411,413] The togaviruses were named for their lipid "toga" that surrounds

the virus. These biologically diverse agents have a complex ecology. They usually infect small mammals or birds and are transmitted from animal to animal by tick or mosquito vectors. Many of these viruses are also maintained within the arthropod lineage by transovarial passage from the gravid female to the larva. Humans are accidental hosts when they are inadvertently infected instead of the usual animal reservoirs.

Three members of these groups do not fit the traditional profile and will be discussed in their traditional context. These are the rubella virus, a togavirus, and hepatitis C and hepatitis E virus, which are flaviviruses.

The other division within the *Togaviridae*, the alphaviruses, contains the agents of eastern equine encephalitis, western equine encephalitis, and Venezuelan encephalitis, as well as the chikungunya virus, which is an emerging pathogen. The flaviviruses include the agents of St. Louis encephalitis, dengue (breakbone fever), yellow fever, and West Nile fever.

Small numbers of human infections with eastern equine encephalitis and western equine encephalitis infections occur each year. Human disease may be presaged by an increased incidence of illness in horses. Epidemics of St. Louis encephalitis occur approximately every 10 years in the southern part of the United States, especially in Texas, in the Gulf Coast states, and in Florida. The reservoir for St. Louis encephalitis virus is birds, and the mosquito vectors vary with the geographic location. Disease is most severe in the elderly, and infections tend to be concentrated in heavily wooded neighborhoods with poor screening of homes.

Dengue fever is prevalent in the Caribbean and in Southeast Asia. Imported cases are seen in the United States with

increasing frequency, and indigenous transmission of the virus in the continental United States has been reported occasionally, most commonly in Texas. A most unusual route of entry into the United States was uncovered when infected mosquitoes were found in stagnant water that had accumulated in tires imported from Southeast Asia. Uncomplicated dengue infection is an undifferentiated febrile disease, sometimes accompanied by a rash and arthralgias or arthritis. The differential diagnosis includes other viral infections, such as rubella. There are four distinct serotypes of dengue virus. As hypothesized by Halstead, serial infections with different serotypes trigger immunopathologic mechanisms that produce the dengue hemorrhagic fever syndrome.[199,308] Formerly limited to Southeast Asia, the lethal hemorrhagic form of the disease has occurred in the Caribbean with increasing frequency and is currently on the rise.

The West Nile virus produces a spectrum of neurologic diseases, including

encephalitis, meningitis, and a poliomyelitis-like syndrome.[417] Until 1999, it had produced localized infections in Africa, the Middle East, and Eastern Europe. In that year, it was discovered in New York City and has subsequently spread throughout the United States. The first patient with encephalitis was thought to have the disease caused by St. Louis encephalitis virus, which is closely related immunologically to the West Nile virus. These two viruses share other characteristics, including having birds as a frequent host (i.e., a characteristic that may be related to the rapid spread of the virus). The importation and spread of the West Nile virus demonstrates how quickly an infectious agent can go from exotic trivia to everyday relevance.[371]

The Zika virus is a flavivirus that is thought to be responsible for the wide-spread epidemic of microcephaly in Brazil and other tropical locales.

Chikungunya virus may accurately be described as an emerging pathogen, which is an often overused term. This is justified by the emergence of a genetic variant which increases the replicative capacity of the virus within the mosquito, as well as other characteristics. Chikungunya virus, as noted above, is a togavirus. Disease is transmitted by the *Aedes aegypti* and *Aedes albopictus* mosquitoes, both of which also transmit the dengue virus. Chikungunya causes high fevers, similar to that produced in dengue, but is more likely to cause severe polyarthralgias. The incubation is usually 3 to 7 days, but may be as short as 1 day or as long as 12 days. The acute infection usually resolves within 10 days; occasionally, complications such as nephritis, meningoencephalitis, and Guillain–Barré syndrome may occur. There is no specific treatment, but supportive care may be needed. Establishing a definitive diagnosis is important as symptoms overlap with dengue, and nonsteroidal anti-inflammatory agents, which may be of use in patients with chikungunya, should not be used in patients with dengue. This and additional information concerning the chikungunya virus is available through the CDC (www.cdc.gov/chikungunya/).

Bunyaviruses

The *Bunyaviridae* are a large and diverse group of viruses that are transmitted to humans by arthropod vectors, principally ticks and mosquitoes, as well as from the excreta of infected rodents. There are four medically important genera: (1) Bunyavirus, including the California encephalitis virus group; (2) Nairovirus, including Congo-Crimean hemorrhagic fever virus; (3) Phlebovirus, including Rift Valley fever virus and sandfly fever virus; and (4) Hantavirus, including the agents of Korean hemorrhagic fever, nephropathia epidemica, and hantavirus pulmonary syndrome.[70,352]

California Encephalitis Viruses. One of the most important of the bunyavirus groups in the United States is the California encephalitis group of viruses. Despite the name "California," these viruses produce disease most commonly in the midwestern and

north central states. The California encephalitis virus group commonly produces aseptic meningitis, a disease that occurs in the summer and is clinically indistinguishable from enteroviral disease.[47,352] Infections by the California encephalitis virus itself are uncommon; the LaCrosse virus is the most common human pathogen in the group. Most patients recover uneventfully from their infection.

Hantaviruses. The hantaviruses have expanded from exotic infectious agents to endemic threats in the United States.[413] The uniform characteristics of the group include asymptomatic, persistent infection of a rodent host, transmission to humans through infected rodent urine and excreta, and infection of the vascular endothelium. The status of the viruses, their rodent hosts, and associated clinical diseases are summarized in Table 23-4.

Severe hemorrhagic fever was recognized among American soldiers during the Korean War. Shock and renal failure, which were prominent symptoms, provided the descriptive name hemorrhagic fever with renal symptoms (HFRS). Fever, thrombocytopenia, and renal failure comprise the classical clinical picture of this disease. In the clinical setting, the return of platelets presages restoration of normal renal function. Shock and multiorgan failure, simulating bacterial sepsis, characterize the most severe cases. Less common manifestations include retroperitoneal hemorrhage and pulmonary edema. Some patients experience undifferentiated febrile illness with normal renal function. A clinical syndrome of severe hepatic disease has been described in Greece, where the prevalent strains resemble the virus associated with infections in Korea.

Subsequent to the documentation of the disease in Korea, it was recognized that this severe clinical syndrome occurred in the eastern Soviet Union and Manchuria. The etiologic agent was not isolated until 1982, when Hantaan virus, which was named after a Korean river where epidemic disease had occurred, was isolated. This virus produces asymptomatic, persistent infection in its rodent host, *Apodemus agrarius*. Subsequently, a second, related agent, Seoul virus, which produces less severe infection, was isolated. The rodent hosts of Seoul virus are urban rats, *Rattus norvegicus* and *Rattus rattus*. Seoul virus has been found in laboratory rats and has produced infection in laboratory workers. There are no documented cases of Hantaan virus infection in the United States, but cases of Seoul virus-induced HFRS have been documented serologically in Baltimore. The rat-associated hantaviruses in the United States have been associated with hypertensive renal disease, but not with other chronic renal diseases.[171] Although hypertensive renal disease is not usually associated with infectious agents, the lesson of *Helicobacter pylori* and gastritis should caution us against dismissing the association out of hand.

TABLE 23-4 Selected Hantaviruses: Hosts and Clinical Diseases

Virus	Geographic Distribution	Rodent Host	Clinical Disease
Hantaan	Far East	*Apodemus agrarius* (Eastern form)	Hemorrhagic fever with renal syndrome (HFRS)
Seoul	Worldwide	*Rattus rattus; Rattus norvegicus* (urban rats)	Mild HFRS
Dobrava	Balkans	*Apodemus flavicollis*	HFRS
Puumala	Scandinavia; Europe	*Clethrionomys glariolus* (vole)	Nephropathia endemica
Sin nombre	Southwestern United States	*Peromyscus maniculatus* (deer mouse)	Hantavirus pulmonary syndrome (HPS)
Bayou	Louisiana	*Oligoryzomys palustris* (rice rat)	HPS
Black Creek Canal	Florida	*Sigmodon hispidus* (cotton rat)	Unknown
New York	Northeastern United States	*Peromyscus leucopus* (white-footed mouse)	HPS
Monongahela	Eastern United States	*Peromyscus maniculatus nubiterrae* (forest form of deer mouse)	HPS
Andes	South and Central America	*Oligoryzomys longicaudatus*	HPS

For information on other viruses, consult reference 441.

A milder version of this infection, known as nephropathia epidemica, is produced by Puumala virus and occurs in Scandinavia (Table 23-4).[413] The virus produces asymptomatic infections in voles; in humans, it is an acute febrile disease with lower back pain and polyuria but with mild or no hemorrhagic manifestations and without shock. Patients recover without sequelae. The symptoms are frequently nonspecific, and the clinical diagnosis was correct in only 28% of cases in one Finnish study.

In 1993, the spotlight focused on hantaviruses when an outbreak of severe respiratory disease occurred in the Four Corners area of the southwestern United States.[370,413] The patients had a prodromal syndrome of fever, myalgia, cough or dyspnea, gastrointestinal symptoms, and headache, which progressed rapidly to irreversible pulmonary edema. With amazing rapidity, the causative agent was recognized as a new member of the hantavirus genus, named Sin Nombre virus (initially named Four Corners virus). The rodent host of Sin Nombre virus is the deer mouse, *Peromyscus maniculatus*. It is hypothesized that heavy rains increased food sources during that or the prior year, causing a great increase in the number of rodents, increasing the frequency of contact between humans and mice, and altering the epidemiology from sporadic, endemic disease to epidemic infection. There is no evidence of human-to-human transmission of hantavirus pulmonary syndrome. Retrospective diagnosis of cases of infection with Sin Nombre or a related virus dating back as far as 1978 has been made by immunoperoxidase staining of archived paraffin-embedded tissue.[505] All of the 12 infections identified retrospectively had occurred west of the Mississippi River, most in the far West of the United States.

Subsequent to the discovery of Sin Nombre virus, other related hantaviruses have been identified in the United States, including Louisiana, Florida, and the eastern United States (Table 23-4). The frequency of hantavirus infection outside of the

endemic areas is unknown.

The lung is the target organ pathologically as well as clinically.[370] Edema and fibrin deposition with the deposition of hyaline membranes in the airspaces are accompanied by an interstitial mononuclear exudate. Large immunoblastic cells infiltrate many organs. The hematologic findings include left-shifted neutrophilic leukocytosis, thrombocytopenia, circulating immunoblasts, and in severe cases, hemoconcentration. Viral antigen deposition is concentrated in endothelial cells, particularly in the pulmonary vasculature, and virus particles may be seen in those cells.[504] Hanta viral antigens are also found in follicular dendritic cells, macrophages, and lymphocytes.

Human Gastroenteritis Viruses

Viral gastroenteritis is second only to viral respiratory disease as a cause of virus-associated morbidity in the United States. The incidence of viral gastroenteritis has been estimated at approximately 11% per year.[114] Mortality is rare. Much has been learned about the etiologic agents of disease, but still only approximately 50% of infectious gastroenteritis can be assigned an etiology. Most of these agents have been identified initially by immunoelectron microscopy, but more commonly now by advanced molecular techniques, including NGS. In immunoelectron microscopy, immune serum is mixed with a virus suspension or a stool specimen. Clumping of the submicroscopic viral particles can be observed when negatively stained preparations are examined with the electron microscope. Most of these agents cannot be cultivated easily. Therefore, details of their composition are either not known or are being described; for convenience, they will be discussed with the most common pathogen, the rotaviruses.

The agents of viral gastroenteritis can be divided into four groups. The classification is fluid because of emerging evidence about some of the agents:

Rotaviruses
Caliciviruses
    Norwalk virus (i.e., Norovirus)
    Sapporo virus
Astroviruses
Enteric adenoviruses

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The frequency of these viruses is summarized in Table 23-5.[195] The data come from a study of fecal specimens submitted to a health maintenance organization in Georgia. The detection of the viruses in this study was entirely by molecular methods

and was performed at the CDC.

Many aspects of these viral gastrointestinal infections are similar. They cause acute vomiting or diarrhea, which is usually mild, self-limited, and noninflammatory. Bloody diarrhea does not occur. Microscopic analysis of biopsies from human volunteers who have ingested these infectious agents show blunting of intestinal villi and mild inflammatory changes in the submucosa, changes that are indicative of small-bowel malabsorption. Damaged cells at the tips of the villi are replaced by cells from the bases of the crypts over 5 to 10 days following the injury. Diarrhea that lasts more than 7 to 10 days is unlikely to be viral. Treatment is by replacement of lost fluid and electrolytes, usually caused by vomiting. Unfortunately immunity appears to be only partial.[114]

Of note is the absence of conventional intestinal viruses from the list of pathogens. There is little evidence that enteroviruses or respiratory adenoviruses cause gastrointestinal disease.

Rotaviruses. Rotaviruses are the most important human pathogens in the *Reoviridae*. The reoviruses themselves are not associated with human disease, but they have been useful in the study of molecular mechanisms of viral pathogenesis. "Reo" stands for *r*espiratory-*e*nteric *o*rphan, from the source of virus isolates and the lack of association with clinical disease. Rotavirus got its name from the wheel-like appearance of the virion in electron micrographs (Table 23-1).

The rotaviruses contain both human and animal pathogens, but animal rotaviruses do not infect humans. Human rotaviruses have been classified serologically, with most pathogenic human strains in the United States and Europe falling into group A. Human rotavirus is a common cause of gastroenteritis in infants and young children.[258] The clinical presentation is varied, but vomiting and dehydration are prominent features in comparison to gastroenteritis produced by other viruses. The combination of vomiting and a seasonal occurrence in the winter months has led investigators to name the condition "winter vomiting disease." The frequency and specificity of respiratory symptoms in rotavirus infection are controversial. It is possible that the prevalence of vomiting in rotavirus infection reflects the age of the patient rather than a property of the virus, because Norwalk virus produces significantly more vomiting in infants than in adults. Uhnoo and Svensson studied the comparative features of group A, subgroups 1 and 2 in Swedish infants.[459] Patients with subgroup 1 strains developed fever up to 39°C significantly more often than those with subgroup 2, but infants who had subgroup 2 infections were sicker, hospitalized more frequently, and more likely to have respiratory symptoms. The frequency of diarrhea and vomiting in the two groups was similar.

TABLE 23-5 Estimated Burden (Percentage) of Acute Gastroenteritis Caused by

Viruses in the Community

Any Virus	26.80%
Norovirus	15.90%
Astrovirus	4.40%
Rotavirus	2.1%[a]
Sapovirus	2.20%
Adenovirus	2.40%
Bacteria	4.10%
Parasites	1.00%
Unidentified	68.30%

[a] Incidence varies by age. The numbers given are for all age groups. In the referenced study, rotavirus was more common than norovirus in the 5- to 15-year age group.
Adapted from reference 195.

Chronic rotavirus infection may occur in immunosuppressed children. In adults, rotavirus infection is usually asymptomatic. These viruses are notorious causes of nosocomial infection, including epidemics, in children.

Caliciviruses. These viruses were originally identified by electron microscopy and characterized descriptively as small, round-structured viruses.[201] There are two major groups of viruses within the *Caliciviridae*: the Norovirus for which the species is the Norwalk virus and the Sapovirus for which the species is the Sapporo virus.

Norwalk Virus. This agent, originally termed the Norwalk agent of gastroenteritis, was recognized as a 27-nm particle in stool specimens from an outbreak of community gastroenteritis in Norwalk, Ohio. Subsequently, similar RNA viruses were isolated from other epidemics. Molecular analysis has revealed that there are two main clusters.[9] These agents are also referred to as noroviruses.[261]

Norwalk viruses have frequently produced epidemics of disease and also cause sporadic infection.[201] These viruses produce infection and active disease in persons of all ages, in contrast to most of the other viral agents, which produce symptomatic disease predominantly in infants and young children. They are the predominant cause of infectious gastroenteritis in adults. The Norwalk-like agents have been associated

with contaminated food and water and are one of the possibilities to consider when a patient has recently ingested shellfish and develops gastroenteritis. Epidemic disease has been described in venues that vary from football games to cruise ships or nursing homes.

Sapporo Virus. These agents resemble the Norwalk viruses morphologically, but differ immunologically and genetically. This virus, like the Norwalk virus, is named for the location of its discovery, Sapporo, Japan, where in 1977 it was discovered as the cause of gastroenteritis in infants. It may cause outbreaks of infantile gastroenteritis; although some food-borne transmission has occurred, this is not the usual route of spread.[81]

Astroviruses. The astroviruses also contain RNA and have been difficult to cultivate in vitro, although a few strains have been successfully grown.[320] They derive their name from their star-shaped ultrastructural appearance. Astroviruses,

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first identified in 1975 as a cause of diarrhea in children, are thought by some to be second only to rotavirus as a cause of gastroenteritis in children.[105] These viruses are also an important cause of infection in a wide variety of animals.[105] They have produced epidemic disease in young children, on pediatric wards, in day care centers, and in nursing homes. Concurrent infection with astroviruses and caliciviruses has been described.

Enteric Adenoviruses. A causative association of the classical adenovirus serotypes with diarrheal disease could not be established, but "noncultivable" serotypes were demonstrated by immunoelectron microscopy. Adenovirus types 40 and 41, which do not grow well in the usual tissue culture cells, are intestinal pathogens.[245] These enteric adenoviruses are the second most common cause of viral gastroenteritis in infants and have been associated with intussusception.[191] They produce sporadic infection, although epidemics have been described. Most patients are less than 2 years of age.

Coronaviruses

The classical coronaviruses are causes of the common cold.[225] Most require use of organ culture for reliable isolation and are unlikely to be encountered in the clinical laboratory.

A dramatic addition to the genus was recognized in 2003 when a novel coronavirus was identified as the cause of a mysterious new respiratory illness in China. This virus

is thought to have been possibly contracted initially from Himalayan masked palm civet cats, but it has also been isolated from other animals, such as Chinese ferret badgers, among others.[190] This infection was called SARS. In contrast to previously recognized coronavirus infections, SARS is characterized by lower respiratory infection that often required admission to an intensive care unit and may be fatal.[33,82]

The incubation period for SARS ranges from 2 to 10 days, with the average between 4 and 6 days.[425] There is no convincing evidence of transmission prior to the development of symptoms or after 10 days following the resolution of fever. There may be an influenza-like prodromal illness that includes fever, myalgias, and headache. As radiologic evidence of pneumonia develops and the patient's condition worsens, additional symptoms, such as chills, dyspnea, and cough, develop. Both leucopenia and lymphopenia are seen. Patients appear most infectious at about day 10 of the illness. Interestingly, watery diarrhea has also been described as a prominent symptom of patients with SARS.[495] Transmission is predominantly via respiratory droplets, but the fomites and the aerosolization of other body fluids or stool can transmit the disease. If the patient survives, then the disease will begin to resolve around the third week of illness. Between 6% and 20% of surviving patients had some type of pulmonary impairment. The virus, curiously, only infrequently infected children, and those infected had mild disease.

Another troubling characteristic of this infection is the propensity for infection of hospital personnel, particularly those who have close patient contact.[219] More that 40% of the infections in Canada and Singapore were nosocomially acquired, which demonstrates the need for strict adherence to respiratory infection prevention precautions for patients with respiratory tract infections.[498] With amazing rapidity and with the collaboration of scientists worldwide using electron microscopy and advanced molecular techniques, the cause of the infection was identified as a novel coronavirus. The significance of an hMPV that was isolated from some patients is unclear, but it likely represented only a coinfecting pathogen.[75] It is of interest that a conventional coronavirus, OC243, recently produced an outbreak of respiratory infections that included pneumonia.

There were 8,096 documented cases of SARS from 26 different countries by the cessation of the epidemic.[425] There was a case fatality rate of 10% during this first pandemic of the 21st century.

Although the severe acute respiratory syndrome coronavirus (SARS-CoV) has retreated into its zoologic niche, another coronavirus, the Middle East respiratory syndrome coronavirus (MERS-CoV), has emerged and is currently (i.e., at the time of this writing) causing infections that are of concern throughout the world.[387] This novel coronavirus, which is zoologically associated with camels, was first described in Saudi Arabia in 2012.[394] It is endemic to the countries around the Arabian Peninsula. A total

of 1,026 infections have been documented as of February 2015, with a 36.7% death rate (i.e., 376 deaths). This infection, like SARS, has spread to distant parts of the world secondary to the ease of air travel. The MERS-CoV has spread to 19 countries and there is an ongoing epidemic in South Korea at the time of this writing.[394]

Currently, there has been a slight male preponderance and the median age of infected individuals is 50 years. These numbers may change, if more asymptomatic or subclinical infections are discovered. There is a high likelihood of transmission of the virus to healthcare providers, unless respiratory precautions are strictly followed. Eighteen percent of infections have been in healthcare workers to date.[496]

Fever, cough, and dyspnea characterize MERS, but patients may also have chills, myalgia, nausea, vomiting, abdominal pain, and diarrhea. As with SARS, patients may develop leukopenia and lymphopenia.[394] Both thrombocytopenia and thrombocytosis have been described, as well as elevated liver enzymes.[394]

Coltiviruses

This group includes the virus of Colorado tick fever, an undifferentiated febrile illness that is usually mild and self-limited, but may on occasion be severe or even fatal.[401] It is transmitted by the bite of ticks. Previously, this virus was included in the Orbivirus group, which is composed of animal pathogens. The orbiviruses are members of the family *Reoviridae*, along with rotaviruses.

Retroviruses

The retroviruses are RNA viruses; they were so named because they contain an enzyme that transcribes RNA into DNA, a reversal of the normal sequence in which DNA is transcribed into RNA (i.e., retro). For many years, these viruses were known to cause tumors in animals. In the 1970s, two viruses that caused T-cell lymphocytic lymphomas in humans were recognized and classified as human T-cell lymphoma virus (HTLV-I and HTLV-II). This discovery

was extremely important biologically, but the tumors are uncommon.[175]

The retrovirus group assumed sudden prominence and increased notoriety in 1983 when a third human retrovirus was identified as the cause of the acquired immunodeficiency syndrome (AIDS).[165] AIDS had been recognized clinically several years earlier when opportunistic infections were detected in young men who were homosexual, bisexual, or drug addicts, but did not have conventional risk factors for *Pneumocystis* pneumonia or candidiasis.

Similar viruses were isolated in several laboratories and named variously HTLV-III, lymphadenopathy-associated virus (LAV), and AIDS-related virus (ARV). An expert panel settled on the name human immunodeficiency virus (HIV). Although HIV was first isolated in 1983, retrospective analysis of frozen serum and tissue suggested that a similar virus had infected a sexually active teenage boy in St. Louis in 1968.[163] Subsequently, a serologically related virus was isolated and named HIV-2. The distribution of HIV-1 is worldwide, whereas HIV-2 is found predominantly in West Africa, where it also causes AIDS. Patients with HIV-2 infection have been reported in the United States, but most patients have had some connection with West Africa. There are immunologic cross-reactions between HIV-1 and HIV-2, but advanced assays have been designed for resolution. These specific immunoassays must be performed both to distinguish between the two infections and also to detect the type-specific antigens with maximum sensitivity. To complicate matters further, at least one patient had been identified who was infected with both viruses. HIV is a member of the lentivirus (*lenti* = slow) subfamily of the *Retroviridae*. Other viruses in this group produce chronic infections with long periods of clinical latency. Some animal retroviruses also produce immunodeficiency, such as the simian immunodeficiency virus and feline leukemia virus. Infection across species has not been demonstrated.

TABLE 23-6 Key Facts: Global HIV Epidemic

- HIV continues to be a major global public health issue.
- HIV has claimed more than 39 million lives to date.
- Approximately 1.5 million people died from HIV-related causes in 2013.
- There were approximately 35 million people living with HIV at the end of 2013.
- There were approximately 2.1 million new HIV infections in 2013.
- Sub-Saharan Africa remains the most affected region, with 24.7 million people living with HIV in 2013.
- Almost 70% of new infections occur in sub-Saharan Africa.
- Approximately 12.9 million people living with HIV were receiving antiretroviral therapy (ART) globally in 2013.
  - Of these, 11.7 million people were receiving ART in low- and middle-income countries.
  - The 11.7 million people on ART represent 36% of the 32.6 million people living with HIV in low- and middle-income countries.
- The treatment of HIV in children is still poor in low- and middle-income countries.
  - Less than 1 in 4 children living with HIV had access to ART in 2013, compared to over 1 in 3 adults.

Modified from http://www.who.int/mediacentre/factsheets/fs360/en/

TABLE 23-7 Major Antigens of Human Immunodeficiency Virus, Type 1

Gene	Gene Products
Group-specific antigen/core *(GAG)*	p(protein)18, p24, p55
Polymerase *(POL)*	p31, p51, p66
Envelope *(ENV)*	Gp (glycoprotein) 41, gp120, gp160

Adapted from reference 57.

In contrast to the other lentiviruses HIV is genetically extremely heterogeneous, to the extent that virtually each isolate is unique (termed a quasi-species). There are, however, genetically related groups, which are in turn divided into clusters or clades. Group M (major) comprises 95% of global isolates and is divided into at least eight clades (A, B, C, D, F, G, H, and J). All clades are represented in Africa; in the United States and Europe, Clade B is the dominant subtype. (It should be noted that genetic clades do not map to distinct viral serotypes.)

Group O (Other) strains, which are found predominantly in West Africa, share less than 50% homology with Group M strains. Group N (non-M or O) viruses are found in Cameroon.

To complicate matters even further, all retroviruses have a propensity for recombination when multiple strains are juxtaposed. It was assumed that patients infected with HIV virus would be resistant to infection with a second strain. Unfortunately, the absence of complete immunity and the frequency of multiple exposures to virus have resulted in infection with several strains, a situation in which recombination can occur.[27]

There is no question that HIV is the most devastating plague of recent epidemics. It has changed our society (i.e., a resource-rich country) and continues to wreak havoc in resource-poor countries, despite international efforts. The advent of effective combination antiretroviral chemotherapy (i.e., highly active antiretroviral therapy) has transformed this infection in many ways into a chronic disease in resource-rich countries that can afford the drugs. Nevertheless, the epidemic continues to spread and in resource-poor countries has enhanced the spread of other infectious diseases, such as tuberculosis. Key facts regarding the global HIV epidemic are summarized in Table 23-6.

The discussion below is a brief attempt to capture some of the most salient features of this multifaceted virus and the diseases it produces. It is a tribute to modern science that an infectious agent was described only 3 years after the first reports of the clinical

syndrome. Without the modern armamentarium of molecular biology and virology, we would still be defenseless against this scourge.

The HIV-1 virion is icosahedral and contains 72 external spikes.[152] It is considerably more complex than HTLV-I and HTLV-II, consonant with its more complicated natural history. The gene products can be divided into three groups. The viral proteins that represent the most important diagnostic antigens are summarized in Table 23-7.

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The gp120 envelope protein facilitates entry of HIV-1 virus into the cell.[152] This protein has a remarkable affinity for a complex of receptors on the surface of cells throughout the body. The primary component of the complex, the CD4 receptor, is found on T-helper lymphocytes, macrophages, and Langerhans cells in the skin. The second part of the receptor complex is a chemokine receptor. Viral strains that interact with the CXCR4 receptor are most likely to infect T lymphocytes in vitro; strains that interact with the CCR5 receptor (R5 virus) are associated with macrophage cell lines in vitro. Some viruses which interact with both chemokine co-receptors can infect both lymphocytes and macrophages.

The enhanced susceptibility to HIV infection in patients with genital ulcers or other sexually transmitted diseases may result from the accessibility of CD4+ macrophages and lymphocytes in the inflamed bases of the ulcers and/or other alterations in mucosal immunity.[418]

The most important cells targeted by the virus, however, are the CD4+ T lymphocytes.[103] These "helper" lymphocytes assist in the organization of many cell-mediated inflammatory responses, such as the production of granulomas. Without them, the body is at increased risk for many opportunistic infections. In addition to the T-cell abnormality, defective response of B cells to T-cell-independent antigens has also been documented. Macrophages are very important, because they provide a place for virus to be sequestered even after infected T cells have been destroyed and/or most virus have been killed by antiretroviral therapy.

The initial encounter with HIV-1 virus produces a transient febrile disease, which may be accompanied by lymphadenopathy, pharyngitis, or a diffuse rash.[90] During the earliest phases of acute illness, high levels of circulating virus are present in the absence of specific antibody. It is at this phase that the diagnosis is established through the detection of the p24 antigen or of viral nucleic acids. Antibody develops within several weeks to several months in the natural course of the infection. The detection of these antibodies is the mainstay of diagnosis, as most patients do not present in the very early stages of the disease (i.e., prior to the production of antibodies).

The ensuing subclinical phase of the disease is accompanied by antibody production, circulating p24 antigen, immune complexes, and low levels of circulating

virus. Testing blood products for virus, using molecular amplification technology, has substantially reduced the risk of transfusing infected blood products. During the chronic viremia, repetitive mutations in the viral genome thwart the attempts of the host immune system to eliminate the infection. The onset of clinical illness is associated with an increase in the quantity of virus, both within peripheral-blood mononuclear cells and in plasma.

A wide spectrum of clinical conditions may result after HIV-1 infection. The CDC classification of HIV disease and associated conditions are summarized in Tables 23-8 and 23-9.[56] Certain diseases (Category C in Table 23-9) are highly associated and are termed AIDS-defining conditions.

The infectious complications of HIV infection include a variety of viral, fungal, mycobacterial, bacterial, and parasitic infections. The practice of infectious disease and clinical microbiology has been irrevocably altered by HIV. Numerous previously uncommon or exotic infections have become commonplace in centers that have large numbers of HIV-infected patients. Old rules concerning the seeking of a single pathogen as the cause of a disease must be disregarded when the patient is as severely immunosuppressed as are patients with AIDS or patients with leukemia and/or stem cell transplants. A wide variety of infections are possible, when the host inflammatory response is significantly diminished or absent. What would be "shotgun" microbiology in a normal patient becomes good practice in this group of patients. Integration of clinical, epidemiologic, and laboratory data is needed to decide which laboratory resources must be utilized for an individual patient.

The most common opportunistic infections that afflict patients infected with HIV are among the AIDS-defining conditions that are listed in Table 23-9. Some of the infections are characteristic of a particular phase of the disease. For instance, oral candidiasis is often a presenting feature of the disease. *Pneumocystis jirovecii* pneumonia first occurs when lymphocyte counts are relatively high early in the disease process. *Mycobacterium avium* complex infections tend to occur late in the disease, when the CD4+ lymphocyte count is low. Although infections with cell-associated pathogens (i.e., facultative intracellular organisms) are most closely associated with defects in cellular immunity, bacterial infections for which humoral immunity is an important host defense are also more common in HIV-infected patients. The search for the cause of an opportunistic infection should not

TABLE 23-8 CDC Classification System for HIV-Infected Adults and Adolescents

CD4 Cell Count Categories	*Clinical Categories*		
	A **Asymptomatic, Acute HIV, or PGL**	**B** **Symptomatic Conditions, not A or C**	**C** **AIDS-Indicator Conditions**
≥500 cell/μL	A1	B1	C1
200–499 cells/μL	A2	B2	C2
<20 cells/μL	A3	B3	C3

PGL, persistent generalized lymphadenopathy.
See Table 23-9 for categorical descriptions.
From reference 56.

TABLE 23-9 Symptomatic Conditions (Category B) and AIDS-Indicator Conditions (Category C) Included in the CDC Classification System for HIV-Infected Adults and Adolescents

Category B (Symptomatic Conditions)

These are symptomatic conditions occurring in an HIV-infected person that are (1) attributed to the HIV infection or indicate a defect in cell-mediated immunity, or (2) are considered to have a clinical course or management that is complicated by the HIV infection.

Examples include:

- Bacillary angiomatosis
- Candidiasis, oropharyngeal (thrush)
- Candidiasis, vulvovaginal; persistent, frequent, or poorly responsive to therapy
- Cervical dysplasia (moderate or severe)/carcinoma in situ
- Constitutional symptoms, such as fever (>38.5°C) or diarrhea lasting >1 month
- Hairy leukoplakia, oral
- Herpes zoster (shingles), involving at least two distinct episodes or more than one dermatome
- Idiopathic thrombocytopenic purpura
- Peripheral neuropathy

Category C (AIDS-Indicator Conditions)

Bacterial pneumonia, recurrent (two or more episodes in 1 year)
Candidiasis of bronchi, trachea, or lungs
Candidiasis, esophageal
Cervical cancer, invasive
Coccidioidomycosis, disseminated or extrapulmonary
Cryptococcosis, extrapulmonary
Cryptosporidiosis, chronic intestinal (>1 month duration)
Cytomegalovirus disease (except liver, spleen, lymph nodes)
Encephalopathy, HIV-related
Herpes simplex: chronic ulcers (>1 month duration); or bronchitis, pneumonitis, or esophagitis
Histoplasmosis, disseminated or extrapulmonary
Cystoisosporiasis, chronic intestinal (>1 month duration)

Kaposi's sarcoma
Lymphoma, Burkitt's, immunoblastic or primary central nervous system
Mycobacterium avium complex or *Mycobacterium kansasii*, disseminated or extrapulmonary
Mycobacterium tuberculosis, pulmonary or extrapulmonary
Mycobacterium, other species or unidentified species, disseminated or extrapulmonary
Pneumocystis jirovecii pneumonia
Progressive multifocal leukoencephalopathy
Salmonella septicemia, recurrent
Toxoplasmosis of brain
Wasting syndrome due to HIV

From reference 56.

stop when the first agent is identified because many patients with AIDS have coinfections.

Not all HIV-related diseases are caused by opportunistic infections. HIV itself is cytopathic and produces clinical disease such as encephalitis and AIDS-related dementia. Collapse of the cellular immune system and its surveillance mechanisms leads to neoplastic complications, some of which may be virus-induced. Lymphomas are frequently encountered.[330] A previously unusual neoplasm, Kaposi's sarcoma, is well described in patients with AIDS, often as an early manifestation of the disease. Other manifestations of the disease may have an immunopathologic mechanism, or the mechanisms are unknown. Some infections that might have been predicted to afflict AIDS patients, such as legionellosis and aspergillosis, are relatively uncommon. The cellular immune system undoubtedly contributes to the control of invasive aspergillosis and legionellosis, but other mechanisms, not destroyed by HIV-1, must also be important.

As therapy for HIV infection and for infectious complications of the immunodeficiency has improved, the pattern of infections encountered has changed. An analysis of autopsies from Los Angeles during the period 1982 to 1993 documented a decrease in the number of fatal infections caused by *Pneumocystis jirovecii*, bacterial sepsis, cytomegalovirus (CMV), *Mycobacterium avium* complex, and *Toxoplasma gondii* from 1989 to 1993 compared with 1982 to 1988.[268] This trend has continued in the United States. Over time, institution of prophylaxis for AIDS-related infections and of more effective therapy has significantly diminished the number of deaths in resource-rich countries due to AIDS.[297] Although AIDS-defining conditions remain important, other chronic diseases and complications of antiretroviral therapy have become just as important.

Guidelines for children are included in the standard, whereas recommendations for screening of pregnant women have been published separately[68] (www.cdc.gov/hiv/risk/gender/pregnantwomen/facts/). HIV-1 is excreted in saliva,

milk, semen, and other body fluids. Breast-feeding is not advisable for women with HIV and those taking antiretroviral therapy according to the CDC (www.cdc.gov/breastfeeding/disease).

The most important groups at risk for HIV infection are those who have shared contaminated needles (drug addicts), those who have had sexual intercourse with an infected individual, and neonates of an infected mother. In resource-rich countries, the transmission of the virus through the therapeutic administration of blood products has been largely mitigated by improved donor screening techniques.

At the outset of the epidemic, most of the sexually transmitted infections in the United States and Europe were between homosexual or bisexual men (i.e., presently described as men who have sex with men [MSM]), but heterosexual transmission was soon documented. Heterosexual transmission of HIV-1 virus has been the dominant form of transmission in Africa from the beginning of the epidemic. The risk of transmission of HIV infection from mother to infant and the severity of disease in the infant are directly related to the severity of infection in the mother at the time of delivery.

Therapy for HIV infection has represented a constant battle between the virus and the pharmaceutical industry, resulting in alternating exhilaration and despair among patients and healthcare workers. By 1996, therapeutic efforts had concentrated on combination chemotherapy, using an inhibitor of RNA-dependent DNA polymerase (i.e., reverse transcriptase) along with an inhibitor of the viral protease.[125] The inhibitor of reverse transcriptase can be either nucleoside/nucleotide (NRTI) or nonnucleoside (NNRTI) in nature. Treating patients with these powerful drugs in combination, known as highly active antiretroviral therapy (HAART), has become a subspecialty of infectious diseases. The virus, of course, has also been resilient, developing resistance to the major classes of antiviral drugs,[87] particularly if therapy is incomplete or inadequate. In such instances, HIV genotyping (i.e., sequencing of the nucleic acid targets that encode for the proteins targeted by the antiretroviral agent) is an important means to determine the predominant and resistant quasi-species. One of the useful applications of NGS is the ability to determine the genotype of both the major and the minor quasi-species present. This affords the determination of which combination of antiretroviral drugs will be active against the predominant quasi-species, as well as those drug-resistance encoding mutations that are present in the mixture of minor quasi-species.

Although no therapy has yet proved capable of eradicating HIV infection, the sentence of death that a diagnosis of HIV infection once connoted has been reversed by HAART in societies that can afford the expensive drugs. When combined with better education of high-risk groups, HAART resulted in a stable or decreasing incidence of new infections for the first time. The ongoing challenge is to find resources to offer the new therapies to countries with meager financial reserves, and much has been undertaken in this regard by philanthropic individuals, societies, and governments. However, without continued vigilance, the epidemic curve may begin to turn upward secondary to a variety of causes, including continuing appearance of drug resistance, difficulty in reaching users of intravenous drugs, and a resurgence in unprotected sexual activity once it is (incorrectly) perceived that the death sentence has been lifted.[66]

The risk of transmitting HIV in blood transfusions has been dramatically reduced by screening all units of blood for antibody and asking members of high-risk groups not to donate blood. The result has been a substantial decrease in the incidence of transfusion-related HIV infection as the sensitivity of the tests used for screening blood products before transfusion has increased. The introduction of highly sensitive nucleic acid amplification assays for screening all units of blood administered in the United States has probably reduced the risk of transfusion to an absolute minimum.

Occupational exposure to infectious agents is a fact of life for medical personnel. Blood-borne pathogens are a major part of the problem, because of the frequency of the pathogens and of exposure to blood. Sharp objects (e.g., needles and surgical instruments) are the most frequent vehicles for transmission. Viral agents represent the most serious occupational risks. Recommendations for management, preferably by prevention, of occupational exposures have been formulated by the CDC.[60–63] A summary of the general recommendations is provided in Box 23-4.

BOX 23-4

Management of Occupational Blood Exposures[a]

Provide immediate care to the exposure site

- Wash wounds and skin with soap and water
- Flush mucous membranes with water

Determine risk associated with exposure by

- Type of fluid (e.g., blood, visibly bloody fluid, other potentially infectious fluid or tissue, or concentrated virus), and

Type of exposure (i.e., percutaneous injury, mucous membrane or non–intact skin exposure, or bites resulting in blood exposure)

Evaluate exposure source

Assess the risk of infection using available information

Test known sources for HBsAg, anti-HCV, and HIV antibody (consider using rapid testing)

For unknown sources, assess risk of exposure to HBV, HCV, or HIV infection

Do not test discarded needles or syringes for virus contamination

Evaluate the exposed person

Assess immune status for HBV infection (i.e., by history of hepatitis B vaccination and vaccine response)

Give postexposure prophylaxis for exposures posing risk of infection, according to current recommendations

Perform follow-up testing and provide counseling

[a]Hepatitis B (HBV), hepatitis C (HCV), and human immunodeficiency virus (HIV).
Adapted from references 67, 73, 74, and 76.

TABLE 23-10 Laboratory Support for Diagnosing and Treating HIV-Infected Patients

Parameter	Primary Approach	Secondary/Confirmatory Approach
Initial diagnosis	Fourth-generation HIV 1/2 Immunoassay	Multi-Spot assay with or without nucleic acid amplification
Prognosis and initiating therapy	Quantitative plasma viral concentration (viral load) and baseline HIV genotyping	
Initiating therapy and prophylaxis for opportunistic infections	Quantitation of CD4 lymphocytes	

Monitoring therapy	Quantitative plasma viral concentration (viral load)	
Evaluating clinical or virologic relapse	HIV genotyping	Rarely, HIV phenotyping

Treatment of patients with HIV infection is complicated and requires support from many areas of the laboratory. In some ways, the expansion of diagnostic possibilities has simplified the approach, as summarized in Table 23-10.

Herpesviruses

Viruses of the human herpesvirus group are among the most frequent isolates in general laboratories. The group consists of the following:

Herpes simplex virus (HSV), type 1
Herpes simplex virus, type 2
Human cytomegalovirus (CMV)
Epstein–Barr virus (EBV)
Varicella-zoster virus (VZV)
Human herpesvirus-6 (HHV-6)
Human herpesvirus-7 (HHV-7)
Human herpesvirus-8 (HHV-8)

Some members of the herpesvirus group can integrate their DNA with that of the host cell. These viruses produce a latent infection in lymphoid cells or ganglia of the CNS and subsequently reactivate to cause recurrent disease. In general, the primary infections are more severe than are recurrences. The primary infections produced by several viruses of this group are more severe when they occur during adolescence or adulthood, rather than childhood (e.g., VZV). The viruses circulate more freely and earlier in life among low socioeconomic groups and not until adolescence in more affluent society. The infections in high socioeconomic groups, therefore, tend to be more severe.

Herpes Simplex Virus. HSV causes a wide variety of infections.[486] Two serotypes of the virus infect humans. Shared antigens between types 1 and 2 complicate the early serologic differentiation of antigens and antibodies, but reliable reagents are now commercially available. In healthy persons, infections of the oral cavity and genital tract predominate. Type 1 infections are most common in the upper body, whereas type 2 infections produce genital lesions; but there are frequent exceptions to the rule. For example, as many as one-third of isolates from the genital tract represent type 1

strains.

The typing of herpes isolates from genital sites is not necessary for treatment, but does provide valuable prognostic information because genital infections caused by type 1 HSV are less likely to recur than are those caused by type 2 virus.[277] In a group of 457 women who had a primary genital infection (defined by absence of antibody to type 2 virus and a positive culture of genital lesions), 89% had a recurrence after a median of 391 days.[23] There were at least 6 recurrences during the first year in 38% of patients, and 20% of individuals had more than 10 recurrences. A sequential study of patients with recurrent infection demonstrated that the patient was asymptomatic and lacked visible lesions during one-third of the period during ongoing viral shedding.[469] Genital infections with either viral type are more likely to recur than oral infections with the same type.[277] In addition, the mechanism of infection may be different for the two serotypes. Exposure to type 1 virus begins in childhood, with antibody having developed in 25% to 50% of college students. Acquisition of antibody to type 2 virus does not begin until the teens and continues throughout the period of sexual activity. Isolation of type 2 virus from a genital site implies sexual transmission, whereas the route of transmission for type 1 virus may be by autoinoculation with oral secretions.

Herpesvirus is passed from person to person by infected secretions or lesions. Infection is usually sporadic, but an epidemic of cutaneous infection at a wrestling camp (*Herpes gladiatorum*) has been described.[21]

The most serious HSV infections are encephalitis, which most commonly affects the temporal lobe and the orbital aspect of the frontal lobe in adults, and disseminated infection in newborn infants, which may be acquired either during or after birth. HSV causes sporadic encephalitis. In the neonatal period, the infecting strains are usually type 2, reflecting their origin in the maternal genital tract. Type 1 strains are overwhelmingly predominant in older children and adults. The diagnosis is made by brain biopsy and culture. Culture of the cerebrospinal fluid (CSF) is too insensitive to be diagnostically useful. HSV is infrequently isolated from CSF. In the national collaborative study, an isolate was recovered from CSF in only 2 of 45 (4%) biopsy-proven cases herpes encephalitis.[371] Serologic diagnosis is too insensitive, nonspecific, and slow to be of use in therapeutic decisions. The widespread availability of HSV PCR on CSF specimen has significantly reduced the need for brain biopsy, which is now rarely performed for this reason. However, in the presence of a negative CSF PCR for HSV, a brain biopsy may still be necessary to establish this diagnosis or to detect other treatable causes of the disease.[487] The advent of sophisticated imaging techniques has also revolutionized the diagnostic approach.

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Magnetic resonance imaging (MRI) is more sensitive than computed tomography. Herpesvirus encephalitis occurs very rarely, if ever, in the presence of a normal MRI study. Lesions are not limited, however, to unilateral temporal lobe lesions. The abnormalities by MRI may be multifocal or diffuse.[412] At the beginning of the illness, the only abnormalities may be in the electroencephalogram.[399]

Herpesvirus remains latent in spinal ganglia after the initial infection. Type 1 virus is most commonly recovered from thoracic ganglia, whereas type 2 is more commonly recovered from sacral ganglia. Herpetic aseptic meningitis does occur uncommonly, usually caused by type 2 virus. Viral meningitis is characteristically associated with a normal level of glucose in the CSF, in contrast to the markedly diminished levels of glucose in bacterial meningitis. The cerebrospinal glucose level in herpes meningitis, however, may be very low, and the cell count may be in the thousands.[337] Early in the course of any viral meningitis, neutrophils may predominate, which confuses the clinical picture and suggests the possibility of a bacterial process. HSV, usually type 2, also causes recurrent lymphocytic meningitis (Mollaret's meningitis); the culture is almost uniformly negative, but the viral genome can be demonstrated in the CSF by PCR.[451]

Neonatal herpes infection is estimated to occur in 1:2,000 to 1:5,000 deliveries. It is almost always symptomatic and is frequently fatal. In a collaborative study, the mortality rate was 50% to 60% in infants with disseminated infection who were treated with acyclovir or vidarabine.[485] In contrast, no fatalities occurred among infants with disease limited to the nose, eyes, or mouth. The diagnosis is suggested by the development of vesicular skin lesions, but these may be absent in 20% of patients. Poor prognostic factors include disseminated disease, comatose state at admission to the hospital, disseminated intravascular coagulation, or prematurity.

The high morbidity and mortality rates of infant disease have prompted great efforts to diagnose maternal infection. Most cases of neonatal HSV infection are contracted during vaginal delivery and can be prevented by cesarean section. Complex protocols for screening pregnant women have been developed but unfortunately have not worked well. Asymptomatic maternal infection at the time of delivery occurs regularly, although the risk for infants appears to be low if the infection is recrudescent and maternal antibody to type 2 virus is present. Many women do not recognize their primary genital infection. In one study, specific antibody to type 2 HSV was detected in 439 of 1,355 pregnant women who had no clinical history of genital herpes (32%).[154] Asymptomatic shedding of virus in late pregnancy and at delivery was detected in 5 of 1,160 cultures (0.43%). During the pregnancy, 43 of the women who had antibody to type 2 virus recognized their first symptomatic genital infection.

Efforts to prevent neonatal infection have shifted to assessment of antibody status in pregnant women and careful examination for active clinical disease at the time of delivery. The commercial availability of reliable assays for type-specific antibody

facilitates identification of women who are at risk for primary infection.[338]

Persons with compromised defense mechanisms are predisposed to herpes simplex esophagitis, tracheobronchitis, or pneumonia and disseminated infection, including hepatitis. Herpes simplex infections may be necrotizing and may suggest a bacterial etiology if the characteristic inclusions are not recognized or viral cultures are not done.[350] Immunosuppression is not a prerequisite for lower respiratory tract infection or esophagitis. The risk factors for lower respiratory tract infection include intubation, suggesting that the pathogenesis may be similar to that of bacterial pneumonia—aspiration of contaminated oropharyngeal secretions.[384] Patients with extensive burns are at risk for infection both of the denuded skin and of the lower respiratory tract.[146]

Infection of the eye includes conjunctivitis, which may be accompanied by fever, photophobia, and regional lymphadenopathy. Herpetic keratitis, which has a branching or dendritic appearance, is the second most common cause of corneal blindness (after trauma) in the United States.

HSV is one of the viral causes of pharyngitis and tonsillitis, usually as a primary infection. The mucosa may be ulcerated, and lesions may be limited to the posterior pharynx.[174] Necrotizing tonsillitis may mimic bacterial peritonsillar abscess. Hemorrhagic cystitis has been reported as a part of disseminated infection,[108] and herpetic proctitis may occur in homosexual men.[179]

Cytomegalovirus. CMV is one of a family of related viruses that are specific for a particular species.[331] Mouse CMV, for example, does not infect humans, and vice versa. CMV was first isolated from mice by Margaret Smith, who subsequently isolated the human virus from the salivary gland tissues of an infected infant. She recognized the species specificity of the virus, although the reviewers of her manuscript, which was initially rejected, did not.[479]Isolation of the human virus was reported concurrently by Smith, Weller, and Rowe. With the generosity of a gentleman, who also happens to be a Nobel Prize winner, Dr. Weller gives Dr. Smith the credit for the primacy of her discovery. CMV is an opportunistic pathogen that may produce persistent, even lifelong, infections.[361] It was recognized many decades ago as a human pathogen by the peculiar cytopathology it produces (Image Plate 23-1D). Initially, investigators considered CMV as a protozoan invader of human tissue.[246]

This virus is associated with leukocytes and may be transmitted with a blood transfusion or transplanted organ. This is the reason for the movement toward leukoreduced blood products. When detected by monoclonal antibodies or genetic probes, CMV is concentrated in the neutrophil fraction of the buffy coat, rather than in the mononuclear fraction. This fact formed the basis for the CMV antigenemia assays that were widely used prior to being supplanted by viral load assays. Multiple

genetic variants of CMV have been found in a single infected patient.[85] Virus is also excreted in saliva and in semen. Venereal transmission has been strongly suggested by epidemiologically related clusters of infections. CMV may be passed from mother to offspring through the placenta, in cervical secretions during delivery, or in breast milk.

The variety of infectious diseases produced by this pathogen is great, and includes congenital infection, neonatal infection, heterophil-negative infectious mononucleosis, hepatitis, pneumonia in immunosuppressed patients, and disseminated infection in immunosuppressed patients.[361] In the past, it was difficult to determine whether an individual patient had CMV-induced disease or was only persistently infected, because replicating virus may be found

in normally sterile organs and body fluids in the absence of clinical disease. The use of quantitative PCR for CMV has largely changed this wherein higher viral loads correlate with histopathologically-proven disease.

It is relatively easy to document infection by culturing the virus or demonstrating the presence of specific antibody. In contrast to documentation of CMV infection, proof that a particular disease process is caused by CMV often requires biopsy and histologic examination, which is the most definitive. Even then, the assessment is not always easy. For example, occasionally, cells with CMV inclusions may be seen in the base of a colonic ulcer. It is not clear if this is ulceration secondary to CMV or local reactivation of virus in an ulcer bed that is due to another cause. In such instances, correlation with the plasma CMV viral load is essential. Similarly, the simple recovery of CMV from bronchoalveolar lavage correlates poorly with CMV pneumonia,[402] but the predictive value is increased if a high CMV viral load is demonstrated in the bronchoalveolar lavage fluid.

Detection of serum antibody is the most sensitive method for determining if the patient has ever been infected by CMV. The presence of serum immunoglobulin M (IgM) antibody is useful for determining recent infection. This is useful for patients with a heterophil-negative mononucleosis-like syndrome, which may be a primary CMV infection, and for screening for primary infections during pregnancy. A latent virus or one that is clinically silent may reactivate to produce disease. An individual patient may experience CMV disease after reactivation of a latent infection or after a primary exposure to the virus. The distinction is prognostically important, because primary infections are clinically more severe and more likely to cause symptomatic disease in a neonate.

Transplant donors and recipients should be serologically screened, so that seronegative patients do not receive blood or an organ from a seropositive donor.

As mentioned above, the primary CMV infection of previously healthy persons

usually manifests as a self-limited mononucleosis syndrome. As in EBV infection, there is evidence for CMV mononucleosis that the symptoms are produced by cytotoxic lymphocytes, which attempt to eliminate the CMV-infected cells.

The spectrum of disease is considerably broader in patients who are immunosuppressed, depending on the extent and type of immunocompromise. In patients who are moderately immunocompromised, such as kidney-transplant recipients, CMV pneumonia is a common manifestation. When immunosuppression is more extreme, extensive, overwhelming infection may result. Recipients of heart, liver, or bone marrow transplants or patients with AIDS may experience hepatitis, or previously unheard of complications such as perforation through an infected intestinal wall.

Most congenitally and neonatally acquired infections are asymptomatic, but subtle symptoms may develop in some infants who appear normal at birth. At the other extreme is disseminated infection with multiorgan disease and severe congenital abnormalities, such as microcephaly. The frequency of intrauterine transmission of CMV during a primary maternal infection has been estimated at 20% to 40%, and the risk of an adverse outcome is increased during the first half of the gestation.

Epstein–Barr Virus. EBV is the primary cause of infectious mononucleosis.[396] This versatile virus produces disease that ranges from acute self-limited infection to malignant neoplasms. The mononucleosis syndrome consists of fever, malaise, exudative pharyngitis, lymphadenopathy, and atypical lymphocytes circulating in the peripheral blood. Splenomegaly is common, and rupture of the spleen is a serious complication, but fortunately not frequently encountered. Acute hepatitis may also be a part of the syndrome.

The virus was originally recognized during studies of Burkitt's lymphoma in Africa and only later associated with mononucleosis.[396] The virus gains entry to the body by infecting the pharyngeal epithelium. Its primary cellular target, however, is the circulating B lymphocyte, which it infects and then immortalizes. The activated B lymphoblast, along with its passenger EBV, matures into a long-lived memory lymphocyte in the germinal center of the lymph node.[455] The result of this interaction is a polyclonal stimulation of the humoral immune system, which produces an array of antibodies to many antigens. At the same time, the cellular immune system is triggered to fight the EBV infection. It is the activated T lymphocytes that are seen in the peripheral-blood smear as atypical lymphocytes. The circle is completed when virus is excreted through the oral mucosa into the saliva. Young children usually have an asymptomatic or minimally symptomatic infection, and the diagnostic heterophil antibody test is often negative.

If integration of the EBV genome into certain types of cells occurs, a neoplasm may result instead of an acute infection. The unregulated growth of EBV in patients with

HIV infection, who have lost the regulatory control of cellular immunity, may lead to increased EBV-related tumors. Most of these tumors are of B-lymphocyte lineage, such as Burkitt's lymphoma and primary lymphoma of the brain, but nasopharyngeal carcinoma is also EBV-associated. A subset of gastric and colonic carcinomas has also been ascribed to infection with EBV. A variety of other processes include lymphocytic interstitial pneumonia and hemophagocytic syndrome. Establishing association of a latent or persistent virus such as EBV is not enough to prove a causal relationship, which can come only from repetitive association and careful molecular analysis, which often employs in situ hybridization studies. The relationship between EBV and lymphoma appears to be particularly complex.[455]

In the mid-1980s, it was suggested that a poorly defined syndrome of chronic fatigue might represent chronic EBV infection. Chronic EBV infection does exist and may be troublesome, but the nonspecific syndrome of chronic fatigue does not appear to be EBV-related.[102]

The cells that best support viral replication with shedding of infectious particles are epithelial cells of the oropharynx. Saliva from acutely ill or chronically infected patients contains viable virus, which can be demonstrated by co-cultivation with normal peripheral-blood lymphocytes. Alternatively, peripheral-blood lymphocytes of infected individuals can be cultured in the presence of an agent that depletes T lymphocytes, such as cyclosporine. The diagnosis of neoplastic manifestations must be by molecular demonstration that the virus has been integrated into the genome of the malignant cells. Cells from EBV-induced tumors can often be cultured as immortalized cell lines in vitro.

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HIV-associated lymphomas are less commonly encountered in resource-rich countries where HAART is readily available. In these situations, the populations at increased risk for EBV driven lymphomas are transplant recipients. The disease, adequately named "posttransplant lymphoproliferative disorder," ranges from poorly characterized lymphoid proliferations to definitive lymphomas. Early detection becomes imperative, as these are EBV driven and reversible or treatable if detected prior to the development of an outright lymphoma. Therefore, quantitative EBV viral load testing has become established as a means to monitor transplant recipients for increasing EBV levels and impending or developing posttransplant lymphoproliferative disease (PTLD).

Varicella-Zoster Virus. VZV causes chickenpox as a primary infection.[11] The virus may remain latent in the sensory ganglia of the spinal cord for many years before reactivating and producing disease for a second time. The lesions of reactivated

disease resemble chickenpox but are limited to the dermatome that is innervated by the infected nerve. The reactivation disease is known as shingles. The dermatome of most concern is the ophthalmic branch of the trigeminal nerve because destructive corneal infection may occur as part of the reactivation disease. Reactivation of the virus in nerves of the head and neck is also associated with subsequent VZV meningitis/meningoencephalitis. Before reactivation was understood, the virus of chickenpox was called varicella virus and that of shingles was called herpes zoster, which explains the curious nomenclature.

VZV produces considerable discomfort in healthy persons and may produce pneumonia, but in immunosuppressed individuals the disease can become a life-threatening disseminated infection. Pregnant women are at increased risk for severe varicella, and neonates who are born within 4 days of their mother's infection are at great risk because maternal antibodies have not yet developed. The risk of embryopathy in the fetus after maternal VZV infection in the first 20 weeks of pregnancy is approximately 2%.

Conventional wisdom states that immunity to varicella is lifelong and that all subsequent disease is recrudescent. Reinfection has been suggested, however, by careful serologic study of immune persons in a house that had an individual with chickenpox.[12] Housemates who had preexisting antibody remained well but developed increased antibody titers, indicating subclinical infection.

There is great concern in hospitals for the safety of immunosuppressed patients who come in contact with an infected person. Exposure may occur unwittingly because patients with VZV are infectious just before the onset of the characteristic rash. Measures to reduce risk include use of immune healthcare personnel for immunosuppressed patients and limitation of visitors. Prompt administration of zoster immune globulin after inadvertent exposure has a protective effect.

Human Herpesviruses-6 and -7. The human herpesvirus-6 virus (HHV-6) was first isolated from patients with lymphoproliferative disorders and named human B-lymphotropic virus.[367] The virus has the morphologic structure of a herpesvirus but is genetically unrelated to the five previously identified herpesviruses. Thus, it was named human herpesvirus-6. There are two currently recognized subtypes, designated A and B; subtype B is predominant. This virus shares with EBV the ability to grow in human lymphocytes. In contrast to EBV, which infects lymphocytes in a latent stage and does not actively grow in the cells, HHV-6 appears to produce only productive infection in human lymphocytes (i.e., mature infectious viral particles are produced in the infected cells).

In 1990, a new herpesvirus was isolated from $CD4^+$ T lymphocytes of a healthy individual, using techniques that cause activation of T cells. The virus, which was named human herpesvirus-7 (HHV-7), is distinct from but closely related to HHV-6.

The two viruses do not produce cross-immunity.

HHV-6 and HHV-7 produce a common childhood disease called exanthem subitum (also known as roseola infantum, roseola subitum, Duke disease, or fourth disease). Exanthem subitum occurs in children between 6 months and 3 years of age. Almost all children over the age of 13 months have antibody to HHV-6. Antibody to HHV-7 appears at a slightly older age.

Exanthem subitum begins with an abrupt fever, which is followed by a rash as the fever subsides on the third or fourth day. Febrile convulsions occur in approximately 8% of acutely infected patients. This infection is very unlikely in an afebrile child. The illness usually resolves without sequelae, but serious disease may result on rare occasions. In immunosuppressed adults HHV-6 has produced serious disease after transplantation of bone marrow and solid organs and in patients who are infected with HIV. These viruses have been suggested as etiologic agents in chronic fatigue syndrome, multiple sclerosis and neoplastic disease, but substantial supportive data has not emerged. The frequent reactivation of herpesviruses during the course of other diseases complicates definitive establishment of an etiologic role.

Human Herpesvirus-8. The next addition to the group of lymphotropic herpesviruses is the Kaposi's-sarcoma-associated herpesvirus, which has been named human herpesvirus-8 (HHV-8). As implied by the name, the virus was first detected in tissue from Kaposi's sarcoma in patients with HIV infection, using molecular techniques.[333] Subsequent investigation demonstrated that the viral gene sequences were present in several clinical types of Kaposi's sarcoma: that occurring in patients with AIDS, classical Kaposi's sarcoma unrelated to HIV infection, and Kaposi's sarcoma in homosexual men who are not infected with HIV. The sequences have been identified in AIDS-related body cavity–based lymphomas, which also contained genetic sequences from EBV. This type of HHV-8-associated lymphoma has been classified as "primary effusion lymphoma." Finally, HHV-8 is associated with a subset of patients with multicentric Castleman's disease, an atypical lymphoid hyperplasia, which may progress to lymphoma. The sequences are also found in nonneoplastic tissue of HIV-infected patients with Kaposi's sarcoma.

B Virus. In the 1930s, a virus of the herpes group was isolated from a patient in whom a fatal neurologic disease developed after being bitten by a monkey. About the same time, Sabin isolated a virus from another patient in whom

transverse myelitis developed after a monkey bite. He named the isolate B virus after the afflicted patient. It has also been called herpesvirus simiae, which is something of a misnomer because a variety of herpesviruses are found in monkeys. The actual

taxonomic name is *cercopithecine herpesvirus 1*, but "B virus" is more commonly used. In one review of herpes B virus infections 16 of the 25 infections were fatal.[488]

The B virus is unusual because it causes infections across species lines. It is indigenous to Old World monkeys, such as rhesus and cynomolgus, in which it establishes latency. Localized human infections have been reported, but most are severe and systemic. An animal bite is the usual means of transmission, but infection after a needlestick injury has been reported. A vesicular eruption often develops around the bite and is followed by regional lymphadenopathy and fever. Encephalitis, transverse myelitis or visceral necrosis accompanies systemic spread.

Of concern was the first documented episode of human-to-human transmission, which occurred as part of a cluster of infections at a research facility. Another concern in the past was the discovery that monkey kidney cells that had been commercially supplied for diagnostic laboratories were contaminated with B virus. Fortunately, no human infections resulted, but the cells were recalled.[482] The ability of B virus to replicate in monkey kidney monolayers has been known for years. In fact, the stress of processing the kidneys may stimulate activation of latent B virus. Cytopathic effect of the virus consists of cell enlargement and fusion, which may spread through the monolayer. Cowdry type A inclusions identical to those of HSV or VZV will be seen in stained cell preparations. Subculture to other monkey kidney cells or to rabbit kidney cells, which are used for isolation of HSV, will transmit the virus.

Adenoviruses

Adenoviruses were originally recognized when spontaneous degeneration occurred in adenoidal tissue cultures. They were called adenoid degeneration (AD), adenoid–pharyngeal–conjunctival (APC) or acute respiratory disease (ARD) agents.[423] These viruses can be present in the pharynx and in the feces without causing discernible disease. Designation of an infectious agent as the etiologic agent must be done carefully so that one is not misled by a fortuitous association. For instance, at one time adenoviruses were considered causes of the pertussis syndrome because they were isolated in the absence of the common bacterial cause, *Bordetella pertussis*. The role of adenovirus in a pertussis syndrome is controversial. It has been suggested that being fastidious, *B. pertussis* may not have been recovered in many cases of whooping cough while the adenoviruses represented incidental isolates or coinfecting pathogens.[233]

Adenovirus disease may be sporadic or may occur in epidemics. These viruses produce approximately 5% of the ARD in children less than 5 years of age and may be responsible for 10% of childhood pneumonia. They produce an exudative pharyngitis that can mimic group A β-hemolytic streptococcal disease. Adenoviruses may cause otitis, pharyngitis and gastroenteritis. Certain serotypes are associated with acute

conjunctivitis, with or without pharyngitis. Adenovirus infections of the gastrointestinal tract were discussed earlier with the other viral causes of gastroenteritis. The major adenoviral infections and their associated serotypes are summarized in Table 23-11.

Poxviruses

The poxviruses are a diverse group.[155] Most are animal pathogens, but some can also infect humans. The most well-known of the poxvirus group was the variola virus, the etiologic agent of smallpox and the sole member of the group that infects humans only. It has now passed from endangered species status, hopefully, into extinction.[132] After the last natural infection was recorded, stocks of viruses were restricted to the United States and Soviet Union; with the dissolution of Soviet Union, however, the possibility of dispersal of viral stocks beyond Moscow has been a concern. The genome of variola and five other pox viruses has been sequenced.[341]

The recognition that variola poses a major threat as an agent of bioterrorism has stimulated renewed interest. The population of the world is now largely susceptible or only partially immune, and physicians are unfamiliar with the

TABLE 23-11 Adenovirus Infections

Syndrome	Age Group	Serotypes
Acute febrile pharyngitis	Young children	1, 2, 3, 5, 6, 7
Pharyngoconjunctival fever	Young children and adolescents	3, 7, 14
Acute respiratory disease	Military recruits	3, 4, 7, 14, 21
Pneumonia	Young children Military recruits	1, 2, 3, 7 4, 7
Epidemic keratoconjunctivitis	All	8, 11, 19, 37
Acute hemorrhagic cystitis	Young children	11, 21
Gastroenteritis	Young children	39, 40
Meningoencephalitis	Children and immunocompromised patients	1, 2, 5
Hepatitis	Children and immunocompromised patients	1, 2, 5

Adapted from reference 245.

clinical presentation and diagnosis of smallpox. The records of historical outbreaks, including an epidemic in Boston early in the 20th century, have been reexamined to refresh faded memories.[3,36] Immunization with vaccinia virus, one of the milestone achievements of modern medicine, was abandoned in the 1970s, because of well-characterized side effects of the vaccine.[38] The balance between cost and benefit of vaccination has clearly been affected by the altered circumstances,[156,157] but the proper course is disputed.[39,302] Residual antibody has been demonstrated in previously immunized individuals, but the efficacy of these antibodies is unclear.[160]

Orthopoxviruses of animals, such as cowpox and monkeypox viruses, can occasionally infect humans. These infections resemble smallpox clinically. A new dimension to the risk of monkeypox was added by the recognition of human infection that was acquired from pet prairie dogs.[389] The pets may have been infected by imported African rodents that are known to be susceptible to monkeypox virus. The differential diagnosis of all poxvirus infections includes VZV.[326] Laboratory support is, therefore, essential whenever the possibility of variola is raised.

The origin of vaccinia virus is unclear. It has many characteristics of a distinct species, but may have been derived from one of several candidate viruses (including cowpox virus) by mutation or recombination.

Members of two other genera, *Parapoxvirus* and *Yatapoxvirus*, are animal pathogens, but can infect humans. Tanapox virus causes human infection in parts of Africa, but it has produced disease in travelers after they have returned from an endemic area.[111]

Molluscum contagiosum virus, the sole member of the Genus *Molluscipoxvirus*, causes a distinctive cutaneous lesion; like variola it infects only humans.[132]

The parapoxviruses include agents causing diseases in sheep (orf or contagious pustular dermatitis virus) and in cows (milker's node or pseudocowpox virus). They may also produce localized infections in humans who are occupationally exposed to the viruses. The poxviruses are unlikely to be isolated in the clinical laboratory.

Papillomaviruses

The human papilloma viruses (HPVs) cause common warts, sexually transmitted venereal warts, and tumors of the respiratory, intestinal, and genital tracts. The viral nature of warts was known early in the 20th century, when they were transferred with cell-free filtrates, and Shope defined the rabbit papillomavirus as the cause of

papillomas in 1933.[298] The extensive and thorough study of HPV was impeded until molecular techniques sufficient for the task were developed in the 1970s.

More than 60 genetic types of HPV have been described. There are strong associations between certain genotypes and clinical lesions (Table 23-12). The common wart is the most common lesion, but most attention has been focused on the genital infections, including the association with neoplasia. Sexual partners of patients who are infected with HPV are also frequently infected with the same genotype.

The causative nature of HPV with cervical neoplasia has been constructed from many studies. Although HPV provides the major risk, additional factors appear to be necessary for the development of cancer. Rather than a dramatic shift from normal to neoplastic epithelium in the cervix, there is a continuous spectrum, recognized as increasingly severe grades of cervical intraepithelial neoplasia. The final stage in the process is the development of overt malignancy, invasive carcinoma, which may be either glandular or more commonly squamous. Likewise, there is a continuum in the expression of HPV genes and antigens. In the normal epithelium the cells of the basal layer progressively differentiate to form nonkeratinized squamous epithelium at the surface. As the cervical epithelial changes progress from mildly dysplastic to severely dysplastic, the following changes occur: (1) the cells become less differentiated throughout the epithelium, (2) the chromosomal content of the nuclei becomes increasingly abnormal, (3) the expression of HPV gene products (antigens) becomes less intense, and (4) the expression of HPV genes is increasingly difficult to demonstrate. A characteristic of the HPV-infected cell is koilocytosis, a perinuclear clearing in the squamous epithelium accompanied by nuclear atypia, although this appearance is not present in all infected cells. The appearance of koilocytic atypia in exfoliated cervical cells is shown in Figure 23-5.

The cellular biology of HPV supports a primary role in the development of neoplasia.[316] The viral types most closely associated with cervical cancer, particularly HPV-16 and 18, are capable of transforming cells in culture so that they lose their normal growth control mechanisms. The DNA from these types integrates into host DNA. The viral integration may cause chromosomal rearrangement and lead to abnormal cellular differentiation in vitro. Zur Hausen has put forth the hypothesis that cervical neoplasia is the result of failure of a primal cellular surveillance mechanism that was designed to control persisting viral genomes.[510]

Papillomavirus DNA has been demonstrated in normal cervical epithelium adjacent to genital warts and cancer. Failure to remove all the infected tissue may lead to recurrences. HPV DNA can also be demonstrated in 5% to 10% of women with normal cervical smears or biopsies. The infection may be transient; the association of even high-risk genotypes with cervical neoplasia is most dramatic when HPV DNA is persistently present.

National consensus recommendations for treatment of patients at risk for cervical

neoplasia, which evolve continually, include morphologic parameters (cervical exfoliative cytology) and viral determinants (detection of HPV). The molecular assessment for the presence of HPV is now part of the standard for screening women for cervical cancer and the precursors thereof. The current approach to classifying endocervical cytology is the Bethesda System.[434] Table 23-13 summarizes the cervical cancer screening guidelines of the American College of Obstetricians and Gynecologists (ACOG).

The role of the laboratory in the management of endocervical disease has many aspects.[101] Recommendations for management of HPV disease continue to evolve. The ACOG now includes the use of an HPV test in conjunction with a Pap test every 5 years, as an alternative to a Pap test alone every 3 years. The molecular methods designed to detect the presence of HPV are more sensitive than cytology, whereas cytology, and in some instances biopsy, provides the

TABLE 23-12 Association of Selected Human Papillomaviruses (HPV) with Clinical Disease

Viral Type	Clinical Disease	Neoplastic Potential
HPV-1	Deep plantar wart	Not described
HPV-2	Common wart Mosaic wart Lip lesions	Not described
HPV-3	Flat wart	Not described
HPV-4	Common wart	Not described
HPV-5	Plaques in epidermodysplasia verruciformis	Squamous carcinoma may develop in sun-exposed areas
HPV-6	Genital: exophytic condyloma; flat condyloma; giant condyloma Respiratory papillomas Conjunctival papillomas	Weak association, especially in vulva
HPV-7	Common butcher's warts	Not described
HPV-11	See HPV-6	See HPV-6
	Focal epithelial hyperplasia of oral	

HPV-13	cavity	Not described
HPV-16	Anogenital: Bowenoid papulosis; flat condyloma	High association in cervix Moderate association in vagina, vulva, anus, and penis Also found in respiratory tract, including larynx, tonsils, and oral cavity associated with head and neck squamous cell carcinoma.
HPV-18	Genital: flat condylomas	High association in cervix; infrequently found in precursor lesions. Associated with head and neck squamous cell carcinoma.
HPV-31	Genital: flat condyloma	High association with cervical cancer
HPV-32	Focal epithelial hyperplasia in oral cavity	Not described
HPV-33, -35, -39, -51, -52, -56, -58, -59, -68	Genital	Moderate association with cervical cancer
HPV-45	Genital	High association with cervical cancer

Adapted from reference 316.

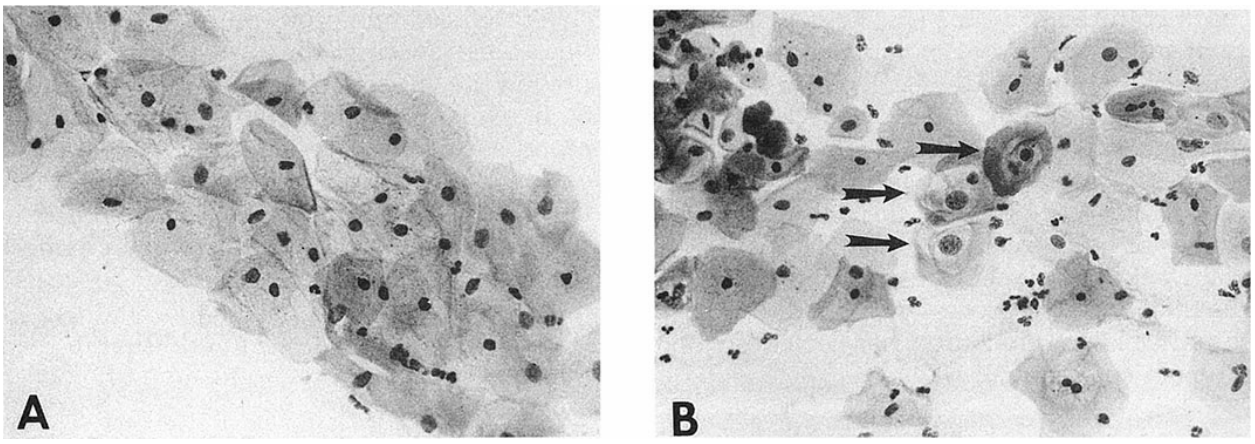

FIGURE 23-5 Cervical cytology. **A.** Normal exfoliated cervical squamous epithelial

cells. The nucleus is small and compact, and the cytoplasm is abundant in these mature, keratinized epithelial cells. **B.** Koilocytic atypia. The nuclei in some of these superficial cells are enlarged with coarse, clumped chromatin, and there is a pallor or clearing in the cytoplasm around the nucleus (perinuclear halo) (arrows) (Papanicolaou stain ×250).

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TABLE 23-13 The Cervical Cancer Screening Guidelines of the American College of Obstetricians and Gynecologists

- Cervical cancer screening should start at 21 years of age.
- Women 21–29 years old should have a Pap test every 3 years.
- Women 30–65 years old should have a Pap test and an HPV test every 5 years. As alternative option is the Pap test alone every 3 years.
- Women without a history of moderate or severe dysplasia or cancer should stop cervical screening after age 65, if they have had either three consecutive negative Pap tests or two negative HPV tests, with the most recent test performed within the past 5 years.

Note: Women with a history of cervical cancer, those with HIV, and those exposed to diethylstilbestrol (DES) before birth should not follow these routine guidelines.
Modified from http://www.acog.org/-/media/For-Patients/pfs004.pdf?dmc=1&ts=20150618T1250230513

specificity needed to accurately characterize the type and extent of disease present. The importance of a team approach to achieve optimal benefits for the patient is evident in this area of medical practice.

Polyomaviruses

There are a wide variety of polyoma viruses, but only a few that are of medical importance to humans. These are small, double-stranded DNA viruses that infect most individuals at some time during their life and usually cause subclinical disease. Discernible disease is caused by the reactivation of these viruses in immunocompromised hosts.

The JC and BK viruses, both named after the initials of the patients from which they were initially isolated, are the most common polyoma viruses encountered in human medicine. The JC virus produces a destructive demyelinating disease termed progressive multifocal leukoencephalopathy in immunocompromised patients.[304] The BK virus causes hemorrhagic cystitis, often in stem cell transplant recipients during

periods of profound neutropenia. It also causes nephropathy of the transplanted kidney in renal transplant recipients. The detection of both the BK and JC viruses relies predominantly on quantitative PCR, although other methods are available.

The Merkel Cell carcinoma virus is a more recently described polyoma virus, and as the name implies is associated with the rarely encountered Merkel Cell carcinoma. This neuroendocrine carcinoma occurs most commonly in immunocompromised individuals, particularly transplant recipients.

Parvoviruses

As the name implies, the parvoviruses are very small; they are unusual in that they contain single-stranded DNA. The only human parvovirus, known as B19, has been demonstrated to cause a common childhood febrile illness with rash. Erythema infectiosum is also known as fifth disease (the fifth childhood disease after rubeola, rubella, varicella, and roseola).[28,503] It is characterized by fever and a characteristic "slapped-cheek" rash in young children. Most persons are immune by the time they reach adulthood, but susceptible persons may experience arthritis or arthralgias. This pattern of mild rash illness in childhood and severe arthritis in adults resembles that of rubella virus. The rash in erythema infectiosum is maculopapular, but a vesiculopustular lesion has been reported in an adult.

Parvovirus B19 has an affinity for erythroblasts (i.e., immature red blood cell precursors). The receptor on human erythrocytes for parvovirus B19 is the P antigen; individuals who lack that antigen are naturally resistant to infection. Unfortunately, very few individuals lack the P erythrocyte antigen. Parvovirus B19 can produce transient anemia in normal persons and severe chronic anemia or aplastic crisis in patients with malignancies, hemoglobinopathies or immunodeficiency. If pregnant women contract a primary parvovirus infection, fatal erythroblastosis fetalis with hydrops may result. In this case the virus produces severe chronic anemia, whereas in immunologic erythroblastosis (Rh disease) antibodies to the fetal red cells do the damage. A viral hemophagocytic syndrome in children and adults has been associated with human parvovirus. Transmission of parvovirus infection from heavily infected patients to susceptible healthcare workers has been documented.

The clinical manifestations of human parvovirus infection during an epidemic in Cadiz, Spain, included hematologic manifestations in 13.9% of 43 patients, dermatologic lesions in 53.4%, arthralgias or arthritis in 20.9% and infection in pregnancy in 7.0% patients (two of these women suffered abortions). Fever and lymphadenopathy were presenting symptoms in 37% of the patients.[162]

Hepatitis Viruses

Many viruses can cause infectious damage to the liver. EBV and CMV occasionally

cause symptomatic hepatitis as part of the mononucleosis syndrome. The primary hepatitis viruses are a diverse group. The "alphabet soup" of hepatitis is summarized inTable 23-14. Several other viruses that have been described as agents of hepatitis are covered more extensively in specialized texts. For example, the hepatitis G virus, also known of as the GB virus, is a member of the family *Flaviviridae* and is an infrequent cause of hepatitis.[295,375,489] Similarly, agents such as the transfusion transmitted virus (TT virus) and SEN virus, both members of the family *Circoviridae*, are more appropriately covered elsewhere. The following sections concentrate on the most important hepatotrophic viruses.

Hepatitis A Virus. Hepatitis A virus produces sporadic and epidemic infectious hepatitis. The disease has been known since antiquity, but the virus was not identified until the 1970s. Hepatitis A virus was first classified as enterovirus 72 based on biochemical and physical features. Subsequently, it was recognized that there were considerable differences from classical enteroviruses. It has now been transferred into a new genus, *Hepatovirus*, within the family *Picornaviridae*.[223] There are seven genotypes of hepatitis A virus; most strains belong to genotype I.

Hepatitis A virus produces acute self-limited hepatitis that may be fulminant. The incubation period between exposure and clinical disease is short (<1 month). For 7 to 10 days before clinical illness, there is noncytopathic viral replication

TABLE 23-14 Primary Viral Causes of Hepatitis

Virus	Taxonomy	Transmission	Chronicity	Neoplasia
Hepatitis A	*Picornaviridae* *Hepatovirus*	Enteric: water, food, shellfish	No	No
Hepatitis B	*Hepadnaviridae* *Orthohepadnavirus*	Parental: blood, sexual, needles, enteric	Yes	Yes
Hepatitis C	*Flaviviridae* *Hapacivirus*	Parenteral: blood; less frequently sexual contact	Yes	Yes
Hepatitis D	*Deltavirus*	Parenteral: blood; requires Hepatitis B virus	Yes	Yes
Hepatitis E	*Hepeviridae* *Hepevirus*	Enteric	No	No

in hepatocytes, viremia, and fecal shedding of infectious virus. A period of viral replication with hepatocyte damage, including classical "ballooning" degeneration of liver cells, follows. At this point the communicable period is almost complete. Hepatitis A virus infection is rarely transmitted by blood transfusions because little virus is present in the blood and the duration of viremia is limited. Transmission of infection to patients with hemophilia in clotting factor concentrate, which contains samples from

many donors, has been described in Europe and the United States. Massive hepatic necrosis and postinfectious cirrhosis are rare. Chronic hepatitis and neoplasia are not complications.

Hepatitis A is transmitted by contaminated food and water. Bivalve shellfish from contaminated waters have produced numerous outbreaks because they are often eaten raw or steamed to a temperature that does not inactivate the virus.[272] In one outbreak in Shanghai more than 300,000 individuals were infected after eating raw clams taken from contaminated water.[223] Sexual transmission also occurs.[255]

The transmission of hepatitis A virus, like EBV and poliovirus, is determined by sanitation and socioeconomic condition. The virus circulates freely under conditions of poor sanitation and infects humans at an early age, when the infection is usually asymptomatic. More affluent citizens escape infection until later in life, when symptomatic hepatitis results[223] (see text on "Poliovirus" and "Epstein–Barr virus").

Hepatitis B Virus. Hepatitis B virus is a DNA hepatovirus. It produces acute and chronic hepatitis and is an etiologic agent of hepatocellular carcinoma.[161] Hepatitis B virus has a long incubation period (45 to 120 days) and is transmitted primarily by parenteral means.[224] Until routine screening of blood products for this virus was commonplace, hepatitis B was the most common cause of transfusion-associated hepatitis. Other parenteral routes have included acupuncture and tattooing. Hepatitis B antigen has been demonstrated in mosquitoes, but transmission of infection by arthropods has not been authenticated. This virus can be transmitted by sexual contact and has produced epidemic disease in male homosexuals, prostitutes, and abusers of intravenous drugs. Perinatal infection occurs, but breast milk does not appear to play a role in transmission. Hepatitis B virus may produce fulminant fatal acute hepatitis, called massive hepatic necrosis. It has been suggested that a mutation in the virus may cause this severe manifestation.

The array of mechanisms for transmission of hepatitis B virus infection recalls HIV. The concentration of hepatitis B virus in blood and body fluids is much greater than HIV, however, and nosocomial infection is consequently a greater problem. Hepatitis B has been an important cause of occupational infections in medical personnel, but the frequency has been greatly reduced by the widespread use of immunization in healthcare workers.

Hepatitis B virus is a 47-nm spherical virus that possesses several antigens of importance for diagnosis and pathogenesis. Several genotypes of hepatitis B virus have distinct clinical implications. Classically, genotype A produces longer survival and less severe disease; genotype B is found more commonly in infections that result in hepatocellular carcinoma; genotype C produces severe disease; genotype D occurs in fulminant hepatitis.[224]

There are three envelope polypeptides of particular importance: HBsAg (hepatitis B

surface antigen), HBcAg (hepatitis B core antigen) and HBeAg (hepatitis B e-antigen). This virus has not been cultured in vitro, but the variety of antigens and corresponding antibodies provide ample tools for documenting clinical disease. Although not absolute, the presence of e-antigen correlates well with the presence of DNA, DNA polymerase and with viral infectivity. The fascinating story of the discovery of Australia antigen (now known as HBsAg) and its relationship to hepatitis has been reviewed by Blumberg.[29] It serves as a reminder that no one can predict where basic research will have its clinical impact. Who would have predicted that a research group interested in genetic polymorphisms of blood proteins would discover the key to an important cause of human hepatitis?

For reasons that are unclear, in some persons who are infected with hepatitis B virus antibody develops and the virus is cleared from their system, whereas other patients continue to have circulating viral antigens. Chronic liver disease develops in a subgroup of these chronic carriers. Another serious complication of this infection is the integration of viral DNA into the genome of the liver cells and the development of hepatocellular carcinoma.

Hepatitis C Virus. After diagnostic reagents for hepatitis A and B viruses became available, it was obvious that there

were other causes of transfusion-associated hepatitis.[305] The unknown virus or viruses were dubbed non-A, non-B hepatitis. Thanks to the sophisticated technology of molecular biology we now recognize hepatitis C as the cause of most cases of transfusion-associated non-A, non-B hepatitis that occurred at that time. The virus, which could not be cultivated, was eventually characterized by molecular techniques, one of the early triumphs of molecular biology. Surprisingly, the newly characterized agent was found to be most closely related to a group of viruses that are transmitted by arthropods; it is now a genus (*Hepacivirus*) in the family *Flaviviridae*. Concepts of genetic relationships among hepatitis C virus strains are evolving. There are six genotypes, 1 to 6, with subtype 1a and 1b in group 1, numerous subtypes within groups 2 to 4, and only one type each of genotype 5 and 6. Patients who are infected with strains of clades 1a and 1b (corresponding to genotypes 1a and 1b) respond less well to interferon treatment and progress more rapidly to chronic liver disease than do individuals who have been infected with other strains. However, newer therapeutics promise to soon make genotypic subtyping unimportant. Otherwise, there appear to be few phenotypic differences among genetically dissimilar strains.

The epidemiology of hepatitis C infections has many similarities to that of hepatitis B, but some significant differences are apparent. The most common route of spread in

the past has been through blood products, including immune globulin. Transmission through blood exposure still may accidentally occur during surgery, and intravenous-drug abusers remain at elevated risk for disease. The screening of blood products for hepatitis C virus has substantially reduced the risk of transmission by this route. Sexual transmission is another means of transmission, but the frequency is much less than in hepatitis B infection. The prevalence of antibody to hepatitis C among healthcare workers in Baltimore was similar to that in the general population, suggesting that workers are exposed to low levels of virus or that the transmissibility is not great.[453]

Acute hepatitis C virus infection is often less severe than that of hepatitis B, but the frequency of chronic hepatitis C disease is high. The subject has been reviewed by Iwarson and colleagues.[244]

The long-term mortality of patients who are infected with hepatitis C virus is similar to that of controls, although infected patients have a slightly increased risk of dying from liver disease. New therapies, recently introduced at the time of this writing, are expected to effect cures in most patients infected with hepatitis C virus. Once a patient has a documented chronic infection, however, the outlook is much worse. Cirrhosis is a significant complication of chronic infection. Hepatitis C infection is an independent risk factor for hepatocellular carcinoma after the development of cirrhosis. Extrahepatic immunologic manifestations, such as cryoglobulinemia and rheumatoid factors, are a prominent part of hepatitis C infection, as they are for hepatitis B.

Hepatitis D Virus. Hepatitis D virus, also known as the delta agent, is a 35-nm double-shelled RNA virus that is incapable of multiplication without the surface antigen of hepatitis B.[169] The maturing delta virus covers itself in the HBsAg coat before infecting other cells. Thus, the biology of hepatitis D is inextricably tied to that of hepatitis B. Hepatitis D can produce infection and disease only in patients who are concurrently infected with hepatitis B or who are producing HBsAg from a previous infection. Not surprisingly, hepatitis D virus infection is concentrated in pockets of populations with hepatitis B virus infection, such as drug addicts and homosexual men.

When coinfection occurs, the clinical disease is similar to that of hepatitis B and is usually self-limited. When hepatitis D infects a patient who is a chronic carrier of hepatitis B, the clinical disease is much more severe, disease becomes chronic in as many as 80% of cases, and fatality rates may be dramatically increased to as high as 12%.

Hepatitis E Virus. Hepatitis E, one of the more recent additions to the hepatitis viruses, is a 27-nm labile RNA virus.[382] Hepatitis E virus produces an enterically transmitted acute hepatitis that most closely resembles that of hepatitis A. The viral genome has been cloned by techniques similar to those used to identify hepatitis C. The taxonomic status of this virus is not yet clear. It has similarities to the

caliciviruses, but its closest genetic relative is rubella virus, currently classified as a togavirus. Large waterborne epidemics of disease are produced under conditions of poor sanitation, but the infection has not yet been recognized as a cause of indigenous disease in the United States. Travelers to endemic areas, however, have returned to the United States with the infection.

Prion Diseases (Transmissible Spongiform Encephalopathies)

The transmissible spongiform encephalopathies are a fascinating group of CNS infections. They are defined by a distinctive cellular pathology, characterized by a vacuolated appearance of the cerebral white matter. The infections and their natural hosts are summarized in Table 23-15. A molecular and clinical classification of prion diseases has been proposed.[466]

For decades it has been recognized that the scrapie agent was novel—an apparently transmissible agent that contained no evidence of either type of nucleic acid. The responsible pathogen was originally considered a "slow virus," but the agent was more resistant to inactivation by X-rays, ultraviolet light, and harsh chemical treatment, including immersion in 10% formalin, than any other known infectious particle. In the 1960s, Pattison proposed that the agent was a basic protein and Griffith suggested several hypothetical mechanisms, one of which proved to be on target.[380] The nature of the infectious particles was painstakingly elucidated by Prusiner. He persisted in his battle to win over skeptical colleagues, and eventually won the Nobel Prize for his efforts. The unique infectious agent, which was named a prion, turned out to be a normal cellular protein (PrP^c) that was modified by a conformational change. The PrP^c protein is converted into the abnormal protein (PrP^{sc}) by refolding of a portion of its α-helical coil structure into a β-sheet.[379,380] Although the infectious-protein hypothesis has been generally accepted, agreement as to the nature of the infectious agent is not universal.[80]

The diseases produced by prions are referred to as transmissible spongiform encephalopathies (TSEs).[252,380,381] They were of veterinary interest until the first human TSE was

TABLE 23-15 Transmissible Spongiform Encephalopathies (Prion Diseases)

Agent	Natural Host	Human Infection
Scrapie	Sheep	No

Transmissible mink encephalopathy	Mink	No
Chronic wasting disease	Deer and elk	No
Kuru	Humans	Yes
Creutzfeldt–Jakob disease (CJD) (familial, sporadic, and iatrogenic forms)	Humans	Yes
Gerstmann–Straussler–Scheinker syndrome (GSS)	Humans	Yes
Fatal insomnia (familial and sporadic forms)	Humans	Yes
Bovine spongiform encephalopathy (BSE)	Cattle	Yes

recognized. Kuru occurred among a remote, isolated tribe of aborigines in the New Guinea highlands.[158] Gajdusek won a Nobel Prize for unraveling the mysterious disease that began with cerebellar ataxia and a shivering-like tremor, progressed to complete loss of motor and speech function, and resulted in death within a year. The agent was transmitted through the practice of ritual cannibalism as part of the mourning ritual for the dead. Transmission of the infection was stopped when the tribe was convinced to alter its practices. Subsequently, it was recognized that two rare human dementias—Creutzfeldt–Jakob disease (CJD) and a variant, Gerstmann–Sträussler–Scheinker syndrome (GSS)—were caused by a similar agent. These degenerative diseases, which occur in familial and sporadic forms, produce the rapid onset of dementia. Memory loss and behavioral problems ensue at some point in most patients. Death occurs within months to a year after onset.

Bovine spongiform encephalopathy (BSE) appeared in southern England in 1986, and approximately 100,000 cases had been documented by the end of 1993.[39] Scrapie has been passed from sheep to cattle experimentally,[170] and it has been conjectured that the disease was transmitted to cattle through sheep brains included in feed as a protein supplement.[37] Alternatively, BSE may have arisen by a chance mutation, as occurs in sporadic human CJD. Whatever the origin of the first case, the inclusion of animal products in animal feed allowed the widespread dissemination of the infection in cattle.[255]

What was an economic disaster turned into political and scientific catastrophes in 1996 when it was announced that some patients with “atypical Creutzfeldt–Jakob disease” in England may have been contracted by eating meat from cows with BSE. The British government underreacted, the European governments overreacted, and a crisis with more politics than science ensued. Soon it became clear that variant

Creutzfeldt–Jakob disease (vCJD) was real. Several authors believe that it resulted from eating contaminated meat,[91,255,466] although Prusiner has been more circumspect in his analysis.[379–381] vCJD differs from the classical form in several ways, including onset at a young age. The epidemic of BSE and apparently of vCJD in Britain was eventually brought under control by widespread slaughter of cattle and prohibition of animal products in animal feeds. The long incubation periods of TSE make caution in declaring victory a wise policy.[243]

The BSE and vCJD saga shattered the previously held and comforting theory that prion diseases were species-specific. Although TSE had been recognized for many years, there had never before been transmission across species lines. It was devastating to realize that the species barrier may not be absolute and that humans may not be immune to risk. Additional concern was raised by the recognition that at least one victim of CJD had participated in wild game feasts.[64] It is not known whether the wild game animals were afflicted with chronic wasting disease, which afflicts herds of elk and deer in the Western and North Central United States. The potential for additional risks to humans is, however, all too obvious. Another comforting illusion was destroyed when BSE was identified in both Canada and the United States.[67,115]

To date, all transmissions of prion disease have been through neural tissue, including transmission of CJD by hormones derived from human pituitary tissue, by transplantation of dura mater and corneas, and through contaminated electroencephalographic electrodes.[381] Infections have become clinically apparent more than 14 years after the putative exposure. Definitive evidence of transmission by extraneural tissue has not been forthcoming,[252] but prions have been documented outside the CNS in classical CJD[172] and in vCJD.[466]

When TSE is suspected clinically, elaborate precautions must be taken in the autopsy room and histology laboratory because the agents of these infections survive formalin fixation and are even demonstrable after tissue is embedded in paraffin blocks.

Clinical Classification of Viral Infections

Although virologic taxonomy is important scientifically, clinical schemes of classification have practical validity when considering an individual patient. The clinical syndromes and associated viruses that are most commonly encountered are summarized in Table 23-16. It is important to recognize that viruses from varying taxonomic groups may produce the same symptoms.

TABLE 23-16 Clinical Syndromes Associated with Viral Infections

Clinical System	Syndrome	More Likely Agents	Less Likely Agents
Respiratory	Coryza (common cold)	Rhinovirus Coronavirus Adenovirus Parainfluenza 3	Influenza A and B Parainfluenza 1 and 2 RSV Enterovirus
	Pharyngitis	Adenovirus Herpes simplex 1 Enterovirus EBV	Influenza A and B RSV Parainfluenza 1 and 2 Rhinovirus Coronavirus
	Croup	Parainfluenza 1 Parainfluenza 2 Parainfluenza 3	Influenza A RSV Measles Coronavirus
	Bronchiolitis	Parainfluenza 3 RSV hMPV	Adenovirus Parainfluenza 1 and 2 Influenza A and B Rhinovirus
	Pneumonia	Influenza A RSV Parainfluenza 3 Adenovirus CMV (immunosuppressed hosts)	Parainfluenza 1 and 2 Rhinovirus EBV Influenza B SARS coronavirus hMPV
	Pleurodynia	Coxsackievirus B	Coxsackievirus A Echovirus
Central nervous system	Aseptic meningitis	Echovirus Coxsackievirus A Coxsackievirus B	Mumps virus LCM Herpes simplex 2 VZV Adenovirus Bunyaviruses Parainfluenza 3 Polio virus (vaccine strains)
	Encephalitis	Herpes simplex 1	Togavirus Flavivirus Bunyavirus EBV Enterovirus Rabies CMV VZV
Gastrointestinal	Diarrhea (infants)	Rotavirus Adenoviruses 40 and 41 Astrovirus	Calicivirus (Norwalk)
	Diarrhea (adults)	Calicivirus	Rotavirus Adenoviruses 40 and 41 Astrovirus
Liver	Hepatitis	Hepatitis A Hepatitis B Hepatitis C	EBV CMV Hepatitis D Hepatitis E
Parotid gland	Parotitis	Mumps virus	Parainfluenza
Cutaneous	Vesicular rash	Herpes simplex 1 and 2 VZV	Echovirus Coxsackievirus A Vaccinia

	Maculopapular rash	Echovirus Coxsackievirus Human herpesvirus 6 and 7	Adenovirus EBV Dengue Measles Rubella
	Petechial rash	None frequent	Adenovirus Echovirus Coxsackievirus Hemorrhagic fever viruses
Urogenital	Hemorrhagic cystitis	BK virus (immunosuppressed host)	Adenovirus Herpes simplex 1
Cardiac	Myocarditis/pericarditis	Coxsackievirus A, B Echovirus	Adenovirus Herpes simplex 1 Influenza A Mumps
Ocular	Keratitis/conjunctivitis	Herpes simplex 1 VZV Adenovirus	Vaccinia Measles

Adapted from McIntosh K. Diagnostic virology. In: Fields BN, Knipe DM, Melnick JL, et al., eds. *Virology.* 2nd ed. New York: Raven Press, 1990:411–440.

Diagnosis of Viral Infections

The primary diagnostic technique for most viral infections has changed over the past decade from the isolation of the virus in cell culture to detection of the nucleic acids of the viruses by a variety of molecular methods, most of which involve nucleic acid amplification. Serologic techniques remain useful in a number of settings. These include determining the immunologic status of donors and recipients prior to transplantation and in the diagnosis of diseases wherein the viremia may have been transient (e.g., West Nile virus infection). The direct detection of viral antigen in body fluids or tissues has also been effective for some viruses, but there are limitations to this approach that are detailed below. The precise role that antigen detection will play a role remains uncertain, as molecular diagnostic assays become ever more user-friendly, some of which are even available as "waived" point-of-care tests. In Table 23-17, the methods most commonly used in hospital laboratories to diagnose viral infections are summarized. The advantages and disadvantages of each of these approaches are compared and contrasted in Table 23-18. Interested readers should consult excellent general references for more detailed discussions.[249,436]

Collection of Specimens for Diagnosis

In general, an attempt should be made to obtain material from the organ or organs that are infected. For the most common viral infections of the skin, the scrapings or aspirates of cutaneous lesions are the best specimens for testing, which depending on the laboratory may be direct immunofluorescence assay DFA and culture, or solely

molecular diagnostics. For diagnosis of infections of the respiratory and gastrointestinal tracts, secretions from those regions should be submitted. As molecular methods were developed at a time when DFA and culture were the standard of care, many of the transport media used for viral culture have been validated for molecular testing. This is not only convenient, but stabilizes the virus, should culture ever be needed for antiviral susceptibility testing or epidemiologic studies. Most viruses enter the body through the respiratory or gastrointestinal tracts. Although the most obvious clinical manifestations of disease may occur in a distant organ, it is often appropriate to collect a specimen from the point of entry. Sampling of multiple sites is particularly useful if it is difficult to obtain a specimen from an internal organ or if the virus is difficult to isolate from the target organ. For example, the skin is most dramatically involved in measles infection, but measles virus may be isolated from the respiratory tract or from the urine. Similarly, the cardiac and CNSs are commonly affected in serious enteroviral infection, but the virus may be isolated from the upper airways or from the gastrointestinal tract.

If the virus is rarely isolated from these nonsterile sites (as is the case with measles), recovery of a virus establishes an etiologic diagnosis. If the virus is recovered from sites where it may be found in the absence of disease (e.g., the enteroviruses in throat or stool specimens), the recovery of a potentially pathogenic virus provides at best a presumptive diagnosis.

Viruses colonize mucosal surfaces less frequently than do bacteria, but may be present more commonly during the months when increased circulation of viruses occurs—the winter for respiratory viruses and the summer for enteroviruses. The frequency with which enteroviruses colonize the gastrointestinal tract is greatest in infants and least in adults. In a study performed in Cincinnati, the carrier rate for echoviruses was 5.2% among 1- to 4-year-old children, 2.6% in 5- to 9-year-old children, and only 0.2% in 10- to 14-year-old children.[404] HSV and CMV may produce chronic infections and be shed intermittently into secretions and fluids.[361,486]

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TABLE 23-17 Methods for Diagnosis of Viral Infections

Virus	Primary Method	Expedited Method	Ancillary Method	Comments
Adenovirus, Non-Enteric	CA-NAAT LDT	FA CA-NAAT LDT	Culture Histology with IHC	
Adenovirus, Enteric	CA-NAAT LDT	FA, EIA	IEM	
Alphaviruses	LDT Culture	LDT	Serology	
Arenaviruses	LDT Culture	LDT EIA	Serology	Tests performed only in BSL-4 laboratories.
Astroviruses	CA-NAAT LDT	CA-NAAT LDT	EIA, IEM, and Serology	
Bunyaviruses	LDT	LDT	Serology	Both serology and molecular tests are recommended for hantavirus.
Calicivirus	CA-NAAT LDT	CA-NAAT LDT	EIA, IEM, and Serology	
Coronaviruses	CA-NAAT LDT	CA-NAAT LDT	Serology	Some of the common coronaviruses are now part of commercially available multiplex respiratory viral panels. Testing for the SARS or MERS coronaviruses is predominantly done in public health facilities.
Cytomegalovirus	CA-NAAT LDT Culture	CA-NAAT LDT	Serology Histology, possibly with IHC.	Serology is to determine immune status associated with transplantation. Quantitative PCR is used to monitor CMV viral loads for patients at risk for CMV disease.
Enteroviruses	CA-NAAT LDT Culture	CA-NAAT LDT	Serology	The large number of serotypes make serology challenging.
Epstein–Barr virus	Serology EIA CA-NAAT LDT	EIA CA-NAAT LDT	Histology with in situ hybridization	The method is dependent on the need. EIA and serology are predominantly used for the assessment of primary infection. Quantitative PCR is used to determine EBV viral loads in an effort to avoid the development of a posttransplant lymphoproliferative disorder.
Filoviruses	CA-NAAT LDT	CA-NAAT LDT	Culture Serology	Tests performed only in BSL-4 laboratories.
Flaviviruses	LDT	LDT	Serology Culture	
Hepatitis A virus	IgM serology		LDT	
Hepatitis B virus	Serology	CA-NAAT	CA-NAAT	Serology is used both for diagnosis and to monitor patients, wherein CA-NAAT testing is used to monitor patients.
Hepatitis C virus	Serology CA-NAAT	CA-NAAT	Genotyping Histology with IHC	Serology with reflex CA-NAAT is used for diagnosis; then CA-NAAT is used for monitoring. The availability of new drugs may make the need for genotyping obsolete.
Hepatitis D virus	Serology		LDT	
Hepatitis E virus	Serology		LDT	
Herpes simplex viruses	CA-NAAT Culture LDT	FA CA-NAAT LDT	Histology, possibly with IHC Serology, type specific	Variations of culture, such as shell vial assay and modified cell lines (e.g., enzyme-linked virus-inducible system [ELVIS]) should also be considered.
Human herpes virus 6 and 7	LDT	LDT	Serology	

Human herpes virus 8	LDT	LDT	Histology with IHC Serology	
HIV	Serology CA-NAAT	EIA	Genotyping	Serology with reflex, as needed, to CA-NAAT for diagnosis. Quantitative RT-PCR is used to monitor the viral load. Genotyping is used to determine the presence of resistance-associated mutations.
Influenza	CA-NAAT	CA-NAAT FA EIA	Culture	Culture-based methods are largely being replaced by CA-NAATs. New, waived, and simple-to-use point-of-care amplification may replace the insensitive EIAs.
Measles	Serology LDT		Culture	
Metapneumovirus	CA-NAAT LDT	CA-NAAT LDT FA	Culture	
Mumps	Culture LDT	LDT	Serology	
Papillomavirus	SA CA-NAAT		Pap test Histology	
Parainfluenza virus	CA-NAAT	FA CA-NAAT	Culture	
Parvovirus B19	LDT		Histology Serology	
Polyomaviruses	LDT Histology, with IHC or *in situ* hybridization.		Serology Cytology	Quantitative PCR is used for the detection and monitoring of the polyoma viruses in patients at risk or suspected of having associated disease.
Poxviruses	LDT Histology Culture		Serology	Serology is performed for determination of immune status; culture is performed in BSL-4 laboratories if smallpox threat is suggested.
Rabies	Culture FA LDT	FA LDT		Tests performed predominantly in public health facilities.
Respiratory syncytial virus	CA-NAAT	CA-NAAT FA EIA	Culture	
Rotavirus	EIA CA-NAAT	EIA CA-NAAT	IEM	
Rubella	Serology	LDT	Culture	
Varicella-zoster virus	LDT FA	LDT FA	Culture	Culture for VZV has poor sensitivity.

CA-NAAT, commercially available nucleic acid amplification test; LDT, laboratory-developed test, which is usually PCR-based; FA, direct or indirect immunofluorescence; EIA, enzyme immunoassay; IEM, immune electron microscopy; IHC, immunohistochemistry; SA, signal amplification.

The association of a clinical illness with a virus that has been recovered from a nonsterile site may be strengthened by demonstration of a serologic response to the isolated virus, although this inconvenient tack is rarely used.

The optimal specimens for viral diagnostics are aspirates of fluids, exudates, or secretions; tissues; washings of the upper airways; stool specimens; and plasma or whole blood. Swab specimens, which are convenient and more acceptable to patients than washings or aspirates, are acceptable in most situations. Swabs and nasopharyngeal washings are generally equivalent for recovery of respiratory viruses. The newer flock swabs are recommended, as these deliver a greater quantity of

clinical specimen compared with more traditional swabs. Some investigators have found that RSV is more reliably recovered from nasopharyngeal aspirates than from swabs,[212] but that experience has not been universal.[429] Virtually any material may be used for the swab tip, with the notable exception of calcium alginate (at least for HSV).[429]

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TABLE 23-18 Comparison of Diagnostic Methods

Method	Time	Advantages	Disadvantages
Culture	Days to weeks	Specificity maximum; sensitivity usually adequate, but depends on the virus; isolate available for characterization	Cell culture facilities needed; time for diagnosis may be long, but may be shortened by spin amplification.
Antigen detection	Hours to 1 day	Speed of diagnosis; used for viruses difficult to culture	False negatives due to limited sensitivity are an issue with non-DFA antigen detection methods (e.g., lateral flow EIA); hard to batch tests.
Molecular diagnostics	Hours to 1 day	Excellent sensitivity and specificity; speed of diagnosis; have become the method of choice for detecting most viruses	Some, but not all, require molecular facilities; potential for cross-contamination exists for some assays
Serology	Within a week	Assessment of immunity; used for viruses that are difficult to culture	Potential cross-reactions; sensitivity and specificity varies between manufacturers.

Viremia occurs frequently during viral infections but is not often detected. HIV, HBV, HCV, and the transplant-associated viruses (CMV, EBV, and BK) cause viremia and can be quantitatively detected by several methods, most commonly PCR-based methods. Blood may also be a useful specimen for diagnosis of enteroviral infection in young children and infants.[376]

To culture vesicular skin lesions, the skin should be cleansed with an alcohol swab and allowed to dry for at least 1 minute. The vesicle should then be unroofed with a sterile scalpel and a sterile swab touched several times to the base. The swab should be placed in a viral transport medium, as described below. Alternatively, the contents of the vesicle may be aspirated with a tuberculin syringe and 26-gauge needle, if the vesicle is sufficiently large. Material for a Tzanck preparation, which is not commonly performed, can be collected by vigorously scraping the base of the lesion with the edge of a scalpel blade; the material on the blade is then touched to a glass slide and allowed to air dry. The Tzanck preparation has largely been replaced by DFA and culture, and more recently by FDA-approved PCR methods for the HSV. These assays are often constructed so that HSV types 1 and 2 are differentiated, without any additional effort or cost.

As a general rule, the frequency with which viruses are recovered decreases as the duration of illness increases, so every effort should be made to obtain specimens as early in the infection as possible. Additionally, there are certain viruses that are notably

difficult to recover and may be lost in transit, if culture-based methods are used. Examples include RSV and VZV. Although the culture for these viruses may fail, the presence of virus can be demonstrated by traditional DFA or by more sensitive molecular methods. For example, when we studied the detection of VZV using four methods, DFA, culture and two PCR assays, we found the sensitivity of the culture was a dismal 46.3% compared with 87.8% for the DFA. The PCR assays had sensitivities of 97.6% and 100%.[491]

Transportation and Storage of Specimens

Aspirates, fluids, and tissues should be transported to the laboratory in a sterile, leakproof container. Every effort should be made to minimize the interval between collection of the specimen and its inoculation into cell culture, if culture-based methods are used. In the past, inoculation of cell cultures at the bedside had been recommended for some fragile viruses, particularly RSV, but is impractical and is usually not performed, particularly in the era of nucleic acid detection.[429]

Swabs should be placed into a transport medium that includes antibiotics; dry swabs are not acceptable. The type of transport medium is probably not critical, although it is difficult to conduct definitive comparative studies in which all variables are controlled. Data are most complete for HSV. Commercial transport systems, such as the Culturette swab products (BD Diagnostics, Franklin Lakes, NJ), have been satisfactory. The sucrose–phosphate–glutamate medium (SPG) that was designed for *Chlamydia trachomatis* (CT) has been effective, as has been a variant without the glutamate (2SP medium).[248] Traditionally, a nutrient medium has been supplemented with a source of protein in an attempt to stabilize fragile viruses and with antibiotics to minimize bacterial contamination. Transport media that are currently commonly used includes M4 and Universal Transport Media (UTM), among a few others.

Specimens should be refrigerated, if cell cultures will be inoculated. To select the best temperature for storage, one must balance the decrease in viral titer that occurs progressively at 4°C against the sudden decrease that occurs when specimens are frozen and thawed. It is clear that (1) the specimens should be maintained at −70°C if they are to be stored for a very long time (weeks or months) and (2) the best temperature is 4°C if the delay will be short (i.e., less than 24 hours). A reasonable recommendation is that specimens be refrigerated for up to 96 hours and frozen at −70°C if the delay will be longer. The worst temperature is −20°C, especially in a frost-free freezer, where repeated cycles of defrosting and freezing are extremely traumatic to all forms of life. Specimens should never be frozen in the freezing compartment of a standard refrigerator. Johnson has thoroughly reviewed the transport of specimens for traditional viral diagnostics.[261]

Isolation of Viruses in Culture

Although a few of the largest research and public health laboratories may have facilities for inoculation of animals, most diagnostic facilities are restricted to the use of in vitro analogs. Even these, in vitro analogs (i.e., cell culture systems)

are becoming less common, as more rapid and user-friendly, often molecular, methods are being employed. The following sections of text are retained for those performing cell cultures.

Preparation and Maintenance of Cell Cultures

Cell culture is the cultivation in vitro of dissociated single cells. Tissue and organ culture is the maintenance in vitro of a portion of an organ, usually for short, delimited periods of time. The use of organ culture has been restricted almost completely to the research laboratory. For example, cultures of trachea have been used to isolate human coronaviruses, most of which do not grow in cell culture. Occasionally, organ and cell cultures must be combined. For example, an explant of infected brain and an indicator monolayer of susceptible cells were used for the isolation of measles virus from patients with subacute sclerosing panencephalitis. Embryonated eggs are an inexpensive method for cultivating selected viruses, such as the orthomyxoviruses, but given the complexity of use and the development of superior methods, they are rarely used in diagnostic laboratories.[285]

Cell cultures are of three basic types (Table 23-19). Primary cell cultures consist of a mixture of cells, usually kidney, lung, or skin, obtained by dissociation of cells from the minced organ. These cells can be maintained in culture for a limited time only. Once subcultured repetitively in vitro, a primary cell culture becomes a cell line. The most commonly used cell lines are composed of fibroblasts, obtained from skin or embryonic lung. A cell line is diploid if at least 75% of the cells have a normal complement of chromosomes. A cell line is heteroploid if more than 25% of the cells have an abnormal complement of chromosomes. Readers interested in detailed choices of cell lines should see the previous edition of this text. Innovative thinking has produced even more variety in the choices for cell cultures. By combining cell lines, the spectrum of viruses detected can be expanded while limiting the number of tubes required and even avoiding the need for primary cell cultures.[238] Some of the combinations of cell lines that have been developed as Fresh Cells or R-Mix (Quidel, Athens, OH) are included in Table 23-19. The mixed cell lines may be combined with other approaches, such as spin amplification (i.e., shell vials) to enhance detection further.[18,123] A further innovation is the storage of the mixed cultures at −80°C for up

to 4 months before inoculation.[240]

Still another innovation has been the engineering of cell cultures to facilitate isolation of particular viruses or to enhance their detection. One of the cell lines that has been used successfully for detection of enteroviruses is buffalo green monkey kidney (BGMK) cells. By engineering BGMK cells to express a receptor for enteroviruses (decay-accelerating factor, DAF) investigators were able to increase the diagnostic yield.[239]

A different approach to cellular engineering is the ELVIS (*e*nzyme-*l*inked *v*irus-*i*nducible *s*ystem) HSV test system (Quidel). In this case, an HSV-specific gene promoter sequence in the baby hamster kidney (BHK) cell line is linked to a reporter gene,β-galactosidase. When the cells are infected with either serotype of HSV, the promoter gene is turned on and the reporter gene is then activated, producing a visible

TABLE 23-19 Types of Cell Cultures

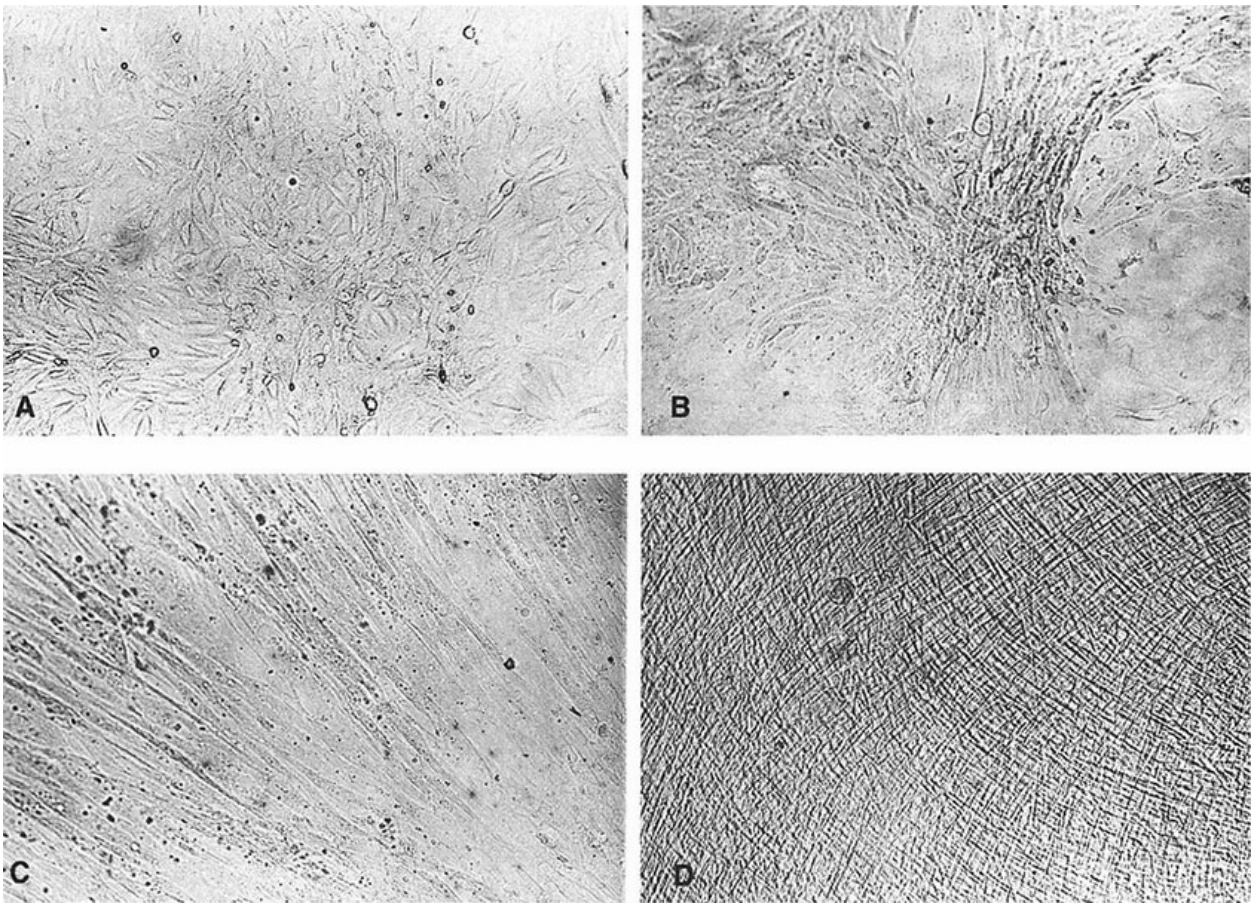
Type of Culture	Characteristics	Examples	Primary Use
Primary	Diploid; mixed cell types; one or two passages	Primary monkey kidney	Influenza; parainfluenza; some enteroviruses
		Primary rabbit kidney	Herpes simplex
Cell lines	Diploid; fibroblastic; limited passages (<50–70)	Human diploid fibroblast (WI-38, MRC-5, HEL, FS-9)	Herpes simplex; cytomegalovirus; varicella-zoster; rhinovirus
Established cell lines	Heteroploid; continuous passage in vitro	HeLa; HEp-2	Adenovirus; RSV; Coxsackie B viruses
		A549	Herpes simplex; adenovirus; varicella-zoster; some enteroviruses
		MDCK	Influenza virus
		LLC-MK2	Parainfluenza virus
		Vero; ML cells	Herpes simplex
		RD cells	Some Coxsackie A viruses
		BGM cells	Coxsackie B viruses
		293 cells	Enteric adenovirus
Mixed cell lines	Combinations of established cell lines	MRC-5 + CV-1	Herpes simplex; varicella-zoster
		ML Cells + A549	Respiratory viruses
		RD + H292; BGMK + A549	Enteroviruses
Genetically modified cell lines	Introduction of a gene to produce a visible indicator after infection	Enzyme-linked-virus-inducible system (ELVIS)	Herpes simplex

change in the color of the monolayer.[362] As with the mixed cell lines, the ELVIS cells can be stored at −80°C until use.[240] HSV is the most common virus sought in diagnostic laboratories, and a targeted approach to detection of this agent is sensible.

The life expectancy of normal diploid cells is approximately 50 serial doublings in

vitro[208]; those cell lines that have been transferred at least 70 times are considered to be established cell lines. These established or continuous lines may be derived from normal tissue, as was the Vero cell line from African green monkey renal cells. Alternatively, they may originate from neoplastic epithelium, such as the HeLa cells that came from a cervical adenocarcinoma and the HEp-2 cells that were derived from a carcinoma of the larynx. The appearance of uninoculated cell cultures is illustrated in Figure 23-6. The fibroblastic cells are usually long, spindle-shaped and oriented in parallel, whereas the established cell lines consist of polygonal epithelial cells. The primary cultures naturally contain a mixture of cell types.

Conditions for growth and maintenance of cell cultures vary considerably, and only a few generalizations are possible. The temperature of incubation is optimal at 36°C to 37°C but may be lowered to 35°C after confluence of cells is achieved. This maneuver may facilitate isolation of viruses, such as myxoviruses and rhinoviruses that grow optimally at 33°C. A physiologic pH must be maintained. Usually a CO_2–bicarbonate buffering system is used, with CO_2 being supplied by the metabolizing cells in a closed tube or flask. In an open system, a CO_2–air mixture must be provided. Buffering compounds that do not depend on CO_2–bicarbonate, such as HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic-acid), may be used in open systems if an incubator with CO_2 is not available. A pH indicator is often included in the media to monitor closely shifts in pH during incubation.



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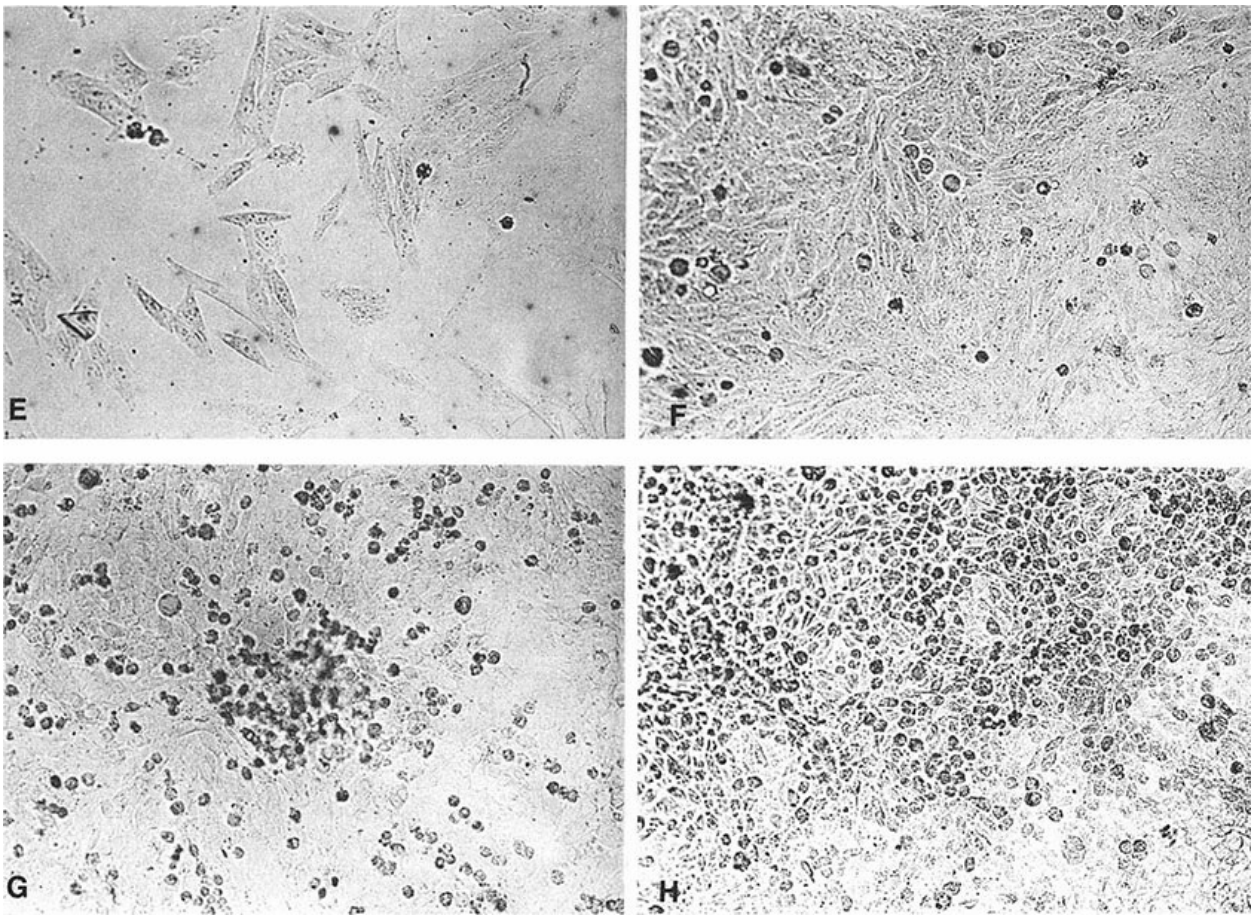


■ **FIGURE 23-6** Normal cell cultures. **A.** Normal rhesus monkey kidney monolayer. This primary culture is just at confluence—there are no spaces between the cells, but cells have not begun to pile on each other. The variability of the cells in the monolayer is a reflection of their origin from diverse cell types (×180). **B.** Normal rhesus monkey kidney monolayer. After continued incubation, areas of more luxuriant growth have become evident. The variability of the cells in the monolayer can still be appreciated (×180). **C.** Normal MRC-5 fibroblast monolayer. These diploid fibroblastic cells have the elongated, spindly character of their type. They are just reaching confluence; a few spaces remain between some of the cells (×180). **D.** Normal MRC fibroblast monolayer. After continued incubation, the crisscross pattern of fibroblasts piled one on top of the other is apparent. The detail of the individual cells is less apparent. Such senescent monolayers should not be inoculated (×180). **E.** Normal HEp-2 cells. An established cell line has been subcultured, and new cells have begun to grow. There are large spaces between individual cells, which have a spindle shape as they spread over the new glass surface (×180). **F.** Normal HEp-2 cells. The subcultured cells have now multiplied to the point of confluence but are still easily visible as distinct polygonal cells. The ideal point for inoculation of most viruses is at this stage or slightly earlier (×180). **G.** Normal HEp-2 cells. After continued incubation, the cell line has multiplied so that cells are piled one on top of the other. Cells that are dividing or detaching from the glass appear rounded. As the monolayers age, they become

less susceptible to the cytopathic effect of viruses, and it becomes increasingly difficult to detect cytopathic effect visually. **H.** Normal McCoy cells. This established cell line, the origin of which is unknown, is pictured at a point at which confluent growth has been obtained. The cells are compact and polygonal. Specimens should be inoculated at this stage or when the cells are slightly less confluent (×180).

Phenol red, which is red at physiologic pH, turning yellow at acidic pH and purple at alkaline pH, is frequently used.

Essential vitamins and amino acids must be supplied in viral culture media.[249] These compounds are stable when stored at 4°C, except for L-glutamine, which must be replenished periodically.[340] Eagle's minimum essential medium (MEM) is commonly used, both for growth and for maintenance of cells. It is usually supplemented with small amounts of serum (up to 5%) for maintenance of a monolayer after cells have reached confluent growth. To enhance initial growth, larger amounts of serum (usually 10%) are used. It is important that the serum be free of infectious agents, including the mycoplasmas, and does not contain antibodies to any viruses that might be present in clinical specimens. For this reason, fetal, newborn, or a gammaglobulinemic calf serum is most commonly used. Other formulations, such as Medium 199 or RPMI 1640, contain a richer mixture of nutrients and may be optimal for maintenance of some cell lines. Two forms of balanced salt solution are most frequently used, both for washing of cells and for incorporation into complete media. The formulations of Hanks' and Earle's solutions differ in several respects, notably the amount of buffering capacity. The two solutions are used virtually interchangeably, but Earle's balanced salt solution with its greater buffering power may be desirable for prolonged maintenance of monolayers.

Contamination of Cell Cultures

Contamination of media is reduced by inclusion of antibiotics. Penicillin (200 mg/mL) and streptomycin (200 mg/mL) or gentamicin (50 mg/mL) is commonly used for suppression of bacterial growth; amphotericin B (1.25 mg/mL) is used for inhibition of fungal growth.[249] The use of antibiotics does not substitute, however, for careful aseptic technique.

Contamination of culture media by *Mycoplasma* species is a common and troublesome problem.[324] *Mycoplasma* species usually are introduced in the serum additives or from contaminated stock viruses; once in the culture, they are very difficult to eradicate. The effects on the cells include suboptimal growth and disturbances in the interactions of cells and viruses. The presence of contaminating *Mycoplasma* species may be monitored by recovering them in culture, by staining for

Mycoplasma DNA, by biochemical measurement of uracil incorporation, or by nucleoside phosphorylase activity techniques. Commercial suppliers of cells should be able to document their efforts at surveillance and quality control.

In addition, cell cultures may be contaminated with viruses (i.e., usually ones that were present in the original tissue)[249] or even with other cells.[351] The most frequent type of viral contamination is the presence of indigenous simian viruses in primary monkey cell cultures, such as the vacuolating agent (SV40), foamy viruses, or the monkey parainfluenza virus (SV5). Simian viruses that have hemagglutinins (e.g., SV5 virus) produced misleading results when hemadsorption tests were performed to detect human respiratory viruses; fortunately, these test have been largely replaced by DFA for those wishing to confirm a cytopathic effect (Individuals interested in hemagglutination and hemabsorption should refer to previous editions of this text) (see Fig. 23-12E). The frequency with which tubes in a shipment of

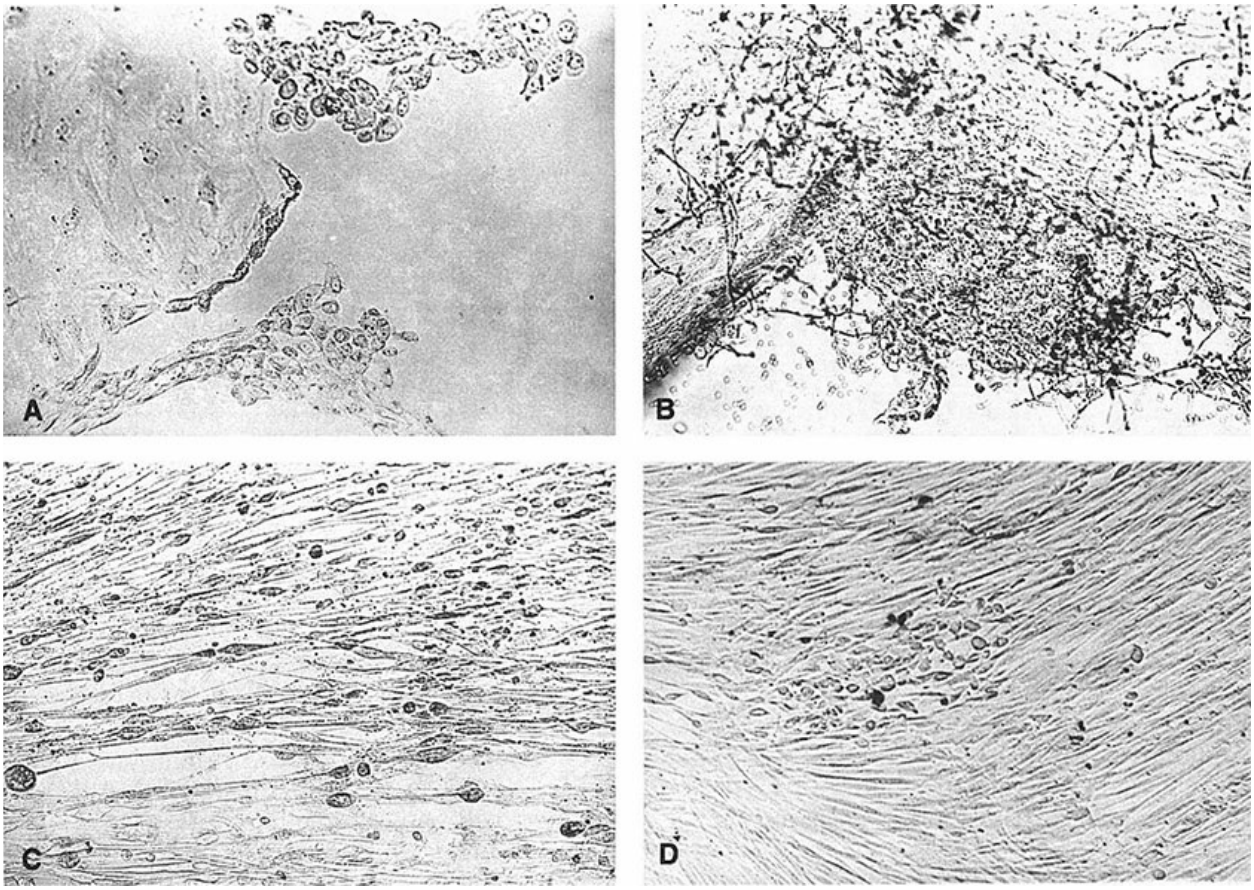
monkey kidney cells are contaminated may vary from 5% to 100%. Reserving a few uninoculated tubes for hemadsorption controls is not foolproof. If an unusual number of patients have hemadsorbing viruses on a given day, the possibility of simian virus contamination should be considered. Contamination of a commercial lot of A549 cells with infectious bovine rhinotracheitis virus has also caused diagnostic problems in several laboratories.[148] An extremely rare but potentially serious problem can be caused by the presence of pathogenic viruses in the tissues of monkeys. Although fortunately very rare, the simian herpes virus and Marburg virus, which produces a hemorrhagic fever, have caused lethal laboratory infections.[89,403,405] These agents provide justification for working with primary cell cultures of simian origin in a biologic safety cabinet.

Contamination of cell cultures with other cells has resulted in the erroneous supposition that a new cell line has been established in more than one research laboratory. In fact, a hardy HeLa cell may have found its way into the flask and taken over.[374] In the diagnostic laboratory, cross-contamination of an established cell line onto a diploid cell line or primary cell culture may give the appearance of cytopathic effects in the original cell culture line (Fig. 23-7D).

Technical Aspects of Cell Culture

A variety of containers are used for the support of cell monolayers. The most common cell cultures in use are commercially available in tubes or vials that are ready for inoculation of specimens. Many laboratories may prefer, for reasons of economy, to maintain cell lines in their own laboratories. T-shaped plastic flasks have generally

replaced glass bottles for the propagation of cell lines (Fig. 23-8).



■ **FIGURE 23-7** Artifacts in cell culture. **A.** Rhesus monkey kidney cells. The tube has been inoculated with a specimen that contained nonspecific toxic material. Cells in the monolayer have rounded up focally, and many cells have detached from the glass. The nonviral effect can be distinguished from the viral cytopathic effect by the overall pattern, by the rapidity of occurrence, and by the inability to reproduce the nonspecific effect after subculture into fresh monolayers (×180). **B.** Rhesus monkey kidney cell culture. A stool specimen was inadequately decontaminated before inoculation. Bacterial and fungal hyphae are evident on the monolayer, which is granular and will soon degenerate. The media may become acidic and cloudy. Retreatment of the specimen with additional antibiotics is necessary. The specimen may also be filtered, but cell-associated viruses may be lost (×180). **C.** MRC-5 fibroblasts. A filtrate of stool that contained *Clostridium difficile* was inoculated onto the monolayer 24 hours earlier. The generalized disorganization of the monolayer with rounding and sloughing of cells is typical of this toxin. Specific identification of the cause of toxicity is provided by neutralization of the effect with specific antiserum. *C. difficile* toxin may give an initial impression of viral cytopathic effect, but the effect will not be reproduced when material from the suspect tube is subcultured onto additional monolayers (×180). **D.** MRC-5 fibroblasts. Focal cell rounding

and enlargement appeared on these fibroblasts that had been inoculated with CSF, suggesting viral cytopathic effect. The atypical progression of cytopathic effect and observation that some of the foci were superimposed on the fibroblasts suggested cellular contamination. HEp-2 cells were subsequently recovered from the tube and demonstrated to have a karyotype that was identical to that of the HEp-2 cells used in the laboratory. The mechanism by which the contamination occurred was never elucidated (×180).

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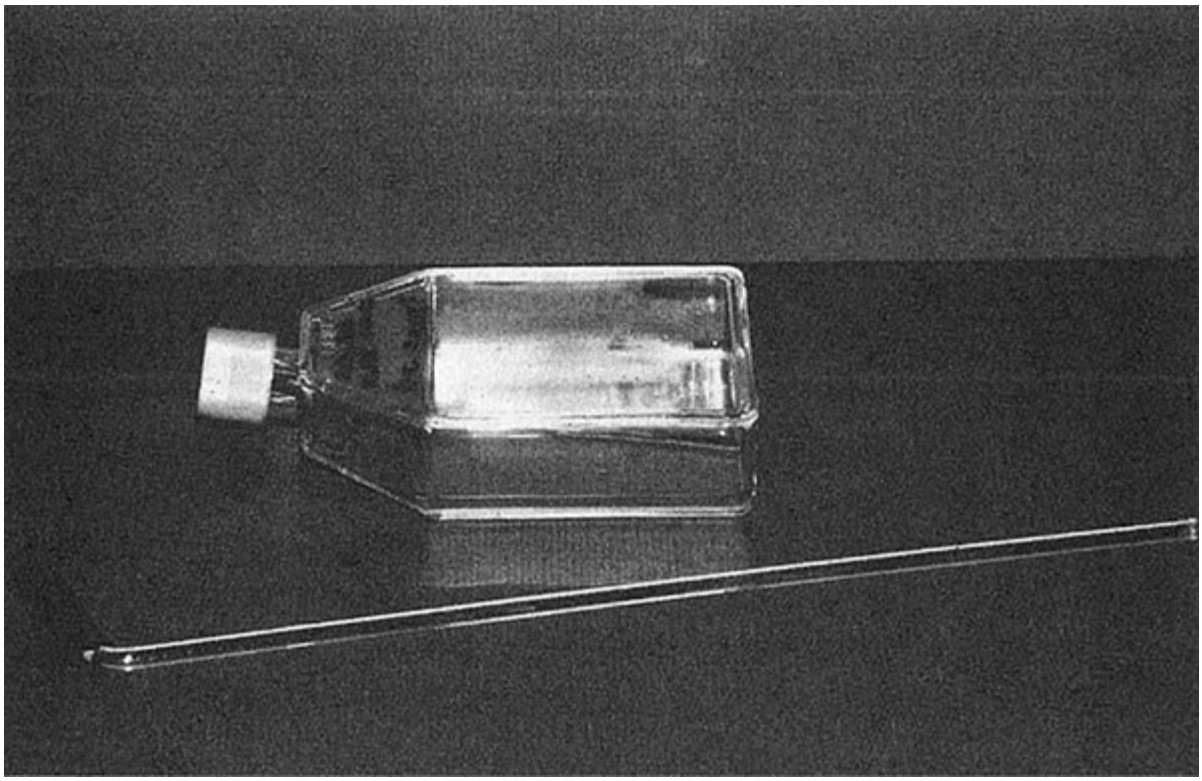


■ **FIGURE 23-8** T-shaped plastic flask and rubber-tipped glass rod, known as a "rubber policeman." Monolayers of cell lines may be grown in large flasks. Many individual tubes for virus culture may be prepared from a single large flask. Mechanical disruption by pipetting or by the rubber policeman helps to dislodge the cells during trypsinization for subculture.

Cell lines may be maintained in the laboratory by dissociation of cells, which are then distributed into new vessels (Box 23-5).

Established monolayers are dissociated by brief incubation in a solution of trypsin or a mixture of trypsin and ethylenediaminetetraacetic acid (EDTA), which is also known as Versene. Damage to cells may occur if the suspension of trypsin is too concentrated

or if the contact is too prolonged. Neutralization of the trypsin is provided by serum in the growth medium that is added after cells have been dissociated. Detachment of the cells from the surface may be enhanced physically by agitation or by scraping with a rubber-tipped glass rod, a device known colloquially as a "rubber policeman" (Fig. 23-8). The dissociated cells are then dispensed into tubes for inoculation of specimens or into additional flasks for continued propagation of stock cells. The density of the suspension can be adjusted to provide confluent monolayers after one or more days of incubation. After inoculation of clinical specimens, the same protocol may be used to prepare infected cells for subculture to additional monolayers.

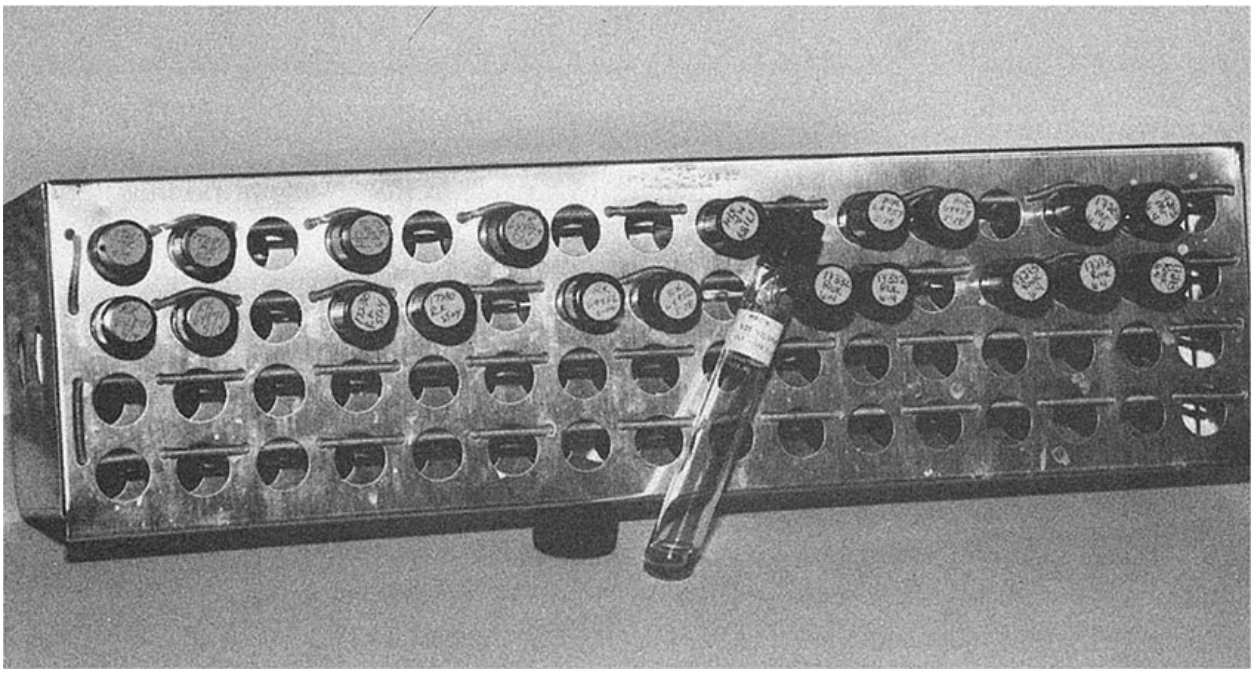
BOX 23-5

Procedure for Maintaining Cell Lines

1. The monolayer is washed with phosphate-buffered saline, pH 7.5.
2. A suspension of 0.25% trypsin or equal parts of 0.25% trypsin and 1:2,000 Versene is added for 15 to 30 seconds, and then removed.
3. The monolayer is incubated at 35°C until the cells have dissociated. Gentle shaking of the flask may enhance dissociation.
4. Growth medium is added and the cells resuspended at the concentration desired for subculture. Alternatively, the cells may be divided arbitrarily (1:2 or 1:3) as dictated by past experience.

Round-bottomed glass tubes (16 × 125 mm) have been traditionally used for viral isolation (Fig. 23-9). These tubes are incubated at a 5-degree fixed angle, so that the monolayer forms on one side of the tube. The heaviest aggregation occurs near the center and at the butt of the tubes; the thinnest layer is at the edges and toward the mouth. Round Petri dishes or glass slides, onto which are affixed plastic chambers may also be used for diagnostic purposes. The slide-chamber system is expensive but useful if morphologic study of an undisrupted monolayer is planned.

The use of shell vials, originally used for isolation of *Chlamydia trachomatis*, have largely replaced tubes, given the decreased space requirements, ease of use and more rapid time to detection that may be achieved when used in conjunction with DFA. A cell monolayer is prepared on a round glass coverslip that has been placed on the bottom of a flat-bottomed vial (Fig. 23-10). If the inoculum is centrifuged onto the monolayer, as is essential for the recovery of *C. trachomatis,* and the monolayer stained with fluorescein-conjugated antiserum or tested with a molecular probe, almost all isolates of HSV can be detected within 24 hours.[134] Presumably,



■ **FIGURE 23-9** Viral culture tubes are slanted at a fixed angle of 5 degrees in a stationary rack. Metal springs hold the tubes in place. Round labels for the tops of the tubes facilitate identification of cultures without removing the tubes from the racks.

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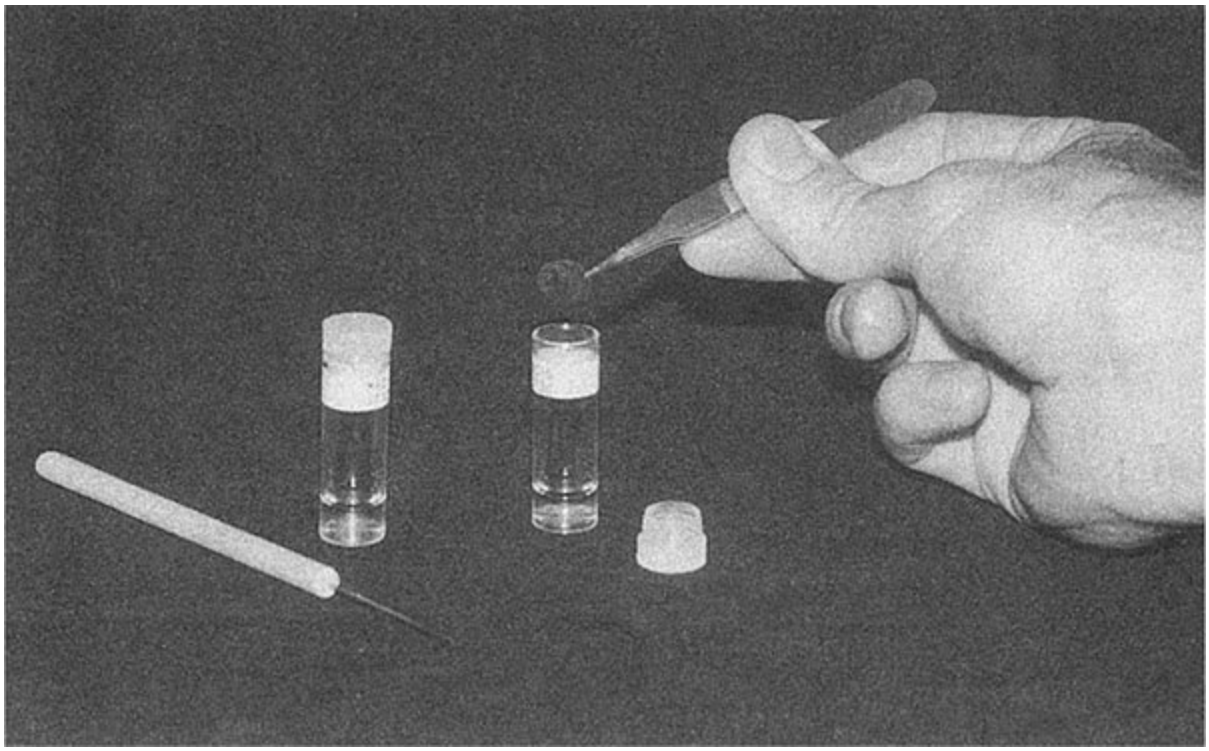

■ **FIGURE 23-10** Shell vials for isolation of *Chlamydia trachomatis* and viruses. The vials are round with a flat bottom and contain a round coverslip on which the cells are grown. The top may be rubber or plastic as shown here. The coverslip is removed with forceps or with a needle.

the shell vial centrifugation technique increases contact of inoculum with the cellular monolayer.

A far more dramatic improvement in the speed of isolation has been reported for CMV.[149,363] The technique is more sensitive than conventional culture for most specimens other than blood, but use of both approaches maximizes results. The preferred method for detecting CMV in blood is quantitative PCR (see below). The gain from shell vial culture comes both from centrifugation and from the use of a fluorescein-labeled monoclonal antibody of high quality. Even supposedly uniform reagents, such as monoclonal antibodies, may vary from lot to lot. Nonspecific staining of cell nuclei may occur with some lots of antisera. The appearance of a shell vial culture containing CMV is shown in Image Plate 23-2F. An advantage of this technique is that infected cells are detected prior to the development of cytopathic effect.

Paya and colleagues[364] have studied the number of shell vials required for each specimen. They found that three vials were required for maximal yield from blood specimens, whereas two vials were needed for urine, bronchoalveolar lavage, and

tissue. Fedorko and colleagues[140] found that the age of fibroblasts in the shell vials was important for recovery of CMV. Old monolayers (8 to 15 days) were less sensitive for virus recovery but more sensitive to toxic components in the specimens than were young monolayers. It is difficult for manufacturers to provide very young monolayers, and unfortunately most laboratories must depend on commercial suppliers for shell vials.

Gleaves and colleagues[173] have reported success with still a third cell-associated herpesvirus, VZV. They examined 68 specimens from 60 patients. VZV was identified by some method in 57% of patients. The sensitivity of diagnostic techniques in decreasing order was direct immunofluorescence of skin scrapings, shell vial culture, and conventional culture. Wilson et al.[491] also demonstrated that DFA was superior to culture for VZV, but also showed that the most sensitive method was PCR.

The shell vial technique has also been applied to viruses other than the herpes group, particularly influenza virus.[123] Other respiratory viruses are also detected using the shell vial technique, but experience is more limited and somewhat less satisfactory than with influenza. The shell vial technique has also been used for BK virus.[311] As noted elsewhere, although the recovery of these viruses is feasible with this method, testing has largely migrated to molecular diagnostic platforms (see below).

Selection of Cell Cultures for Isolation of Viruses

The basic requirements for isolation of viruses in culture are a primary culture of simian kidney cells (for isolation of myxoviruses and enteroviruses) and a diploid cell line of human origin (essential for isolation of CMV, rhinovirus, and VZV; useful for isolation of HSV). The addition of an established cell line of human origin (e.g., HEp-2 or A549) is also useful for isolation of adenovirus and RSV.

Other cell lines may provide satisfactory substitutes for these three basic cell types if isolation of a specific virus is desired (Table 23-19). The quality and provenance of the cells used for isolation are important. Careful studies of cloned fibroblasts from a single fetus demonstrated a large variation in the degree to which the clones supported the growth of respiratory viruses.[458] Variants of established cell lines, such as HeLa and HEp-2, differ also in the sensitivity with which they support some viruses. It is important to ensure that the selected cell variant is of adequate sensitivity.

Once a cell line has been found acceptable, multiple aliquots should be frozen in liquid nitrogen. Cells for daily use may be maintained in continuous culture. The original stock should be used periodically to replenish the working stock so that the cells do not mutate to a state that is less susceptible to viral growth.

Rhesus and cynomolgus monkey kidney cells are comparable for isolation of myxoviruses. Medical Research Council 5 (MRC-5) fibroblasts, on the other hand, have been reported to be more sensitive than WI-38 fibroblasts for the isolation of

CMV.[187]The sensitivity of all diploid cell lines decreases as the number of passages increases. The lowest passage level from a reliable supplier should be sought. If cell sheets, particularly those of established cell lines, are too thick, recovery of viruses may be reduced and evaluation of cytopathic effects is difficult. For some viruses, islands of cells or a barely confluent monolayer are preferable to a fully confluent cell sheet.[457]

In addition, the cells must be in sufficiently good condition to last the several weeks necessary for isolation of slowly growing viruses. Contamination with *Mycoplasma* species should be avoided if maximal isolation rates are to be ensured. Viruses, such as VZV, that spread directly from cell to cell rather than through the culture medium require a healthy monolayer.

Use of growth medium with 10% serum may enhance development and progression of cytopathic effects caused by these viruses,[480] although not all are convinced that this manipulation is necessary. In contrast, serum contains inhibitors of myxoviruses and paramyxoviruses. The maintenance medium in the cells used for these viruses (usually primary monkey kidney) should be free of serum.

Inoculation and Incubation of Cell Cultures

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Transport vials that contain swabs should be vortexed and material from the swab expressed into the medium, which contains antibiotics. Tissues are minced and homogenized in a small volume of transport broth. It may be necessary to suspend fecal specimens in 10 volumes of transport medium. Feces, tissues, and respiratory aspirates that contain excessive debris or mucus may be clarified by low-speed centrifugation. Antibiotics are present in most transport and tissue culture media. If bacterial or fungal contamination of the specimen is expected, additional antibiotics should be added to the specimen before inoculation (penicillin and streptomycin or gentamicin plus amphotericin at twice the concentration used in cell culture media).[249] After processing, the specimen may be inoculated into the culture media. Alternatively, the inoculum may be placed directly onto the monolayer after most of the culture medium has been removed. The more direct contact of viral particles with cells enhances infectivity and may increase both the number of isolates and the speed with which they are recovered. After adsorption of the inoculum onto the monolayer for 30 to 60 minutes at 36°C to 37°C, the medium is replaced and incubation of the culture is continued. Toxic substances in the specimen may also be more evident after adsorption of the inoculum. If two tubes of each cell type are used, one may be inoculated by introducing a specimen into the medium and the other by adsorption. Any remaining specimen should be retained at 4°C or frozen at −70°C in case toxicity

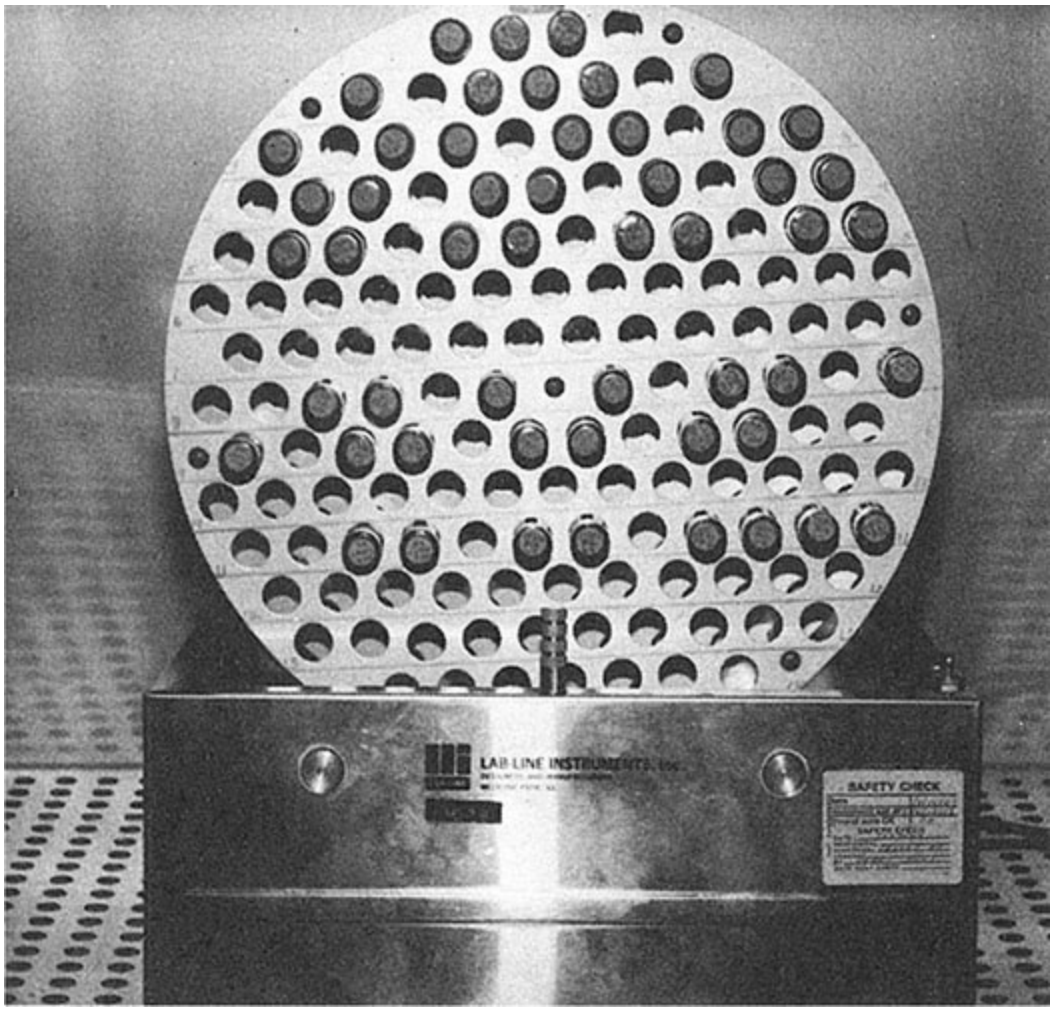
or bacterial contamination occurs. If bacterial or fungal growth results, additional antibiotics may be added to the specimen. The specimen may be passed through a 0.45-μm micropore filter before additional cultures are inoculated, but viruses that are cell-associated, such as CMV and VZV, may be lost when infected cells are trapped in the filter.[291] Nonspecific toxicity to the monolayer is usually evident within 24 hours; the specimen may then be reinoculated after making a 1:10 dilution in balanced salt solution. The pH of the specimen should also be checked and adjusted to neutrality if the specimen is very acidic or alkaline. If nonspecific degeneration of the monolayer occurs after incubation for several days, the cells and fluid may be subcultured onto fresh monolayer.

Monolayers should be incubated at 36°C to 37°C for recovery of most viruses. The optimal temperature of incubation for respiratory viruses, especially rhinovirus, is 33°C, but recovery of other viruses may be reduced at this temperature. Use of a roller drum (Fig. 23-11) facilitates isolation of pathogens in primary monkey kidney and diploid fibroblast cells, especially from respiratory specimens.

Two weeks of incubation is adequate for the recovery of most other viruses. CMV may require longer incubation, but Gregory and Menegus found that 92% of CMV isolates were recovered within 14 days when MRC-5 fibroblasts were used.[187] If the recovery of only HSV is sought, the period of incubation may be further abbreviated to 7 days. Centrifugation of the inoculum and detection of antigen will dramatically shorten the detection time for several viruses, including CMV and VZV.

Detection of Virus and Provisional Identification

Cytopathic Effect. The most common method for detecting virus in culture is examination of cultures for virus-induced damage to the monolayers or cytopathic effect. Monolayers should be examined daily if HSV is sought and at least three times a week if more slowly growing viruses are suspected. Glass tubes can be examined with a conventional light microscope, using simple holders to prevent the round tubes from rolling on the microscope stage (Fig. 23-12). If the monolayer is to be examined in shell vials, an inverted microscope must be used.



■ **FIGURE 23-11** Roller drum for viral cultures. The cytopathic effect of many viruses, especially those that cause respiratory infections, is enhanced by slow rotation of the tubes, so that one revolution occurs every 3 minutes.

Provisional identification of the specific virus (or at least the virus group) can often be provided on the basis of the

■ **FIGURE 23-12** Examination of cultures for cytopathic effect. Examination of cultures for cytopathic effect is usually performed with an inverted microscope, but may also be performed using a traditional microscope, as shown here. A rack, which fits in the specimen holder of the microscope stage, prevents the round tubes from rolling. High magnification is not possible, but is not needed for recognition and characterization of cytopathic effect.

type of cytopathic effect and the type of cell that is affected. Typical patterns of cytopathic effect and the rate of isolation for viruses most commonly isolated in hospital laboratories are summarized in Table 23-20.

When one examines a culture in the laboratory, the pattern of cytopathic effect is known but the name of the virus is not. A flow chart for analyzing cytopathic effect in a clinical specimen is presented in the previous edition of this text for those interested in additional information on this subject.

The effects of the virus on the cells may be subtle initially. Although quantitation of

the viral inoculum based on the extent of cytopathic effect is not precise, it is useful to make a laboratory record of the semiquantitative estimate for comparative purposes (1+, <25% of the monolayer affected; 2+, 25% to 50%; 3+, 51% to 75%; 4+, >75%). These estimates make it easier to document progression of cytopathic effect and establish the presence of a cytopathic agent. It may be necessary to subculture the cells from a tube with a possible cytopathic effect onto fresh monolayers. If possible, such a subculture should be performed after cytopathic effect has reached 2+, using trypsinization, physical disruption of the monolayer with a rubber policeman, or several cycles of quick freeze/thawing. Then, an aliquot is inoculated onto the type of cell in which cytopathic effect was originally observed and any other cell type that might aid in making a provisional identification.

Viral cytopathic effects for three commonly used cell cultures are demonstrated sequentially in Figures 23-13 through 23-16. Although cell culture is largely being replaced by molecular methods, we have chosen to retain these images through this transitional period. Additionally, cell culture is still used for "test of cure" in some settings, in laboratories that have not introduced molecular diagnostics, and in select public health and reference laboratory settings. In most cases early and late changes have been depicted.

TABLE 23-20 Differential Diagnosis of Viral Cytopathic Effect

Virus	Cell Culture			Days of Isolation		Description
	PMK	HDF	HEP-2	Mayo Mean[a]	Mt. Zion Mean[b]	
Influenza virus	+++	–	–	4	3.8	None or focal enlarged granular cells followed by sloughing; rapid progression
Parainfluenza virus	+++	+	+	11	6.4	None or focal rounding and multinucleated giant cells (types 2 and 3)
Respiratory syncytial virus	++	+	+++	8.3	6.1	Enlarged, glassy syncytial giant cells or granular, rounded cells
Mumps virus	+++	+	–	6.9		Enlarged, syncytial giant cells
Measles virus	+	–	+			Vacuolated, syncytial giants cells; rarely isolated
Poliovirus	+++	+++	+++	4.6		Random, swollen, glassy cells; rapid progression and detachment of cells from glass
Coxsackievirus B	+++	+	+++	3.5	4.2	Focal, swollen, glassy cells; detachment from glass
Echoviruses	+++	+++	+	3.9	4.2	Focal, swollen, glassy cells; detachment from glass
Rhinovirus	+	+++	–	6.6		Focal, swollen, or granular cells
Adenovirus	++	+	+++	6.2	6.4	Enlarged, clustered cells (bunches of grapes or lattice)
Herpes simplex virus	++	+++	++	3.5	2.7	Enlarged or shrunken granular cells starting at edge; rapid progression and sloughing; may have giant cells
Varicella-zoster virus	++	++	–	7.6	6.1	Discrete, elongated foci of enlarged or shrunken cells; slow, contiguous progression enhanced by use of growth medium
Cytomegalovirus	–	+++	–	10	5.8	Compact foci of enlarged cells; slow, contiguous progression

[a] From the Mayo Clinic, abstracted from Herrmann EC Jr. Experience in providing a viral diagnostic laboratory compatible with medical practice. Mayo Clin Proc 1967;42: 112–123; and Herrmann EC Jr. Efforts toward a more useful viral diagnostic laboratory. Am J Clin Pathol 1971;56:681–686.
[b] From Mount Zion Hospital and Medical Center, San Francisco, abstracted from Drew WL. Controversies in viral diagnosis. Rev Infect Dis 1986;8:814–824.
+++, Optimal cells for detection of cytopathic effect (CPE); ++, CPE frequent, may be the best available system for detection of CPE; +, CPE may occasionally be observed; –, CPE does not usually occur; PMK, primary monkey kidney cells; HDF, human diploid fibroblast cells; HEp-2, human continuous cell line.
Adapted from McIntosh K. Diagnostic virology. In: Fields BN, Knipe DM, Melnick JL, et al., eds. Virology. 2nd Ed. New York, NY: Raven Press, 1990:411–440.

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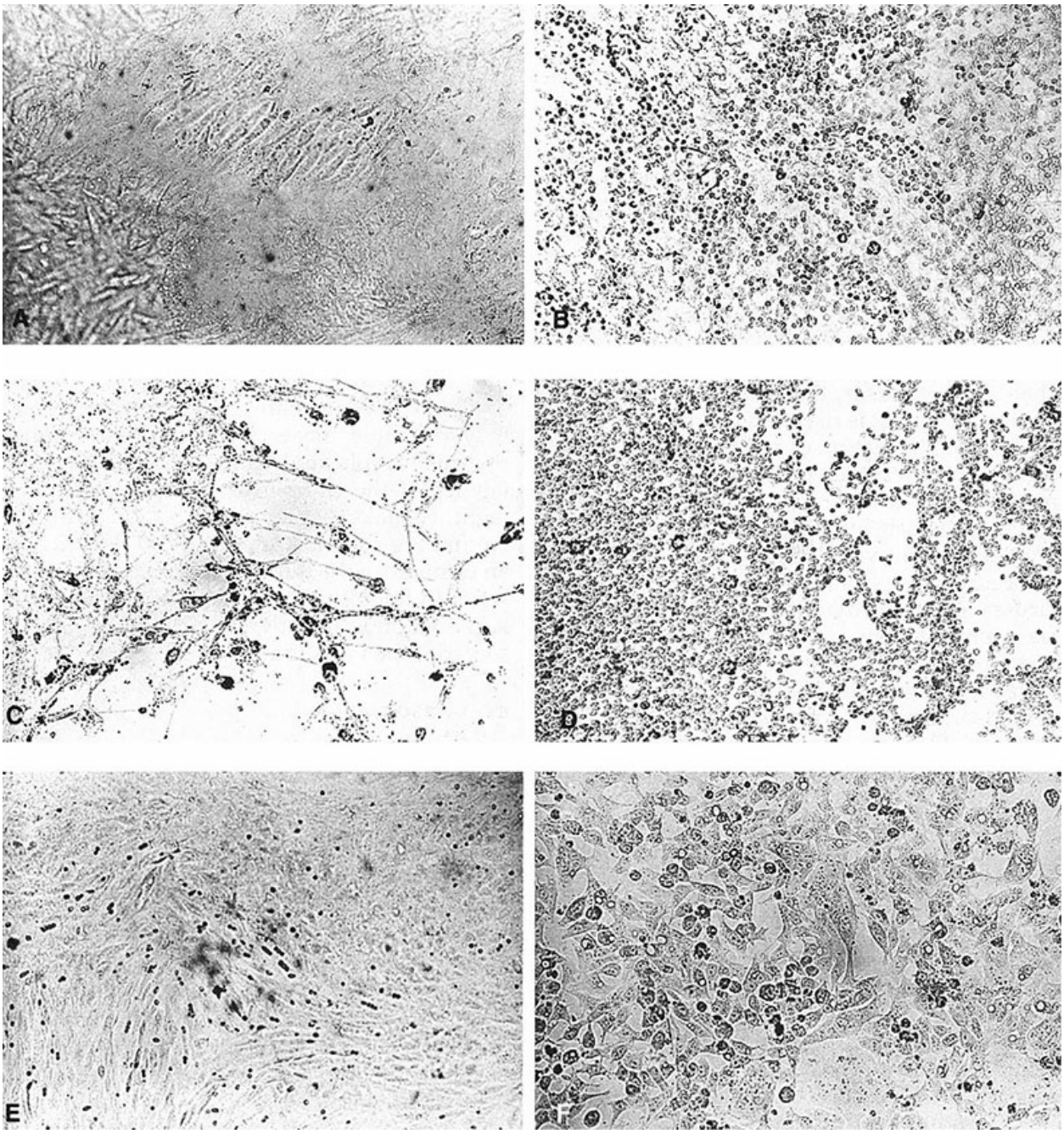

■ **FIGURE 23-13** Cytopathic effect of myxoviruses. **A.** Rhesus monkey kidney cells infected with influenza A virus. This isolate produces no cytopathic effect in the monolayer. A hemadsorption test identified the presence of a virus. Many of the current strains of influenza A virus and most strains of influenza B virus produce cytopathic effect, which often develops more rapidly than that produced by the parainfluenza viruses (×180). **B.** Rhesus monkey kidney cells infected with influenza A virus. The monolayer is intact but is covered with numerous guinea pig red cells. The red cells have adhered to the viral hemagglutinins on the surface of the infected cells. Hemagglutination of red cells in the fluid phase by free virus can often be detected but is not shown in the photograph (×180).

C. Rhesus monkey kidney cells infected with parainfluenza virus 3. The monolayer has degenerated, and cells have fallen off the glass. Many of the remaining cells are enlarged or granular. Often, isolates of parainfluenza viruses do not produce obvious cytopathic effect and must be detected by hemadsorption (×180). **D.** Rhesus monkey kidney cells infected with parainfluenza virus 3. A hemadsorption test has been performed. The remaining cells are covered with guinea pig red cells that have attached to the viral hemagglutinin on the cell membranes (×180). **E.** Rhesus monkey kidney cells that are uninfected demonstrate a negative hemadsorption test. A few guinea pig red blood cells have stuck to the monolayer, but the massive adherence of red blood cells that characterizes a positive test is not present. The settled cells are also dislodged more easily from an uninfected monolayer than from a myxovirus-infected monolayer by shaking the tube. **F.** HEp-2 cells infected with parainfluenza virus 3. Large multinucleated giant cells (known as syncytial cells) have formed in the monolayer by fusion of adjacent cells. This type of cytopathic effect is characteristic of parainfluenza viruses 2 and 3 in monkey kidney cells and parainfluenza virus 3 in HEp-2 cells. Syncytial cells are also formed by other viruses, notably respiratory syncytial virus. The parainfluenza viruses can be differentiated from respiratory syncytial virus easily by performance of a hemadsorption test (×180).

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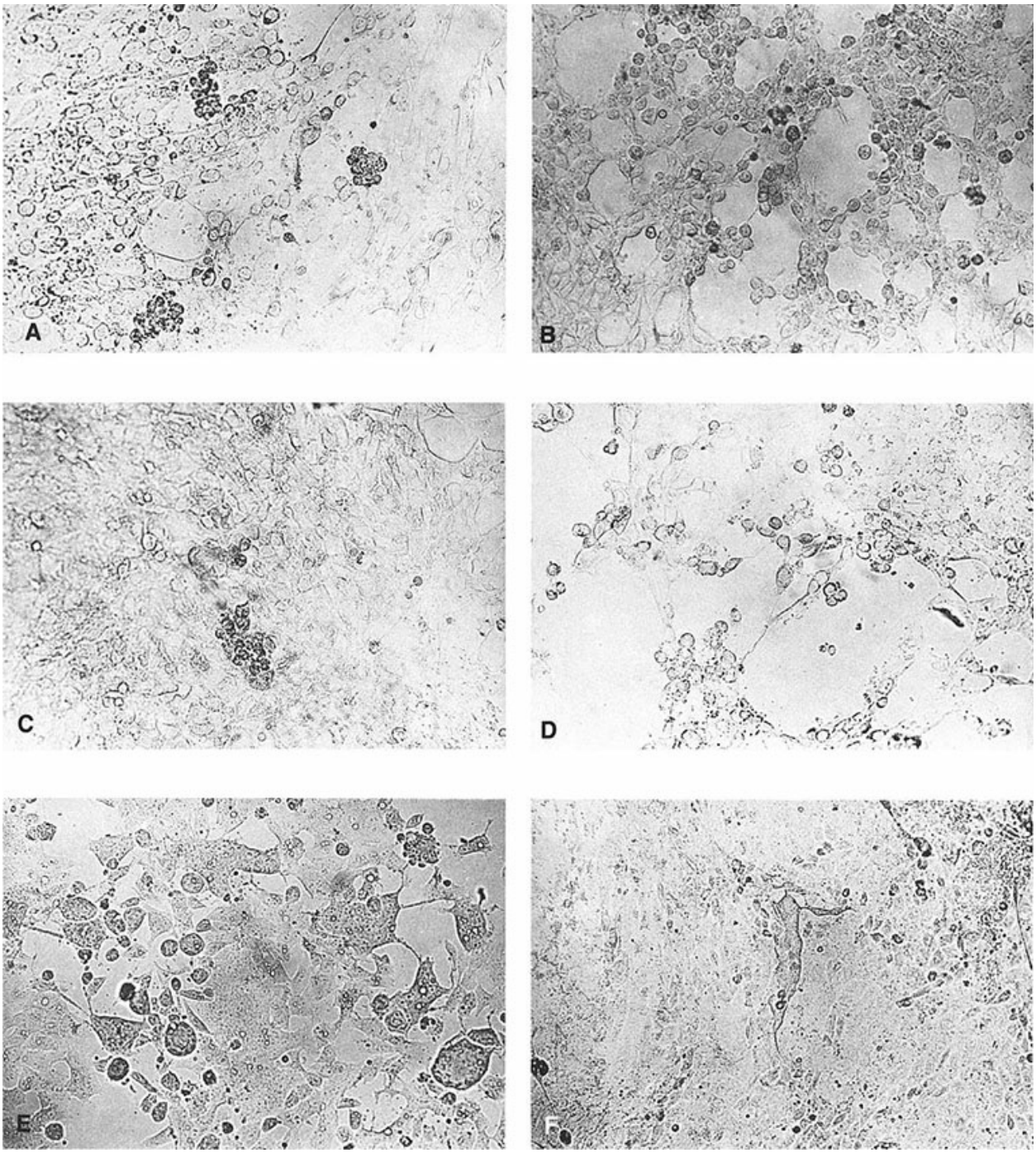

■ **FIGURE 23-14** Cytopathic effect of other respiratory viruses. **A.** HEp-2 cells infected with adenovirus. Several foci of enlarged, granular cells are evident. Adenovirus cells are often clustered like bunches of grapes, much as are staphylococci in a Gram stain (×180). **B.** HEp-2 cells infected with adenovirus. Most of the cells in the monolayer are now rounded; many cells have detached from the glass, leaving multiple holes. In several places, clustering of cells can be seen. Strands of cytoplasm connect some of the residual infected cells, producing the beginning of a lattice effect. (×180). **C.** Rhesus monkey kidney cells infected with adenovirus. One cluster of granular, enlarged cells is evident. A preliminary

report could be issued if multiple foci were present in the culture. If only a few are present, it would be best to observe the monolayer for progression of cytopathic effect. (×180). **D.** Rhesus monkey kidney cells infected with adenovirus. The extent of cytopathic effect has increased considerably. Clustered, swollen cells with intercellular cytoplasmic bridges are evident. Many cells have detached from the glass. Eventually, there will be only scattered cells left on the glass tube as evidence that a monolayer was once present (×180).**E.** HEp-2 cells infected with respiratory syncytial virus. Many of the cells in the monolayer have been fused by the virus to form syncytial giant cells, much like those formed by parainfluenza viruses. Other cells are enlarged or have detached from the glass. A hemadsorption test on this monolayer would be negative. (×180). **F.** Rhesus monkey kidney cells infected with respiratory syncytial virus. One large syncytium has formed in the monolayer. One could differentiate this isolate from parainfluenza virus by hemadsorption or by identification of specific antigen.

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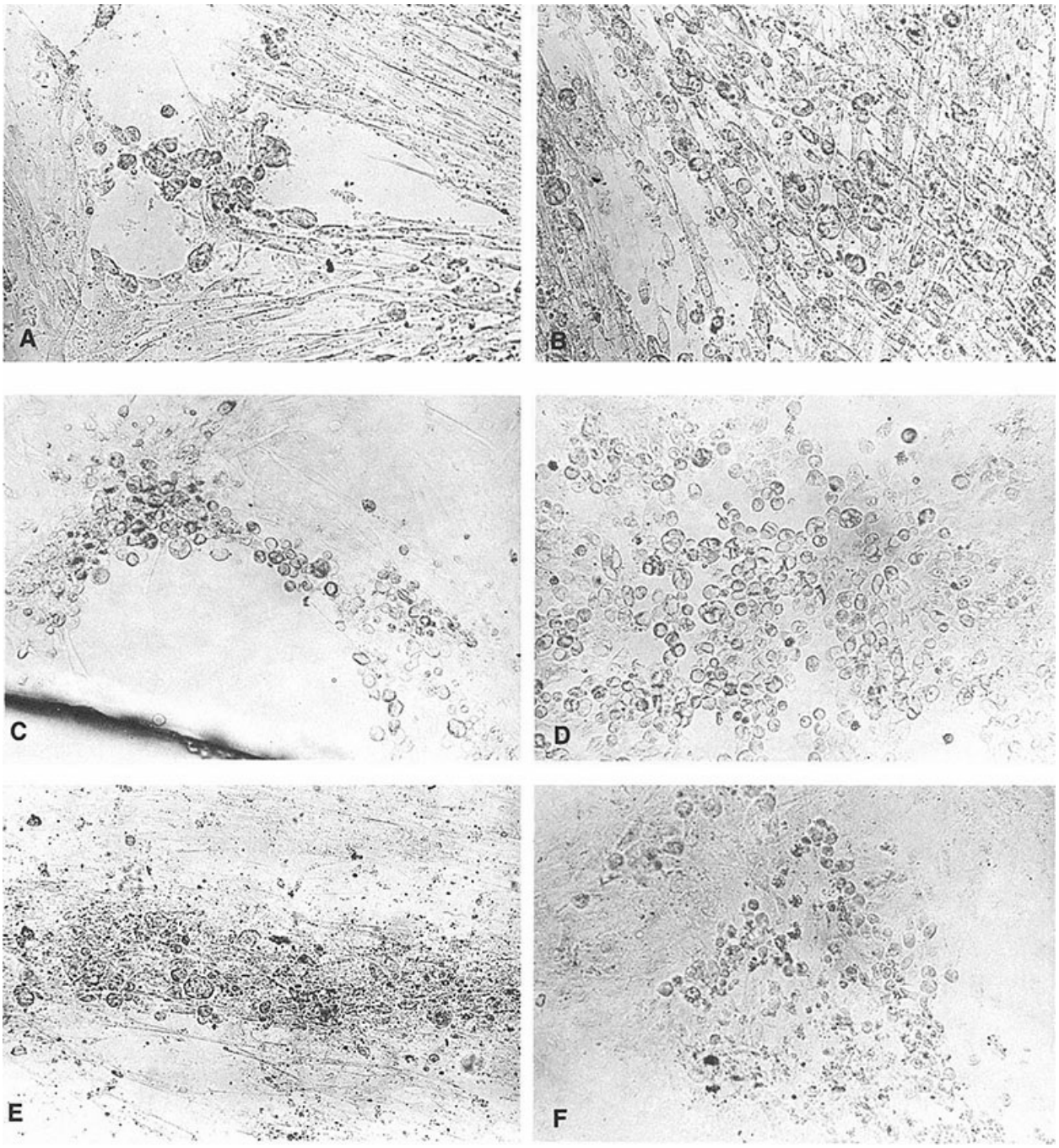

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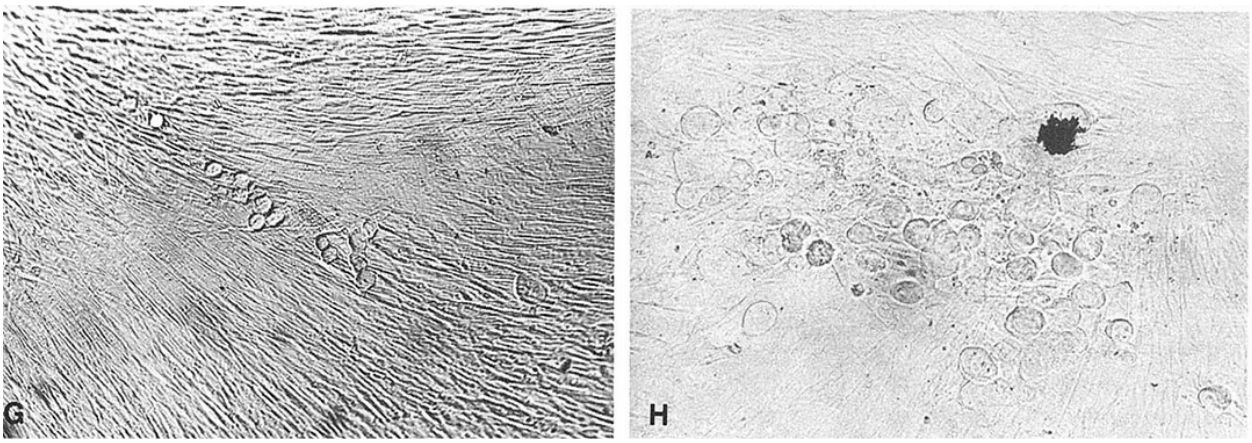
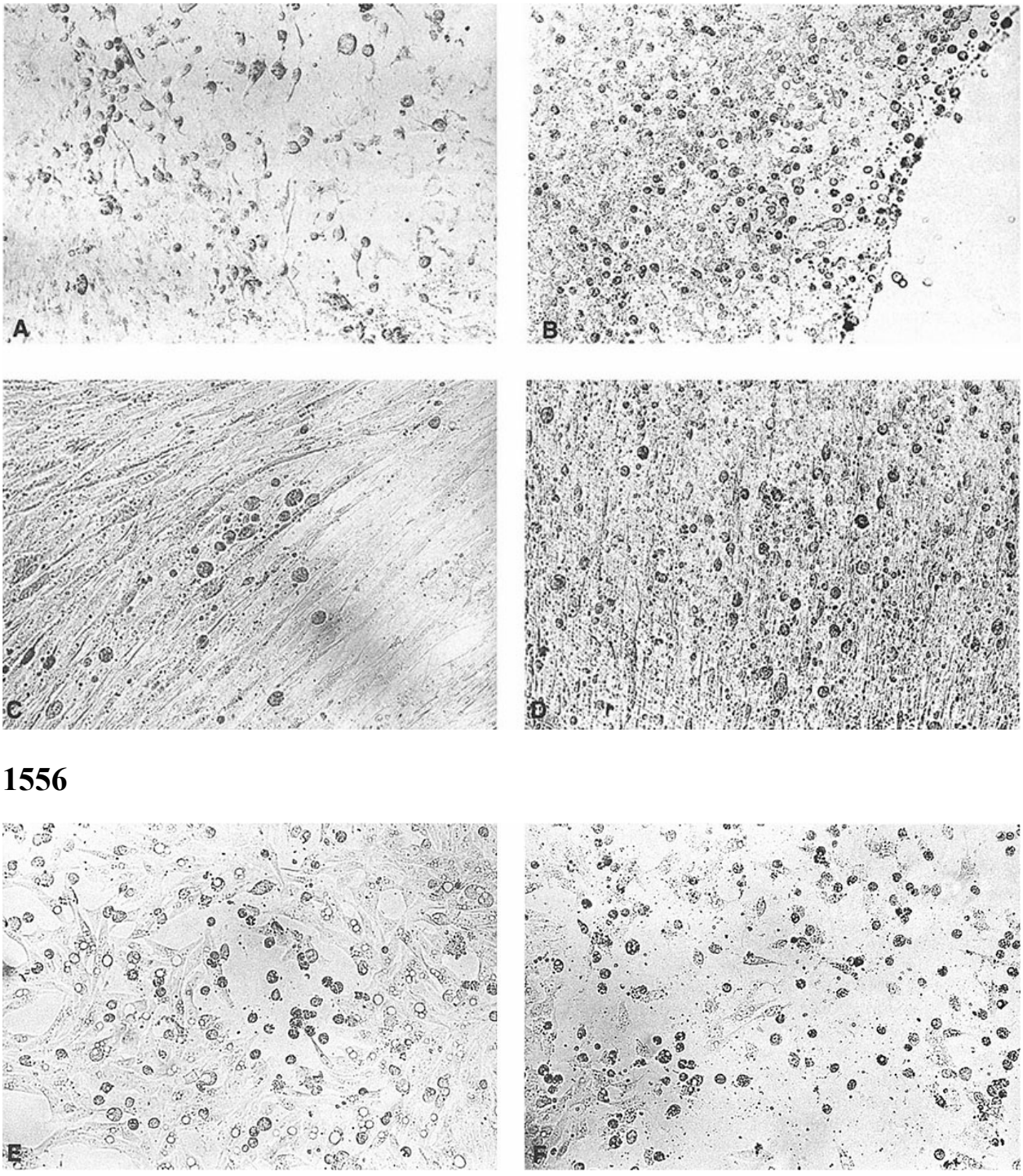


■ **FIGURE 23-15** Cytopathic effect of herpesviruses. **A.** MRC-5 fibroblasts infected with herpes simplex virus. There is a focus of enlarged, rounded cells in the monolayer. Many cells have detached from the glass. Such foci will enlarge rapidly, and the entire monolayer is usually involved within a few days (×180). **B.** MRC-5 fibroblasts infected with herpes simplex virus. Cytopathic effect has progressed so that virtually every cell in the monolayer is affected. Again, the cells are enlarged, even ballooned and granular. This type of generalized cytopathic effect can occasionally be seen with cytomegalovirus if very large quantities of virus are present. Cytomegalovirus can be distinguished from herpes simplex virus by identification of specific antigens (×180). **C.** Rhesus monkey kidney cells infected with herpes simplex virus. As often happens at the outset, this focus of cytopathic effect is at the edge of the monolayer. Enlarged, ballooned cells surround the center of the focus, where the cells have already detached from the glass (×180). **D.** HEp-2 cells infected with herpes simplex virus. There is a large focus of swollen cells in the monolayer. Some of the cells have the appearance of multinucleated giant cells, although it is difficult to delineate the cellular structure in an unstained preparation at low magnification. (×180).. **E.** MRC-5 fibroblasts infected with varicella-zoster virus. A cytopathic focus consists of large swollen cells and granular degenerated cells. The focus is elongating in the direction of the underlying fibroblasts. The slow appearance and slow progression of the foci are characteristic of this virus. Transfer of cytopathic effect requires the inclusion of infected cells. Cytopathic effect has some similarities to that of cytomegalovirus; the foci of cytomegalovirus tend to be more compact and spread centrifugally without such a pronounced linear orientation (×180). **F.** Rhesus monkey kidney cells infected with varicella-zoster virus. A focus of enlarged, swollen cells is less distinctive than cytopathic effect in MRC-5 cells. It can be differentiated from that produced by herpes simplex virus by the speed with which it appears and progresses. Cytomegalovirus does not produce cytopathic effect in monkey cells (×180). **G.** WI-38 fibroblasts infected with cytomegalovirus. There is a small focus of enlarged, granular cells in the monolayer (×180). **H.** MRC-5 fibroblasts infected with cytomegalovirus. A developing focus of cytopathic effect is evident. The cells are swollen, and the focus is quite compact. Cytopathic effect of cytomegalovirus appears late and progresses slowly. It tends to be more compact and less linear than that of varicella-zoster virus. Differentiation from

varicella is by clinical history, by demonstration of multinucleated giant cells in varicella monolayers, and by demonstration of specific antigen (×180).



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■ **FIGURE 23-16** Cytopathic effect of enteroviruses. **A.** Rhesus monkey kidney cells

infected with coxsackievirus B. There is a focus of cytopathic effect in which cells are either small and shrunken or enlarged. Some of the cells have a refractile or glassy appearance that is typical of enteroviral cytopathic effect. Elongated "tadpole" cells are also typical of the cytopathology produced by this group of viruses (×180). **B.** Rhesus monkey kidney cells infected with coxsackievirus B. Cytopathic effect progresses relatively rapidly with the enteroviruses; poliovirus may cause complete destruction of the monolayer overnight. In this photomicrograph, virtually every cell in the monolayer has been damaged, and large segments of the cell sheet (lower right) have peeled off entirely (×180). **C.** MRC-5 fibroblasts infected with echovirus 11. An early focus of enteroviral cytopathic effect is evident. The cells appear enlarged and granular. It would be very difficult at this stage to guess the identity of the virus. Most isolates of echovirus grow well in human diploid fibroblasts, whereas isolates of coxsackievirus do not (×180). **D.** MRC-5 fibroblasts infected with echovirus 11. Cytopathic effect has extended to involve almost the entire monolayer. Virus is spread extracellularly through the medium so that cytopathic effect tends to change from focal to generalized in nature relatively rapidly (×180). **E.** HEp-2 cells infected with coxsackievirus B. A small focus of cytopathic effect in the HEp-2 cells is manifested by rounded, shrunken granular cells and detachment of cells from the glass. Early cytopathic effect is much more difficult to recognize if the cell sheet is too heavy and the cells are piled one on top of the other (×180). **F.** HEp-2 cells infected with coxsackievirus B. Almost all of the cells in the monolayer have detached from the glass. Those that remain are abnormal. Most coxsackievirus B isolates grow well in HEp-2 cells, whereas coxsackievirus A and echovirus do not (×180).

There are relatively few ways in which cells can express cytotoxicity. Rounding is a common expression of damage, but cells may appear either swollen or shrunken and granular or glassy. Some viruses produce factors that cause cell fusion, leading to the formation of multinucleated giant cells, which are also called syncytial cells; RSV, parainfluenza viruses 2 and 3, measles virus, and mumps virus can produce syncytial giant cells. In addition, HSV and VZV produce smaller multinucleated giant cells.

Not all isolates of RSV produce syncytial cells. Production of the F(usion) glycoprotein by the virus is required for cell fusion. Shahrabadi and Lee demonstrated that calcium must be present in the culture medium for RSV-induced fusion of HEp-2 cells to occur.[422] The formation of syncytial giant cells also appears related, at least in part, to the concentration of glutamine in the culture medium.[309]

Cytopathic effect usually starts focally and often is most readily seen at the edges of the monolayer, where the density of cells is low. For viruses that spread from cell to cell through the culture media, cytopathic effect progresses from focality to totality, sometimes with great rapidity. If the number of virions in the specimen is very large, most of the monolayer may be infected simultaneously; in this case, widespread degeneration must be differentiated from nonspecific cytotoxicity by subculture of fluid to fresh cell cultures. For viruses that spread directly from cell to cell (CMV and

VZV), cytopathic effect progresses more slowly and by local extension of the initial foci. When a very large amount of CMV is present in the specimen, the cytopathic effect may be produced through the whole monolayer, mimicking the effect produced by HSV.

It is, of course, somewhat artificial to try to capture the considerable variability of a biologic process in a single photographic frame. In a clinical laboratory, it will be possible to examine other parts of the monolayer, view other cell cultures, and even reexamine a suspect culture after incubation for a few more hours. It will often be possible, therefore, to detect cytologic changes that are even earlier than those that make convincing photographs.

The majority of respiratory viruses are now detected by direct molecular methods rather than culture. However, in some instances, a "test of cure" may be requested by clinicians, particularly in immunocompromised patients who are having difficulty clearing the virus. Additionally, viral culture for these pathogens has been retained in highly specialized laboratories and other settings. Figures 23-13 and 23-14 have, therefore, been retained in this addition of the text. The use of guinea pig red blood cells (i.e., the hemadsorption test), however, has been replaced by direct detection of antigen (i.e., DFA) using high-quality, commercially available product.

Light Microscopy. As they replicate, some viruses produce masses of nucleoprotein and virions in various stages of assembly. The anatomic pathologist or cytologist uses these viral inclusions to detect the presence of a viral infection and identify the agent presumptively.[446,494] Currently, the anatomic pathologist supplements the morphology features present in routinely stained histologic sections or cytologic preparations with the use of highly specific immunohistochemical stains. These stains employ antibodies, often monoclonal antibodies, that have a high degree of specificity for the particular viral target.

The virologist also uses light microscopy. They use it to examine unstained cellular monolayers for cytopathic effect caused by the replicating virus. Although the characteristics of cytopathic effect in unstained monolayers are usually sufficient to render a provisional diagnosis, the virologist may gain additional information using simple stains (Image Plate 23-1). Although feasible, this is rarely performed in practice. Today, virus-specific immunofluorescent stains are used to determine the cause of the cytopathic effect.

Electron Microscopy. For laboratories with access to an electron microscope, the ultrastructural morphology of an isolate may identify the group to which it belongs.[4,344] These methods were used with much greater frequency in the past, prior to the availability of specific immunohistochemical and immunofluorescent reagents. Negative-staining electron microscopy is simple and may be accomplished rapidly on supernatant fluid or lysed cells. This technique may be particularly useful for

differentiation of nonspecific toxicity from a large viral inoculum. Currently, electron microscopy is most useful for the categorization of emerging viruses, as was accomplished with the SARS outbreak.[190,425]

Biochemical Differentiation. The cytolytic changes produced by rhinoviruses may resemble those of enteroviruses,

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although rhinoviruses replicate less rapidly and demonstrate the lability to an acid environment. To make this assessment, aliquots of infected cell culture are adjusted to pH 3.0 or 7.4 for 3 hours. Then an estimate of the infectivity is obtained by inoculating serial 10-fold dilutions of the aliquots into cell cultures. A reduction in infectivity of 2 to 4 $\log_{10}$ should be observed if the virus is a rhinovirus.[249] This is currently only performed in highly specialized laboratories, but remains important to investigate the cross-reactivity of molecular assays for enteroviruses and rhinovirus, which occurred recently with the outbreak of enterovirus, EV D-68.

Cell Association. The demonstration of the association of virions with infected cells is useful to differentiate members of the herpesvirus group. HSV is released freely into the culture medium, whereas CMV and VZV remain cell-associated. HSV rapidly infects the whole monolayer (Fig. 23-15B). Alternatively, the cytopathic effect of CMV and VZV is usually focal (Fig. 23-15E through H), because virions do not spread through the medium. The type of cytopathic effect present informs the virologist regarding the type of DFA that should be selected for confirmatory studies. Occasionally, however, large numbers of viral particles of CMV or VZV in the inoculum may produce widespread cytopathic effect resembling that of herpes simplex. In this instance, the DFA stains for HSV would be negative, whereas the subsequent studies with DFA reagents for CMV or VZV should confirm the identification.

Detection of Viral Antigens. In addition to confirmation of the identity of a cytopathic agent, the demonstration of specific antigens in culture has been used as a method for detecting the presence of a virus, even in the absence of cytopathic effect. Tests for antigen may be performed after a brief incubation if rapid diagnosis is desired, an approach that has been used more commonly with the use of shell vials. The most dramatic improvements in the rapidity with which isolates are detected will occur with viruses that have the slowest growth in conventional systems, such as RSV, CMV, and VZV (Image Plates 23-2D, E, and F).

The literature is replete with disparate reports on the sensitivity of such expedited approaches to viral diagnosis. For instance, the percentage of influenza virus isolates

recovered after spin amplification, incubation overnight, and staining with monoclonal antibodies has ranged from 56%[135] to 98%.[123] Differences in operational variables, such as cell lines and source of monoclonal antibodies as well as varied patient populations, make universal recommendations treacherous. Perhaps it is fortunate that the detection of this virus in particular is now predominantly performed using molecular methods.

It is clear that expedited culture techniques can contribute useful information but that there is a trade-off between sensitivity and rapidity when deciding on the length of incubation. Including conventional cultures and/or sampling shell vials at several times will increase sensitivity, but also increase costs. Although it is difficult to document, provision of results more rapidly may have clinical and financial impact.[17] These techniques have also been used to facilitate the detection of fastidious viruses, such as the rubella virus.[414] The convenience of using DFA reagents for the confirmation of cytopathic effect is that these are usually the same reagents employed in the laboratories performing these tests for the direct assessment of virally infected cells in clinical specimens; ergo, there are two uses for one reagent.

Artifacts and Non–Virus-Induced Changes. As cells age in culture, morphologic changes unrelated to viral infection may occur (Fig. 23-7). The density of cells in diploid cell lines and, in particular, established cell lines may become very high; dying or dividing cells may appear rounded or granular. Scattered atypical cells occur in any culture and are especially noticeable in less-dense areas, such as the edge of the monolayer, where viral cytopathic effect may also become apparent first. It is important, therefore, to maintain uninoculated controls of cells from the same batch as those used for specimens.

Primary monkey kidney cells may be infected with simian viruses that produce a variety of degenerative changes,[249] including a foamy or vacuolated appearance in the monolayer.

The presence of extraneous cells that settle on the monolayer may also cause confusion (Fig. 23-7D). Cytopathic effect may be mimicked because the contaminating cells differ in appearance from the original monolayer. Usually a virus that produces focal abnormalities in the monolayer, such as CMV, is simulated. The artifact may be suspected because the cells are on top of the monolayer, rather than becoming an integral part of it. Contaminating cells may be of human origin, such as epithelial or mesothelial cells present in the specimen, or may have originated in other cell cultures, particularly if a subculture from one cell line to another has been performed.

A variety of nonviral agents or substances in clinical specimens may be toxic to cell monolayers (Fig. 23-7A). If bacterial or fungal growth occurs (Fig. 23-7B), the nature of the problem is obvious; the specimen must then be treated again with antibiotics or

filtered and inoculated into additional cell cultures.

Usually the nature of the cytotoxicity is unknown. The ordinary remedy is to dilute the specimen and inoculate additional monolayers. Howell and associates[236] found that the toxicity of semen from patients with AIDS could be avoided by inoculating the monolayer with a cell pellet, obtained by high-speed centrifugation. CMV, which is a frequent pathogen in patients with AIDS, is associated with leukocytes. Conversely, toxic substances were concentrated in the supernatant fraction.

On occasion, cytopathic effect may be "specific," although the cause is unexpected and perhaps unwanted. *Clostridium difficile* toxin, for example, in fecal specimens (Fig. 23-7C) may produce cytopathic effect resembling enteroviral damage.[415]

Gentry and associates[168] reported the isolation of *Trichomonas vaginalis* in BHK cell cultures. These flagellates produced a cytopathic effect that mimicked that of HSV; cytopathic effect was reproduced when viable *Trichomonas* or lysates from axenic cultures were added to cell cultures. Motile trichomonads could be seen when the cultures were inspected closely.

Isolation of *Pneumocystis jirovecii* from immunocompromised patients with AIDS has been reported. It is difficult

to propagate this atypical fungus serially in the laboratory, but organisms have been grown in cell cultures, including MRC-5 human diploid fibroblasts.[286] The *P. jirovecii* isolates produced a cytopathic effect that resembled viral effects. *Toxoplasma gondii* may also be recovered in cell culture lines, such as human diploid fibroblasts, although the sensitivity of culture has not been evaluated.[79]

Definitive Identification of Isolates

In a diagnostic laboratory, the combination of clinical history and cytopathic effect provides sufficient information to identify some viruses, including HSV, CMV, VZV, adenovirus, and RSV. Enteroviruses can usually be identified to the genus level. Unfortunately, too often, limited clinical information is available. The complete identification of isolates, including typing, requires immunologic characterization of the viral antigens or viral nucleic acid.

Immunologic tests can be adapted either for identification of antigens or antibodies against those antigens. Antisera, traditionally polyclonal, are increasingly monoclonal (see Chapter 3). The most specific immunologic test for the majority of viral infections is the neutralization assay, but this is no longer performed in most clinical laboratories. A review of these methods may be found in previous editions of this text.

Many viral tests are important for epidemiologic analysis, but are rarely necessary

for routine diagnostic purposes. In instances in which complete characterization is necessary (e.g., paralytic disease), the isolate may be sent to a reference laboratory. A thorough discussion of antigenic characterization, which is beyond the scope of this chapter, may be found in textbooks of virology and viral diagnosis.[237,271,436]

For many hospital laboratories immunofluorescence assays provide a viable alternative by which definitive identification of many viruses to the genus and/or species level can be made. Fortunately, the expanding catalog of commercially available reagents, which are of increasingly high quality, simplifies the task. The enteroviruses and rhinoviruses are notable exceptions because their complicated antigenic composition makes definitive identification difficult without neutralization tests.

The immunofluorescence test has been used for many years and in many variations. The DFA assay is the simplest to perform, but specific conjugated antisera are required. Indirect immunofluorescent tests (IFA)—using antiglobulin, staphylococcal protein A, or complement—are more sensitive but often suffer from lower specificity. In contrast to the direct test, multiple conjugated reagents are not needed to perform the indirect test, but the procedure is more time-consuming because of the serial steps required. DFA assays are simple to perform and in the hands of a skilled microscopist deliver very high sensitivity and specificity, in some instances, rivaling PCR. These assays represent a viable alternative to molecular diagnostics, if the clinical setting and test volumes dictate. The limitations of these tests are that they require fluorescent microscopy, and if test volumes are high (e.g., during influenza season), then the test may be tedious and time-consuming, ergo, the migration to molecular diagnostic methods.

Storage of Viral Isolates

If the necessity for additional characterization of an isolate seems probable, infected cell culture fluid should be frozen at −70°C. A general rule is that mammalian cells should be frozen slowly, whereas infectious agents should be frozen quickly. The dramatic decrease in viability that accompanies the freezing process is minimized if the cultures are snap-frozen and if a stabilizing agent is included in the medium. A mixture of dry ice and alcohol is a convenient means for snap-freezing viral isolates. Many stabilizing agents have been used and many are commercially available. Howell and Miller found that the sucrose phosphate broth that is used for transport of swabs for isolation of *Chlamydia* functioned as well as 70% sorbitol for maintenance of some of the most fastidious viruses, including CMV and RSV.[235]

Summary of Culture-Based Detection and Identification of Viruses

The orthomyxoviruses, paramyxoviruses, and RSV (Figs. 23-13 and 23-14) produce a

variety of cytopathic effects in cell culture. Many isolates of influenza and parainfluenza viruses produce no visible changes in the monolayer and must be detected by other means. Cytopathic strains can be differentiated from other viruses with similar effects by noting the restriction of cytopathic effect to monkey kidney cells, in the past by hemadsorption, and currently through the use of virus-specific immunofluorescence reagents. Multinucleated giant cells occur in monolayers that have been infected with measles, respiratory syncytial (Fig. 23-14E and F), parainfluenza (Fig. 23-13F), and mumps viruses; they are produced by virus-directed fusion of adjacent cells (syncytium formation). Such cells may also be seen in the clinical specimens from infected patients (Image Plate 23-1F and G).[446,494]

Isolates are now definitively identified by immunologic means. Antisera of good quality are available for most of the common myxoviruses. The genus or type of virus (e.g., influenza A virus or RSV) may be identified by immunofluorescence of infected cells.

Adenoviruses (Fig. 23-14A through D) can be identified presumptively by observing the characteristic cytopathology in cell cultures. Clusters of swollen infected cells or a lattice appearance to the monolayer are typical. Group-specific antigens that are common to all adenovirus serotypes have been identified, and monoclonal antibodies to the common determinants have been prepared. For most clinical applications, confirmation of the genus of adenovirus by immunofluorescence is sufficient. If the type of adenovirus must be determined, neutralization tests are available in specialized laboratories.

The herpesvirus group produces a variety of cytopathic effects in cell culture. EBV does not grow in the cell cultures commonly used in clinical laboratories; human infections with this virus are usually diagnosed serologically. CMV and VZV are highly cell-associated (Fig. 23-15E through H). They are difficult to grow in cell culture, and they grow slowly. Successful subculture requires transfer of infected cells. Both CMV and VZV produce swollen cells in foci that slowly enlarge by direct spread; the plaques of VZV, in

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particular, tend to follow the orientation of the fibroblastic tissue culture cells. In contrast, HSV is spread extracellularly (Fig. 23-15A through D); cytopathic effect starts focally but spreads rapidly to affect other parts of the monolayer.

Centrifugation of the inoculum onto the monolayer has accelerated the recovery of HSV, VZV, and CMV. Monoclonal antibodies have proved valuable for both detection and identification of CMV, VZV, and HSV. Separation of isolates of herpes simplex into types 1 and 2 can be accomplished reliably with fluorescein-labeled monoclonal antibodies.[294]

Enteroviruses grow relatively rapidly in cell cultures, producing a widespread cytopathic effect (Fig. 23-16). Although there is an FDA-approved RT-PCR assay for enterovirus, it is only approved for CSF, so stool and other specimens are usually submitted for enteroviral culture. In cell culture, affected cells may be shrunken and granular or may have a refractile, glassy appearance. Most serotypes of coxsackievirus A do not grow well in commonly used cell cultures. Isolates of coxsackievirus B usually do not grow well in human diploid fibroblast lines; echovirus strains do not grow well in HEp-2 cells. Definitive identification of an enterovirus requires immunologic identification. Fluorescein-labeled monoclonal antibodies directed against a genus-specific antigen and selected serotypes are available commercially. Immunofluorescence is not foolproof, especially for nonpolio enteroviruses.[397] The reference method for identification is the neutralization test, but differentiation is now more commonly accomplished by nucleic acid sequencing.[307]

Direct Detection of Viruses in Clinical Specimens

The desire for greater rapidity in diagnosis of viral infections has been stimulated by the availability of rapid diagnostic methods in other areas of the laboratory and by the development of effective antiviral chemotherapy. A variety of methods are available, ranging from the traditional to the experimental. The greatest area of growth, which is discussed thoroughly below is the introduction of advanced and often easy-to-use molecular methods of detection.

Light Microscopic Detection of Inclusions

The detection of viral inclusions in smears or tissues by light microscopy has been the traditional means of directly demonstrating viral infections.[446,494] In general, viruses that are assembled in the nucleus (i.e., usually DNA viruses) produce intranuclear inclusions, whereas cytoplasmic assembly (i.e., predominantly RNA-containing viruses) yields cytoplasmic inclusions. Fortunately, some viruses produce obvious cytopathic changes in routinely stained specimens. The identity of these, which may be highly suspected based on the routine H&E findings, may be definitively confirmed using specific immunohistochemical stains.

Intranuclear inclusions may be produced in cells that have been infected by HSV, VZV, CMV, adenovirus, papilloma virus, and the BK and JC polyoma viruses. The inclusions of HSV (Image Plate 23-1A, B, and C) and VZV are indistinguishable in routinely stained material, but can be differentiated with specific immunohistochemical stains. The infected nuclei have a homogeneous, eosinophilic, or slightly basophilic appearance, and the granular, basophilic nuclear chromatin is pushed against the edge

of the nuclear membrane (i.e., marginization of the chromatin). Other infected cells may have a more eosinophilic inclusion that is accentuated by a halo between the inclusion and the nuclear membrane. This halo is an artifact of fixation, but is useful diagnostically. Ultrastructurally, the inclusions are composed of a mixture of deoxyribonucleoprotein and assembled virions (Fig. 23-3A); as the virions pass through the nuclear membrane, they acquire a lipid membrane. The infected cells may be mononuclear or multinuclear.

CMV produces basophilic nuclear inclusions in an enlarged cell (Image Plate 23-1D). These distinctive cells, which are essentially pathognomonic of CMV infection, were originally believed to be protozoa that were invading tissue.[259] A halo is often present around the inclusion. Granular, basophilic inclusions may be found in the cytoplasm of some infected cells.

A variety of inclusions may be found in adenovirus infections. Early inclusions are eosinophilic and may closely resemble herpesvirus-infected cells. Inclusions become more basophilic as they mature and increasingly fill the nucleus (Image Plate 23-1E). Eventually, they become extremely basophilic and may completely distort the cell; these diagnostic cells are referred to as "smudge cells" and must be distinguished from uninfected cells with hyperchromatic nuclei. The adenovirus inclusions are composed of nucleoprotein and numerous virions, which often demonstrate a paracrystalline array because their icosahedral shape permits tight packing of the particles (Fig. 23-3B).

The intranuclear inclusions of the papilloma viruses will not be encountered frequently by clinical virologists because these viruses are difficult or impossible to grow in culture. The inclusions in human warts begin as granular eosinophilic matter, later condensing into rounded, basophilic masses; cytoplasmic inclusions in the epidermal cells of warts are condensed keratin and are not virus-specific. In cervical cytology specimens, the nuclei of koilocytes are enlarged and hyperchromatic with cleared perinuclear halo.

The intranuclear inclusions of the BK and JC polyoma viruses will also not be encountered by clinical virologists, as these viruses do not grow in commonly used cell lines. The inclusions in progressive multifocal leukoencephalopathy occur in oligodendroglia (nonnerve cells that perform a supportive function); the inclusions vary from small granular material to large basophilic masses that fill the nucleus. The BK viral inclusions appear similarly but occur in tubular epithelial cells and urothelial cells. Immunohistochemical or immunofluorescent studies or in situ hybridization should be used to confirm the nature of these inclusions.

Intracytoplasmic inclusions may be found in cells that have been infected by RSV, rabies virus, and viruses of the pox group. The intracytoplasmic inclusions of RSV are brightly eosinophilic (Image Plate 23-1G); they are present regularly in cell culture but less frequently in clinical specimens. Ultrastructurally, the inclusions of RSV consist of

fibrillar ribonucleoprotein (Fig. 23-3C). The intracytoplasmic

inclusions of rabies virus are known as Negri bodies (Image Plate 23-1H). They may be single or multiple and occur in neurons that appear otherwise normal. The edges of the inclusions are sharply defined, as if bounded by a membrane. Clear spaces or basophilic stippling, which may be seen within the inclusion, result from the incorporation of some cytoplasmic material into the mass of viral ribonucleoprotein (Fig. 23-3E). Fortunately, the intracytoplasmic inclusions of the poxviruses, known as Guarneri bodies, are unlikely to be encountered today.

Intranuclear and intracytoplasmic inclusions are found in measles infection. During the prodromal stage of measles, giant cells appear in lymphoid tissue throughout the body. These so-called Warthin–Finkeldey giant cells contain as many as 100 nuclei and rarely may contain inclusion bodies. A second type of giant cell may arise later from epithelial tissue, such as that lining the lower respiratory tract. There are fewer nuclei in these giant cells, but inclusions are almost constant. The intranuclear inclusions resemble those produced by the herpesviruses, may be single or multiple, and vary in their degree of eosinophilia (Image Plate 23-1F).

Detection of inclusions provides valuable information in selected clinical situations. The Tzanck test, performed by preparing a Giemsa-stained smear of vesicular scrapings, can be used to document the presence of a herpesvirus by demonstrating multinucleated giant cells (Image Plate 23-1C). Although this approach is less commonly performed, hematoxylin–eosin or the Papanicolaou stain may also be used with the submission of the specimen to surgical pathology and cytology, respectively. The nuclei should have a ground-glass and molded or multifaceted appearance.[433] Intranuclear inclusions, which are accentuated in histologic sections by the artifactual halo produced by the fixation of tissues in formalin, may not always be visualized in the smears. A presumptive diagnosis of herpes infection can be made, although differentiation between HSV and VZV is not possible by morphology alone.

Solomon and colleagues found positive Tzanck tests in 11 patients with clinical chickenpox (varicella virus) and in 12 of 15 patients (80%) with shingles (herpes zoster virus). However, varicella virus was recovered in only 7 of the 11 patients with chickenpox, which corroborates the insensitivity of culture for VZV.[433] Multinucleated giant cells or inclusions or both have been demonstrated in skin lesions from 18 of 21 patients (86%) from whom HSV was isolated.[342] An analysis of the proficiency with which dermatologists correctly interpreted a study set of 10 smears demonstrated a "reverse learning curve."[188] Second- and third-year residents correctly interpreted a mean of 91% of the slides; dermatologists in practice for less than 10 years, 84%; and dermatologists in practice for more than 10 years, 67%.

Although the direct smears have worked well in the study of skin lesions, the sensitivity has unfortunately been low for other applications. Only a minority of culture-positive specimens from the genital tract[452] or the respiratory tract[349] contained inclusion-bearing cells. Similarly, Nahmias and colleagues[348] found intranuclear inclusions in only 56% of 113 cases of herpes simplex encephalitis. The specificity was considerably higher (86%) than the sensitivity in this collaborative study of antiviral chemotherapy, but false-positive results are potentially dangerous if therapy for another condition, such as bacterial brain abscess, is omitted. Intranuclear inclusions were reported in 10 patients from whom HSV was not isolated; in 7 of these patients, an alternative diagnosis not associated with inclusions was established. These results emphasize the importance of verifying the nature of the inclusion using immunohistochemistry, immunofluorescence, or other assays.

Histologic detection of CMV inclusions in tissue is less sensitive than culture, but may be equivalent using more "sophisticated" techniques, such as immunohistochemistry and in situ hybridization, for revealing CMV in tissue.[447] Morphologic methods have the advantage of better correlation with disease produced by CMV than does the more sensitive culture, since low levels of virus may be shed without producing overt disease.

In parvovirus B19 infection, intranuclear inclusions are also found in giant pronormoblasts, which are found in the bone marrow.[274] These characteristic cells may also be seen in peripheral blood and other tissues in infected fetuses.

Electron Microscopic Detection of Viral Particles

Electron microscopy can be applied to the study of both clinical specimens and cell cultures, either in thin sections of tissue or by negative stain microscopy. The inaccessibility of electron microscopes to most clinical laboratories and the sampling errors inherent in the examination of small samples of tissue have limited the routine application of this technique in making diagnoses of viral disease. The use of electron microscopy was specific for the diagnosis of herpes simplex encephalitis in one study (98%), but herpes virions were detected in only 45% of the infected patients.[348] The technique has been used effectively in the detection of viral agents of gastroenteritis, especially those that are not recovered by conventional cell culture (rotavirus, Norwalk-like agents, and enteric adenoviruses). However, ultrastructural diagnosis remains a research technique in most laboratories.

Negative stain electron microscopy has drawn renewed interest as a result of increased concern about bioterrorism. When examining a specimen with the electron microscope, there is no requirement for predetermining the antigens or nucleic acids to be detected. The diagnosis of important viral infections, such as smallpox, can be made quickly and safely, as the specimen is rendered non-infectious before

examination.[209]

Immunologic Detection of Viral Antigen

A variety of immunologic methods have been used to expedite diagnoses of certain viral infections. Immunofluorescence stains of clinical specimens and immunohistochemical stains of tissues are frequently used techniques. These techniques have many advantages and have functioned well when applied by trained persons. The risks of erroneous results are great in the absence of proper equipment and training.[116] The sensitivity of direct immunofluorescence studies varies by pathogen, as will be covered more thoroughly below. An understanding of the test performance helps the laboratory director determine whether these tests are sensitive and specific enough to "stand alone," or whether they should be performed in conjunction with culture or, more recently,

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molecular studies. Immunoassays, often lateral flow immunoassays, have been used in physician's offices and other laboratories for many years, as some of these are CLIA-waived tests. Although these assays are usually chosen because of the rapid result that is achieved and the ease of use, the results may be incorrect. Therefore, many of the package insert denote a need to follow-up negative test results with a more sensitive assay. These tests are best used when there is a high prevalence of infected individuals (e.g., respiratory virus season) wherein the tests have a high positive predictive value (i.e., a positive test is likely valid and diagnostic of the condition). The real limitation of these assays is the sensitivity (i.e., negative results may be falsely-negative). It is possible to reduce the likelihood of obtaining a falsely-negative specimen by collecting the highest quality specimen possible. Nevertheless, the package inserts must be followed and specimens that yield negative test results must be submitted for testing by a more definitive method.

Respiratory Viruses. The most reliable antigenic method for detection of respiratory viruses is direct immunofluorescence (DFA). In one study, DFA was as efficient as culture for all respiratory viruses except adenovirus. When the slides for immunofluorescence were prepared in a cytocentrifuge, less than 10% of the specimens had an inadequate number of respiratory cells.[280] In contrast, the sensitivity of enzyme immunoassays, particularly the rapid formats, has been considerably lower in most reports.[1,73,281,391] In addition, some immunoassays have yielded sufficient numbers of false-positive and false-negative reactions that confirmation by a second method has been recommended.[1]

Although direct immunofluorescence is clearly the better of the two approaches, practical considerations often lead to the selection of enzyme immunoassays particularly for in-office testing. These tests can be performed quickly and simply without the need for a fluorescent microscope or extensive training of personnel. Although positive tests are very useful during the respiratory virus season, it is imperative that clinicians know the limitations of these assays and the requirement for reflex testing of specimens for which a negative result has been obtained.

Herpes Group Viruses

Herpes Simplex Virus. Direct immunofluorescence has generally performed well in comparison with culture, particularly when performed on visible lesions and enhanced by cytocentrifugation.[282,392,406] Varying results may reflect different reagents, patient populations, types of lesions, and serotypes of virus. DFA testing for HSV on CSF specimens should not be performed because of poor sensitivity. Excellent immunohistochemical stains are available for the anatomic pathologist who is examining a tissue specimen with pathologic changes suggestive of HSV. HSV grows quickly, however, so the gains from expedited diagnosis are not as great as with more slowly growing viruses.

Varicella-Zoster Virus. VZV grows slowly in culture. Not surprisingly, direct immunofluorescence performs comparatively better for VZV than with HSV. In fact, DFA of skin lesions may be the superior means for making this diagnosis compared to culture.[97] Wilson and colleagues[491] found that the DFA had a sensitivity of 87.8%, whereas the sensitivity of culture was only 46.3%; only PCR was more sensitive than DFA, but also more expensive. In addition to direct examination of smeared material, staining of cytocentrifuged viral transport medium has proved useful.[322]

Cytomegalovirus. Monoclonal antibodies have facilitated the rapid immunologic diagnosis of CMV infections in patients' specimens as well as in cell culture. Hackman and colleagues[194] were able to detect CMV by immunofluorescence in 25 of 27 lung biopsy specimens from which the virus was subsequently cultured; light microscopic inclusions were demonstrated in only 20 of the specimens. Preliminary studies with bronchoalveolar lavage specimens from immunosuppressed patients[94,127,312]have yielded greatly differing estimates of sensitivity, varying from 31.6% to 100%. The numbers of patients in these studies were small, the antibodies were different, and the composition of the patient groups may have been different. The correlation of immunologic detection of viral antigen in tissue (without inclusions or histopathologic abnormalities) and clinical disease is not clear. Currently, excellent immunohistochemical stains are available for CMV for the anatomic pathologist.

Rotavirus. Human rotavirus is difficult to recover in cell culture. Infections by these viruses can be diagnosed effectively either by an enzyme immunoassay (EIA) or by latex agglutination. The sensitivity for both methods has ranged from 80% to 90%; the specificity has been 90% to 100%.[86,126,454] Krause and colleagues[275] have questioned the reliability of the Rotazyme immunoassay in neonates.

Human Immunodeficiency Virus. Antigen detection is a relatively new component in HIV screening. The new fourth-generation HIV test includes the detection of p24 antigen, in addition to the detection of antibodies directed to the HIV-1 and HIV-2.

This is designed to detect HIV-infected individuals who are early in the course of disease and have not yet generated an antibody response. The CDC recommended testing algorithm may be found at www.cdc.gov/hiv/pdf/hivtestingalgorithmrecommendation-final.pdf.

Molecular Techniques

The molecular era has been inextricably intertwined with infectious diseases from the outset, and the pace of innovation shows no sign of slowing. We have passed through several stages already, beginning with nucleic acid hybridization and advancing to molecular amplification technology and now NGS (see Chapter 4). The PCR and other molecular diagnostic approaches have made it into the mainstream of clinical laboratories. A substantial number of commercially available, high-quality molecular diagnostic products are available for the detection and, in some instances, quantitation of critical pathogens.[464] The pace is so rapid in this area that this section will be dated before the ink of this publication has dried. There are hospital laboratories that have replaced the entire virology section with molecular testing for viruses. Most laboratories, however, are somewhere in the middle, utilizing molecular diagnostics where most appropriate, while retaining DFA, immunoassays and culture for the areas where these make the most sense. The laboratory director

will need to evaluate the clinical needs and test volumes at a local level to determine which test is best to employ.

Human Immunodeficiency Virus. Testing for HIV includes serologic and antigen testing, as well as quantitative RT-PCR. In brief, the current recommendations for screening for HIV infection uses what has been termed a fourth-generation assay; CDC recommendations may be found at www.cdc.gov/hiv. This assay assesses for antibodies to HIV-1 and HIV-2, and includes p24 antigen detection. The latter was added in an effort to detect more individuals in the window period. For indeterminate results with the screening assay, the recommended algorithm (Fig. 23-17) reflexes to HIV RNA detection and quantitation. Similarly, if there is a high index of suspicion and the screening assaysare negative, many clinicians will request HIV RNA studies.

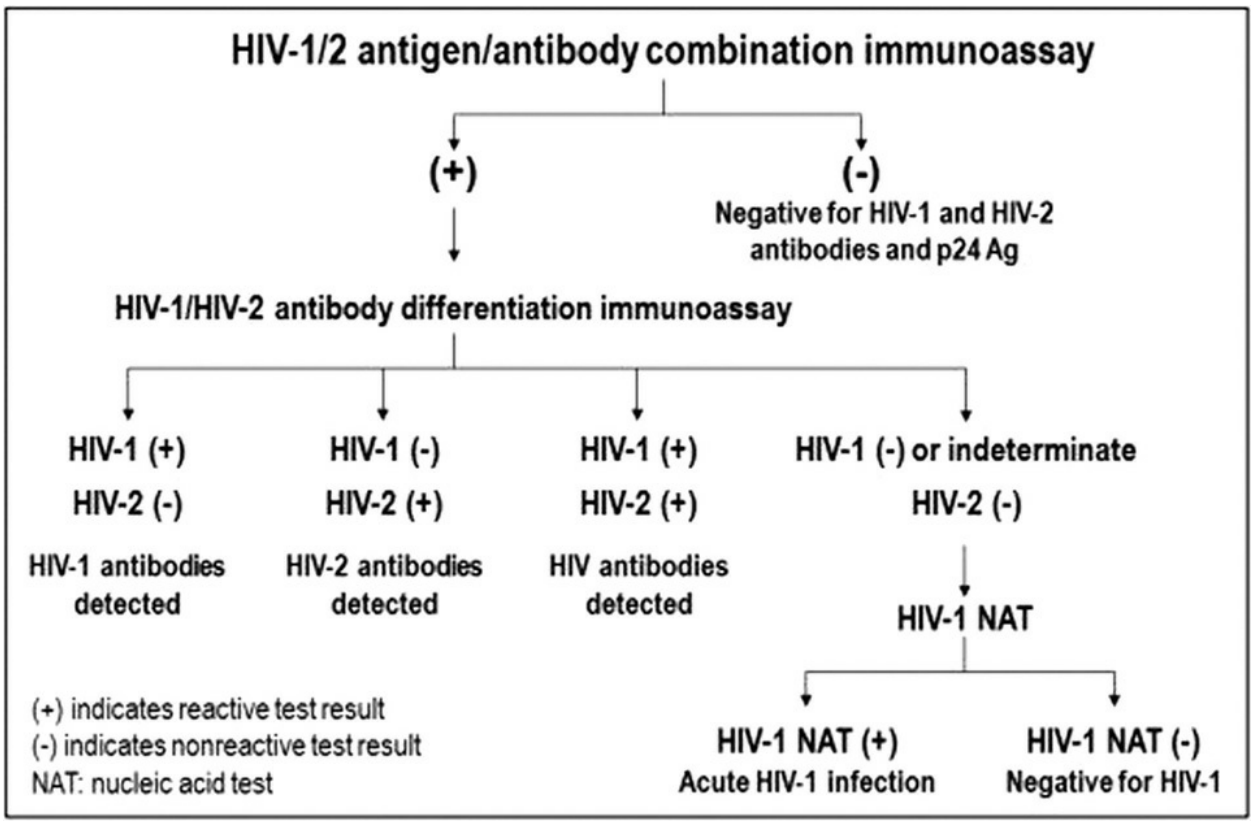
Once the diagnosis has been made, management of HIV-1 infection is based entirely on molecular methods. A quantitative viral load determined by an RT-PCR assay is the standard of care for the monitoring of patients with HIV. Although RT-PCR is the usual method used to determine the viral load, at that time of this writing a small minority of laboratories are still using a bDNA method. The manual and automated versions of RT-PCR that have been developed over the past decade perform

similarly.[227] An increasing viral load may disclose noncompliance with the therapeutic regimen or may indicate the emergence of resistance in the virus to the current antiretroviral regimen. There are a number of commercial vendors that offer HIV RT-PCR assays. In many instances these are FDA-approved, which is often preferred by users. The reporting of HIV has migrated from copies to Log_{10} values. Although there may be variation by clinicians, a 0.5 log change is usually considered clinically significant, as is a change from an undetectable viral load to a detectable viral load.

Both plastic and glass Vacutainer tubes for collecting blood for viral load testing have been evaluated and function equivalently.[283] Vacutainer plasma preparation tubes (PPTs) are a convenient method for collecting blood if it is to be frozen and/or shipped to a reference laboratory for testing.[226] A wide variation in patient reports provided to physicians by clinical laboratories, including reports that were incomplete or unhelpful, has been noted.[151]

There are two situations wherein which viral load testing is appropriate for initial diagnosis. In both situations, there may not have been time for a serologic response to develop. The first situation is acute infection with HIV, which may manifest itself as a multifaceted febrile illness with symptoms referable to any organ system.[256] This initial illness is transient, usually lasting no longer than 14 days. Large quantities of virus may be present before the immune response has been generated. The second clinical scenario is neonatal infection when the mother is known to be infected. Molecular detection of virus is required, because antibody passively transferred across the placenta to the neonate complicates serologic analysis.

The HIV virus is difficult to eradicate because it integrates into the human DNA and will persist as proviral DNA. There are PCR assays that are used, usually in newborns of infected mothers, to determine whether pro-viral DNA (i.e., integrated HIV DNA) is present. The only company (Roche) that manufactures such an assays has changed it to a qualitative HIV assay that will detect the presence of both integrated HIV DNA and HIV RNA, which is an improvement in testing.



■ **FIGURE 23-17** The updated HIV screening algorithm from the Centers for Disease Control and Prevention and the Association of Public Health Laboratories.

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With the advent of effective antiviral chemotherapy, detection of resistance in clinical isolates has become a priority. The clinical relevance of resistance testing soon became evident,[124] and evolving consensus recommendations have been promulgated and continue to evolve.[218] Resistance testing is recommended in pregnant women and after failure of antiviral chemotherapy, either primary therapy or subsequent regimens. It is now clear that evaluation of resistance is useful for treating newly infected patients (infected within 12 months of testing), particularly if the individual who is the source of the infection is known to have been taking antiretroviral drugs. This provides the clinician with baseline information concerning the genetic determinants of resistance that may be present in the patient's strain. In addition, testing of patients who have been infected for up to 2 years (and perhaps longer) has been found to be useful for designing therapeutic regimens.[217]

Two approaches to the detection of resistance have been used. Evaluation of phenotypic resistance involves isolation of virus and traditional assessment of inhibition by antiviral drugs. The complexity of viral isolation has led to an alternative

mechanism in which plasma RNA of the polymerase and protease genes is amplified and used to form a recombinant virus with laboratory constructs. By using a standard virus backbone into which the two most important genes of the patient strain have been inserted, standardization of the assay has been improved. Drug susceptibility testing can be performed within several weeks by using automated methods and reporter genes. Phenotypic assays include PhenoSense (Monogram Biosciences, LabCorp Specialty Testing Group, South San Francisco, CA) and The Antivirogram (Virco, Mechelin, Belgium).

In contrast to phenotypic assays, which measure viral susceptibility, genotypic assays pinpoint the mutations that confer phenotypic resistance.[421] The initial step in PCR-based assays is similar to the recombinant phenotypic approach—amplification of HIV-1 sequences from plasma samples that contain 500 to 1,000 copies per milliliter. As a result, this genotypic approach will not work with samples that contain a small quantity of virus. The next step involves either genotyping all relevant codons or detecting selected codons by hybridization.[201,218] Several companies have attempted to predict the phenotype by analysis of the genotypic result, a so-called virtual phenotype. Genotypic assays most commonly used at the time of this writing are TruGene (Siemens), ViroSeq (Celera), or laboratory-developed sequencing assays. Phenotypic assays have the attractiveness of more direct (and traditional) assessment of resistance, although the clinical interpretation of the results is neither completely defined nor straightforward. In addition, these are very expensive, time-consuming, and some argue provide little, if any, value over genotypic testing.

Genotypic assays are more straightforward, more rapid, and less expensive; the concern has been that important phenotypic resistance may not be pinpointed and that the significance of new mutations may not be clear. For both approaches, reproducibility and sophistication of analysis represent important factors. An additional concern is that a minority of resistant virions in the diverse population of virions that comprise the infecting strain in any individual patient may be missed (analogous to the problem with detecting resistant cells in a population of heteroresistant bacteria, as discussed in Chapter 17).[421]

Although the concern about genotypic assays has not been dissipated, comparative studies have demonstrated good correspondence of genotypic and phenotypic assays for the clinically important resistance mechanisms.[40] As experience has been gained and commercial products have been fine-tuned, the reproducibility and reliability of genotyping has been established.[159,183]

There is evidence that resistance testing can predict the response to antiviral therapy or time to suppression of virus.[182,509] The factors that determine success or failure in treatment of any infection are complex, however. As with all laboratory reports, the results of HIV resistance testing should be interpreted by an experienced clinician in light of all available data. Attempts to develop useful algorithms to facilitate clinical

decisions continue.[421] HIV genotyping involves the assessment of four genes of the HIV virus, which are initially amplified using RT-PCR. The DNA sequence of the genes are determined and the sequences are then compared with genetic databases that include known resistance-inducing mutations. HIV genotyping helps the clinician tailor or individualize therapy. Some of the advantages of NGS are expected to enhance this even further. NGS will provide not only the sequence of the predominant quasi-species of HIV, but also the sequences of less abundant quasi-species. In this manner, therapy can be tailored to address not only the major quasi-species, but also the minor quasi-species, which may represent the next emerging resistant clone.

Hepatitis C Virus. The diagnostic approach to hepatitis C virus and HIV infections has been similar.[22,373] Identification of infected patients is best accomplished serologically. To differentiate patients who have cleared their infection from those who are chronically infected and at risk for chronic disease, however, it is necessary to determine if circulating virus is present.[373] Molecular detection is the only option, because hepatitis C virus has never been cultured in vitro.

The quantitative detection of HCV is currently the standard of care for infected patients.[273,334] The viral loads are used to monitor the response to therapy and also provide an amplicon for HCV genotyping. Currently, the majority of the quantitative assays in use employ RT-PCR (Roche and Abbott), but there are still some laboratories using bDNA (Siemens). As with HIV, the standard reporting is in Log_{10} values.

There are currently 6 HCV genotypes, and genotype 1 has traditionally been subtyped into 1a and 1b for therapeutic/prognostic purposes. HCV genotyping can be performed using a variety of methods.[24,164,354] Current assays include real-time PCR (Real-Time HCV Genotype II; Abbott Molecular), a reverse hybridization assay (VERSANT HCV Genotype 2; Siemens), DNA sequencing (laboratory-developed assays and TRUGENE HCV genotyping assay; Siemens), and most recently by bioelectric array (GenMark). The need for and utility of HCV genotyping and subtyping remains fluid as new therapeutics are introduced. For example, a new therapeutic consisting of multiple antiretroviral agents in combination has been approved for the treatment of HCV genotype 1, which obviates the need for subtyping.

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Hepatitis B Virus. Molecular diagnostic tests for hepatitis B virus have not achieved the currency of those for hepatitis C, because the variety of serologic tests available for hepatitis B has served much of the need. Detection of viral DNA in blood, the definition of active infection, is more sensitive than the presence of HBeAg, however,

and may be found with pre-core mutants in the absence of seroconversion to HBeAg.[24]

The molecular detection HBV DNA is useful in few scenarios. Most commonly, it is used to distinguish chronic active infection from inactive infection. This is of prognostic significance, since patients with chronic active infection are at higher risk for more serious sequelae. The quantitative measure of HBV DNA is also used to monitor the response of the patient to therapy, with the end goal being long-term viral suppression with undetectable an undetectable viral load. An elevating viral load may herald the emergence of a resistant strain. Finally, as with HIV RNA, the detection of HBV DNA is possible in the window period, before the emergence of HBsAg.

Quantitative rapid-cycle PCR is the test most commonly used by laboratories for HBV. Commercially available FDA-approved assays are available (e.g., Roche Diagnostics and Abbott Molecular). In addition, several genotyping assays have been developed commercially. As experience with therapy of chronic hepatitis B infection expands and resistance becomes more prevalent, these tests will undoubtedly assume greater prominence.

Human Papillomaviruses. The molecular detection of HPV is now part of the cervical screening guidelines for women 30 to 65 years of age. Women in this group should receive either cervical cytology every 3 years or they may lengthen the screening interval to 5 years if both cervical cytology and high-risk HPV testing are performed and these are negative. The consensus recommendation for testing is that tests should include only high-risk HPV subtypes.[499]

The use of molecular diagnostics is the only practical approach for the detection and characterization of this group of viruses. The earlier, labor-intensive techniques, such as nucleic acid hybridization by the Southern blot method (see previous edition of this book), have been replaced by more convenient and sensitive methods. There are now four FDA-approved methods for the detection of high-risk HPV subtypes.

The hybrid capture assay (digene HC2 High-Risk HPV DNA Test, Qiagen, Gaithersburg, MD) was the first assay to market, and was the only option available for quite some time. Subsequent generations of this test have greatly improved sensitivity over the initial version.[51,52] The digene HPV Genotyping PS Test (Qiagen) is available as a reflex test for determining if types 16, 18, or 45 are present.

Target amplification methods, such as transcription-mediated amplification (TMA) and real-time PCR, have now been cleared by the FDA for high-risk HPV detection. The Aptima HPV assay (Hologic) is the assay that utilizes TMA technology and takes the novel approach of targeting the E6/E7 mRNA of HPV. The proponents of this approach claim that by detecting the E6/E7 mRNA of actively oncogenic HPV, the user is more likely to detect HPV causing dysplasia in contrast to that causing a transient infection that would naturally be cleared. This assay is performed on their

highly automated Tigris and Panther instruments, with the latter allowing for random access. Cervista HPV is another Hologic assay that target high risk HPV. This assay uses Invader amplification technology.

The cobas HPV test is the first assay that has been cleared by the FDA for primary screening. Although this is true, the recommendations from professional societies such as the ACOG and the American Society of Cytopathology (ASC) have not yet embraced this use of the test in the absence of Pap testing. One of the claimed advantages of this assay is that in addition to detecting the myriad of high-risk HPV genotypes that cause dysplasia, it also reports the presence of the more highly oncogenic types 16 and 18, without the need for subsequent, reflex testing. This assay is performed on the highly automated cobas 4800 System.

Parvovirus B19. Parvovirus infections are usually diagnosed clinically or serologically; assessment of risk is based on immunologic (i.e., immune) status. Sometimes, viral inclusions may be seen in erythrocytic precursors in bone marrow biopsy specimens. Occasionally, however, it is useful to document a serious infection virologically. Parvovirus does not grow in culture, so molecular detection is the only approach available. Several amplification assays have been developed[98,109,202]; although not cleared by FDA. Assays are available as Analyte Specific Reagents (ASR) or primers, and probes may be derived from the literature. Regardless of the approach, the testing laboratory is responsible to undertake a full and thorough validation, as the test is considered an LDT. Additionally and as test volumes in any one location are usually low, the option to send these to a high-quality reference laboratory may prove the best option.

Influenza, RSV, and Other Respiratory Viruses. Likely one of the greatest impacts in recent years has been the expanded availability and expanded use of molecular diagnostic products for influenza A and B viruses and RSV. It was not long ago that these tools were unavailable and patients were being screened by insensitive immunoassays that required labor-intensive DFAs or time-consuming culture to render the final result. The earliest assays targeted influenza A and B; these were offered as an influenza RT-PCR alone or in combination with RSV. The RSV assay was also offered as a stand-alone test. These assays, originally produced by Prodesse, are now supplied by Hologic, which is working to automate the amplification process, as they have done so successfully with their other assays. These assays, and many of the others that followed, have received FDA clearance. Cepheid followed with products that targeted these viruses using their Xpert system, which was extremely attractive to laboratories with limited molecular diagnostics experience. Still other groups have made attempts to address both the need for quick time-to-result and, if desired, batch testing. For example, the Focus group offers two such applications on their integrated cycler. The first is an eight-well cartridge wherein each well may be used individually,

until all the eight have been utilized. This affords the opportunity to perform testing as the specimens are received, similar to the Cepheid applications. Conversely, they also offer a reaction disc that accommodates many more specimens for batch-style testing.

The next substantial application that has been introduced is the multiplex respiratory panel. In some instances, these

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assays include the most commonly occurring respiratory viruses, as well as the bacterial respiratory pathogens *Mycoplasma pneumoniae, Bordetella pertussis,* and *Chlamydophila pneumoniae*. These assays, too, have received FDA clearance and are relatively simple to perform. The appropriate use of these assays is important, as they are more expensive than the assays directed toward a single target (e.g., RSV). However, they may be more cost-effective for those instances wherein all the analytes present would have been ordered individually. These have largely replaced the traditional virology tests for respiratory viruses in many laboratories. Additionally, there are antimicrobial stewardship opportunities (i.e., to discontinue unnecessary antibiotics once a viral etiology is identified), but this requires substantial coordination with an antibiotic stewardship team and exclusion of the possibility of a bacterial superinfection.

Herpes Simplex Virus. The primary diagnostic approach to HSV infections has been culture, but rapid, cost-effective and FDA-approved assays are challenging this approach. HSVs are the most rapidly proliferative of the viruses commonly recovered within the clinical laboratory. Results in cell culture based on cytopathic effect may be available within a few days, whereas results based on modified culture systems, such as the ELVIS system described above, may be complete within 24 hours. In addition to these assays, the HSV DFAs for use on specimens from vesicular lesions are very sensitive and have a high positive predictive value.

There are now several commercially available PCR assays that are FDA-cleared for use on genital specimens. In addition to the high level of sensitivity offered by these assays, they are attractive in that the turnaround time is within the same day, and the testing not complicated. This is in contrast to culture-based methods wherein after the cell culture is inoculated, it must be incubated and subsequently examined or processed further. These nucleic acid amplification assays have proven more sensitive than culture for detecting HSV in mucosal infections.[468]

In the past, the diagnosis of HSV encephalitis was substantially more challenging. After highly suggestive radiologic findings were denoted, then a brain biopsy was generally required to get an adequate specimen for the culture-based diagnosis of herpetic encephalitis or histopathologic diagnosis. The use of PCR on CSF specimen is sufficiently sensitive to detect viral DNA in both in encephalitis and in recurrent

meningitis for most affected patients.[329,426] A number of HSV PCR assays have been described, some of which have been validated as LDTs. Recently, however, the first PCR-based assay has been FDA-approved for use on CSF for the diagnosis of meningitis/meningoencephalitis, and other assays are currently in clinical trials.

Preparation of DNA for amplification can be accomplished equally well by manual or automated methods.[133] Fortunately, DNA is sufficiently stable that specimens can be stored without a decrease in sensitivity at 4°C, either as extracted DNA or as an unextracted specimen, for as long as 16 months before testing by real-time PCR.[245] The use of laboratory resources can be increased by screening specimens for pleocytosis in the CSF before testing, because neutrophils have been demonstrated in all positive specimens, at least in adults.[424] Children with encephalitis, however, may have very few neutrophils in CSF and initial PCR examinations may be negative if performed very early in the course of the infection.[106] The use of PCR on CSF for the diagnosis of HSV CNS disease represents a considerable advance in diagnostics over intrusive methods, such as brain biopsy.

Cytomegalovirus. The ability to determine the amount of CMV present in the blood of the patient in a reliable and reproducible way afforded the opportunity to change from prophylactic antiviral therapy to preemptive antiviral therapy for patients at risk for CMV. CMV antigenemia assay was an early reference method, but it was time-consuming and visually interpreted, and therefore especially susceptible to operator errors. Subsequently, quantitative assays of several varieties correlate well with clinical disease and with the antigenemia assay. The methods that were used successfully include PCR, real-time PCR, and hybrid capture.[46,112,221,293]

One caveat must be kept in mind for users of molecular amplification. In contrast to HSV, early studies demonstrated that the quantity of CMV detected in plasma increased over time when the specimen was stored at 4°C, apparently by leakage of nucleic acid from infected leukocytes.[409] For specimens that were positive in the antigenemia assay, there was little effect on the amplification result; if the patient was latently infected and the antigenemia test negative, however, the amount of plasma virus increased significantly over time.

The detection of quantitative CMV is now uniformly or nearly uniformly accomplished by rapid-cycle PCR methods. Although there are a minority of laboratories that utilize LDTs, there are commercially available FDA-approved products available that are often preferred. There are a number of ASRs that facilitate the validation of LDTs. If laboratories desire to extend the acceptable transport time beyond that which was cleared by the FDA, then stability validation studies should be performed.

Enteroviruses. Culture, which has been the mainstay of enteroviral diagnosis, has

been suspected to be relatively insensitive. This impression was validated soon after the appearance of amplified genetic tests. There is one FDA-cleared assay; this or validated LDT assays are available in many reference laboratories. Early assays used traditional RT-PCR and nucleic acid sequence-based amplification (NASBA), whereas current assays are predominantly RT-PCR assays modified for a real-time platform.[34,278,284] As there are no generally used antiviral agents for enteroviral infection, prompt diagnosis is not quite as imperative as for HSV, but prompt laboratory diagnosis can be used to facilitate clinical management.

There is known cross-reactivity between rhinovirus and enterovirus in molecular assays. In some instances, manufacturers simply result a positive reaction as positive for rhinovirus/enterovirus. Others have gone to great lengths to try to construct a rhinovirus-specific assay, just to have it demonstrated that there is still cross-reactivity with some enterovirus strains.

SARS and MERS Coronaviruses. During the SARS outbreak, it was discovered that viral RNA was detectable in clinical specimens for more than 1 month in some patients. Recovery of nucleic acid was found to increase progressively

until a peak about 11 days after the onset of clinical illness.[84] Viral nucleic acid could be detected in 60% of patients with clinically diagnosed infection and in a very small number of patients without clinically evident disease. Nasopharyngeal aspirates, throat swabs, and sputum were the most useful specimens in the first 5 days of illness. Later in the illness, virus was more readily detected in stool specimens.[84] Real-time PCR assays for this virus have also been developed.[508] At this juncture, diagnostic tests are largely confined to public health laboratories.

The MERS-CoV is also detectable using a CDC-developed rapid-cycle RT-PCR assays that is available at the CDC and in most state public health laboratories. Testing is restricted to patients with appropriate travel and/or exposure history, and appropriate clinical findings.

Other Viral Infections. Molecular amplification assays have been developed for virtually every medically important virus, many of which are real-time PCR assays. Examples include exotic viruses, such as hemorrhagic fever viruses,[117] and highly virulent agents that have potential for bioterrorism, such as smallpox.[432] Assays have also been developed for less commonly considered viruses, such as JC and BK polyomaviruses,[483] measles,[122] rubella,[122]and mumps.[374] These assays are available through reference laboratories, and in some instance materials for the assays may be purchased from commercial vendors. More recently, RT-PCR assays have been

developed for the chikungunya virus, which may be used for detection in the first 8 days of illness. The first RT-PCR for the diagnostic detection of the Zika virus was recently developed and made available from researchers at Texas Children's Hospital and Houston Methodist Hosptial.

In the past, nucleic acid amplification-based assays were also predominantly available only through reference or university laboratories. With the increasing availability of reliable commercial reagents, more and more of which are receiving FDA approval, we are witnessing the same kind of expansion that occurred after immunofluorescence and immunoenzyme reagents became widely available. These include the detection of virus that were routinely detected by DFA and culture, such as respiratory viruses and other herpes group viruses.[95,189,462,465,484] These are available from a number of vendors in a variety of formats, so most laboratories should be able to find a platform to fit their specific needs. Most recently, at least one molecular platform has received "waived" status and another is seeking this certification. It is likely that molecular virology for select pathogens will be performed at the point-of-care for many of our patients in the very near future.

Selection of Tests for Rapid Diagnosis

The choice of a technique for direct detection of virus must depend foremost on the performance of the assay, but also on the availability of equipment, the experience of personnel, and the number of specimens to be tested. The microscopic detection of inclusions is simple and inexpensive, but the sensitivity compared with culture or PCR varies depending on the application. For example, it works well for documentation of herpetic skin lesions, but does not perform as well for influenza viruses compared to RT-PCR and culture. Electron microscopy has restricted indications and is not considered rapid or cost-effective for common pathogens. Microscopic solid-phase immunoassays (immunofluorescence and enzyme assays) have advantages over assays for soluble antigens: (1) the location and type of staining can be assessed morphologically and (2) small numbers of specimens may be processed quickly. These attributes may be outweighed by the disadvantages of subjectivity in interpretation and the need to acquire an expensive microscope for fluorescence.

EIA may be performed in batches, but in most instances wherein they are used a rapid result is desired so they are often performed soon after collection and often at the point of need. The end point for many of these is visually determined at a particular time, which introduces error and subjective decisions. There are newer assays available that are associated with small, inexpensive instruments that perform automated interpretation at the appointed time, which reduces the errors associated with manual visual interpretation. One of these assays, the Sofia (Quidel, San Diego, CA), utilizes fluorometric detection, rather than colorimetric detection, and claims enhanced sensitivity due to this method. To study tissue sections for viral elements,

immunoperoxidase methods are most commonly employed. Endogenous peroxidases in the sections must be blocked completely. Antigen-recovery, using either microwave techniques or treatment with a proteolytic agent, such as trypsin, improves sensitivity.[76,228] In situ hybridization has also been used for the detection of many viruses. However, the commercial availability of high-quality commercial antibodies and the ability to perform staining on an automated platform usually tip the balance in favor of immunohistochemistry.

There has been and will be continued significant advances in molecular products available for the clinical laboratory. By the next edition of this text, we will have experienced many of the contributions of NGS, and we will understand how it best fits into the clinical laboratory. Currently, molecular applications are commercially available for almost every laboratory setting for the most common pathogens. Now, patients in the 200-bed community hospital will have the same benefits as those in a tertiary care medical center and university hospitals. The promises of rapid molecular diagnostics are being realized and what is most exciting is that there is more to come.

Serologic Diagnosis of Viral Infections

Serologic tests are the mainstay of diagnosis for certain viral infections, such as those caused by hepatitis viruses, EBV, and rubella virus. Whenever recovery of the virus in culture is difficult or impossible, documentation of an immunologic response to the agent will continue to be important. In certain cases, such as the evaluation of rubella infections, serologic diagnosis may be as rapid as culturing the virus, even if one has to wait 2 or 3 weeks for collection of a convalescent serum specimen. In situations in which culture of the virus is reliable and readily available, serologic diagnosis has a supportive or adjunctive role. Even in the molecular era serology retains an important role. For example, the viremia and viral RNA in the plasma and CSF is relatively short-lived for West Nile virus, so serology is an important diagnostic tool for the diagnosis of disease caused by this and similar viruses.

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General immunologic principles also apply when establishing a diagnosis of viral disease using serologic methods. Antibodies to many viral antigens remain for months or years after an acute infection. Demonstration of a significant increase (i.e., generally considered a fourfold rise) in titer of antibody is considered diagnostic of recent infection with the agent, unless the possibility of an immunologic cross-reaction exists.

If antibodies were not present in the initial specimen, this diagnostic increase in antibody titer is referred to as a seroconversion. In this case, the infection probably

represents a primary encounter with the virus. The infection may be either a primary infection, a repeat infection, or reactivation of a latent infection if antibodies are present, even in low titer, at the time of initial testing.[348] The serologic diagnosis of herpes infections is frequently complicated by recrudescent disease. For example, in a patient with CNS disease, oral herpes lesions may be reactivated by the stress of the acute illness. A serologic response that may result from the reactivated oral lesions could be misinterpreted as evidence that the CNS disease was caused by herpesvirus.

Serologic cross-reactions within many virus groups and even across groups do exist, notably, among enteroviruses, paramyxoviruses and togaviruses. Therefore, all serologic diagnoses must be considered presumptive to some degree.

A fourfold or greater decrease in antibody titer suggests an infection at some time in the past. Most antibodies disappear slowly, however, so that it is usually difficult to determine the time of the original infection.

A significance advance since the last edition of this text has been the rapid development and validation of the specific IgM and IgG assays. These largely negate the traditional need for an acute and convalescent serum titer comparison.

In general, IgM antibody appears earlier than IgG after an acute infection and is more transient. In addition, IgM, unlike IgG, does not readily cross the placenta, so demonstration of this antibody in a neonate indicates a congenital or perinatal infection, rather than passive transfer of antibody from the mother.

Therefore, the demonstration of IgM antibody to any microbial antigen suggests a recent infection. It is extremely important that the procedure for detection of the IgM class be demonstrably specific.

If an anti-IgM antibody is used to determine the acuteness of an infection, the specificity of the antiglobulin must be documented. False-negative results may be generated if there are very high levels of IgG, which may block the sites that would have reacted with IgM. False-positive results may be encountered if antiglobulins, such as rheumatoid factor, are present in the patient's serum. If a serum reacts with multiple antigens (i.e., rubella, CMV, HSV, and *Toxoplasma gondii*), an antiglobulin should be suspected.

Finally, although IgM antibody in serum is usually transient, there are documented instances in which IgM antibody has persisted for months or even years. Furthermore, it is now recognized that IgM can be detected when some latent infections are reactivated.[116]

The clinical setting must be considered when assigning a diagnosis. Even a seroconversion documents only a recent infection with the agent; association with the clinical illness is by inference.

Human Immunodeficiency Virus

HIV persists in patients in whom antibody to the virus has developed. Serologic tests

for HIV have been used as a screening tool because isolation of the virus in cell culture is difficult and not readily available. The molecular tests for detection of viral nucleic acid are predominantly used for monitoring patients who already have serologically documented infection, but may also be used to document early infection (i.e., prior to antibody development).

The traditional serologic method, repeatedly reactive EIA followed by Western blot testing, has been replaced by the fourth-generation assay, which includes the detection of antibodies directed against HIV-1 and HIV-2, as well as the p24 antigen (Fig. 23-17). The ARCHITECT HIV Ag/Ab Combo (Abbott Park, IL) and the GS HIV Combo Ag/Ab EIA are the two FDA-approved fourth-generation HIV assay. The Western blot has been replaced by the Multi-Spot assay (Bio-Rad) as a confirmatory test. Specimens from screening tests that cannot be confirmed by the Multi-Spot are submitted for nucleic acid amplification studies. This new method of standard screening is usually performed in batches at reference or larger laboratories (i.e., the results are not rapidly available for infection prevention purposes [e.g., assessment of individuals following a needlestick]).

There are two FDA-approved HIV tests that have been approved for home use, the Home Access HIV-1 Test System (Home Access Health Corporation, Hoffman Estates, IL) and the OraQuick In-Home HIV test (OraSure Technologies, Inc., Bethlehem, PA). The Home Access HIV-1 Test System uses a blood sample obtained from a finger stick that is mailed to a licensed laboratory. There are two options for this test, the Standard version, wherein results are available within 7 days after the specimen arrives in the laboratory, or the Express, wherein results are available the same day when it arrives in the laboratory. The OraQuick In-Home HIV test uses a mouth swab to collect oral secretions, similar to their other products. The results from this test are available in about 20 minutes. Both manufacturers provide counseling, and follow-up confirmatory laboratory testing is recommended.

Additional rapid immunoassays are available for use within the laboratory. The VIKIA HIV 1/2 (bioMérieux) uses blood from a finger stick and claims sensitivity and specificity >99%. Although widely used throughout the world, it has not been FDA-cleared for use in the United States.

Assays that are characterized as rapid immunoassays by the FDA include the Multi-Spot HIV-1/HIV-2 Assay (Bio-Rad Laboratories, Redmond, WA), which uses plasma or serum; the SURE CHECK HIV 1/2 ASSAY (Chembio Diagnostic Systems, Medford, NY), which is approved for use with blood from a fingerstick, venous whole blood, serum, or plasma; HIV 1/2 STAT-PAK (Chembio Diagnostic Systems), which is approved for the same specimen types as the CHECK HIV 1/2 ASSAY; the OraQuick ADVANCE Rapid HIV 1/2 Antibody Test, which in addition to venous whole blood or plasma, may be used with oral fluid; the Uni-Gold Recombigen HIV 1/2 (Trinity Biotech, Jamestown, NY); and a rapid immunochromographic assay, the Chembio DPP HIV 1/2 Assay, which like the OraQuick ADVANCE in addition to whole blood

and plasma can be performed on oral

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fluid. A review of comparisons of these assays is beyond the scope of this text. However, it is recommended that all patients tested with positive rapid immunoassays should have follow-up traditional laboratory studies. Negative results should be followed-up with new fourth-generation HIV testing. These rapid assays are particularly useful in infection prevention situations (e.g., following a needlestick) to determine if antiretroviral therapy is needed for the person at risk.

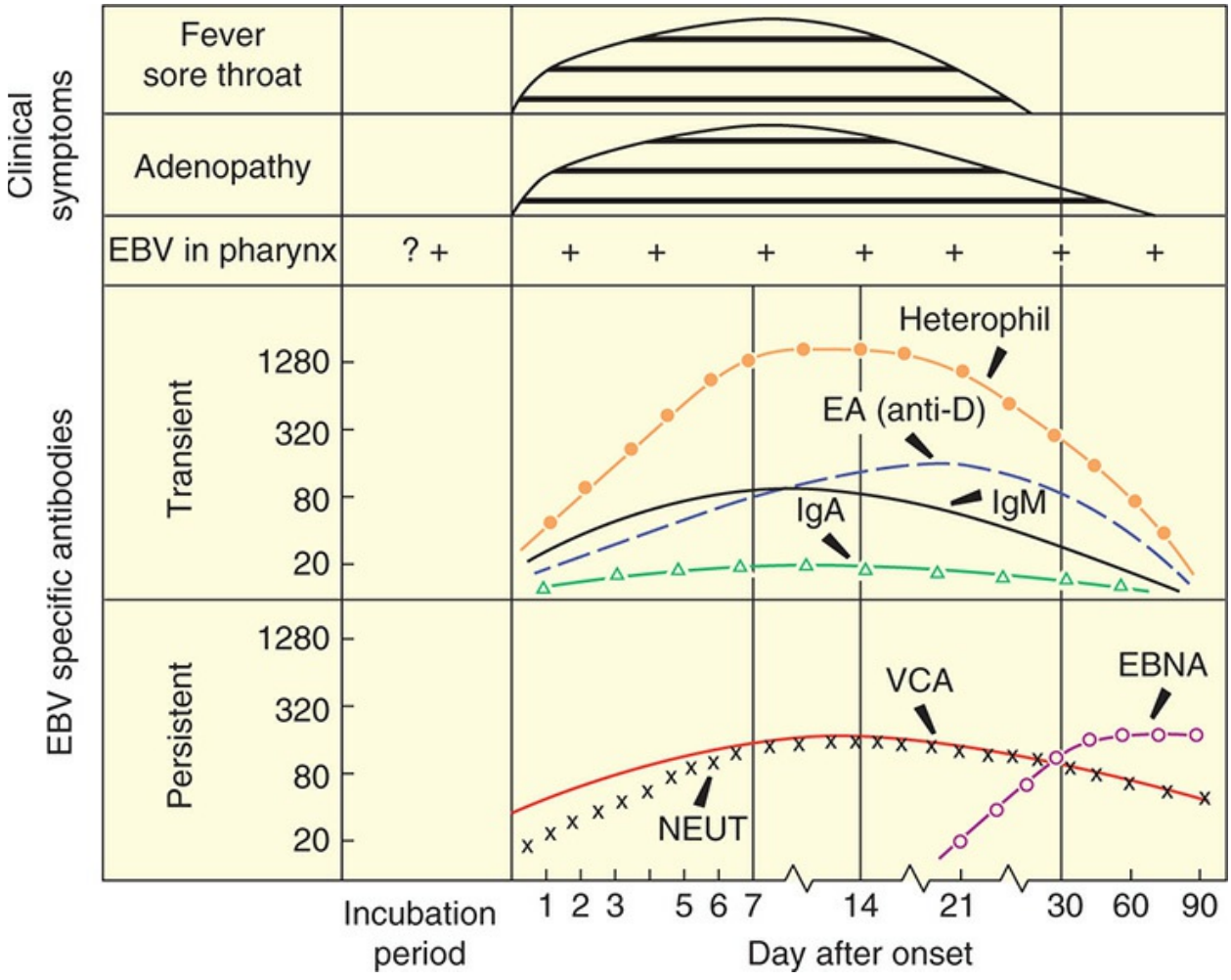
Hepatitis B Virus and Epstein–Barr Virus

There are a few exceptions to the general rule that a seroconversion must be documented to establish a diagnosis. In a few viral infections, antibodies to a variety of antigens appear at different times after infection and persist for varying lengths of time. It may be possible to establish a definitive diagnosis with a single serum when an antibody is detected that appears only acutely. The two prime examples are infectious mononucleosis caused by EBV (Fig. 23-18) and hepatitis B (Fig. 23-19) infections. If the infectious agent is not eliminated by the immune response, the presence of antibody means that the patient may still harbor the microbe. For instance, patients who have antibody to CMV, which often produces a latent infection, are more likely to transmit the virus than if blood or an organ is transplanted from a seronegative individual.[220]

Knowledge of the sequence of events is particularly important for the diagnosis of hepatitis B virus infection. Detection of viral antigen, especially the surface antigen (HBsAg), plays an important role in the diagnosis of acute infection. The e-antigen of hepatitis B virus is detected at the same time as DNA polymerase activity, which is a marker for infectious virus; the presence of e-antigen has correlated with acute, communicable disease in many studies,[346] as discussed above. Antibody to the viral core (HBc) is important diagnostically during the time when surface antigen has been cleared from the circulation and antibody to the surface antigen is not yet detectable. The presence of core antigen correlates with circulating viral DNA.[266] In some patients, a chronic infection develops in which antigenemia persists in the absence of serum antibody; this group has an increased risk for the development of chronic liver disease. It is now apparent that many of these patients have circulating antibody, which is not detected by commercial systems because it is complexed with antigen.[316] In addition, it has been demonstrated that some patients who have circulating antibody and no detectable surface antigen have circulating viral DNA detectable by amplification techniques for as long as 5 years.[327]

Likewise, the diagnosis of infectious mononucleosis can be made on a single serum if antibody to early antigen (EA) and IgM antibody to viral capsid antigen (VCA) are present. A high titer of IgG antibody to VCA in the absence of both antibody to EA and IgM antibody to VCA suggests a past infection. It is important to know the time course of the infection when interpreting the serologic results.[388]

The most common test for detecting infections by EBV is the heterophil antibody test. Heterophil antibodies are immunoglobulins that react with substances from another species. In the course of an EBV infection, antibodies are produced to a variety of extraneous antigens. Agglutinins



■ **FIGURE 23-18** Time course of an Epstein–Barr virus (EBV) infection and serologic response. Although virus may be identified in oral secretions, the techniques for culture are difficult and are available only in research laboratories. The heterophil antibody response (i.e., the monospot test) is the classical means of documenting infectious mononucleosis and is still the most useful test. The transient appearance of heterophil antibody, IgM antibody to viral capsid antigen, and antibody to early antigen (EA) allows one to associate

the presence of antibody with the current illness. Neutralizing antibody and IgG antibody to viral capsid antigen (VCA) persist for months or years. These tests are useful to determine that the patient has been infected previously, and is, therefore, immune; they may also be useful diagnostically if the first specimen is collected sufficiently early and a seroconversion is documented. If the patient is seen late in the course of the illness, diagnosis may be made by detection of a seroconversion to viral nuclear antigen (EBNA). (Modified with permission from James C. Niederman, Yale University School of Medicine, New Haven, CT.)

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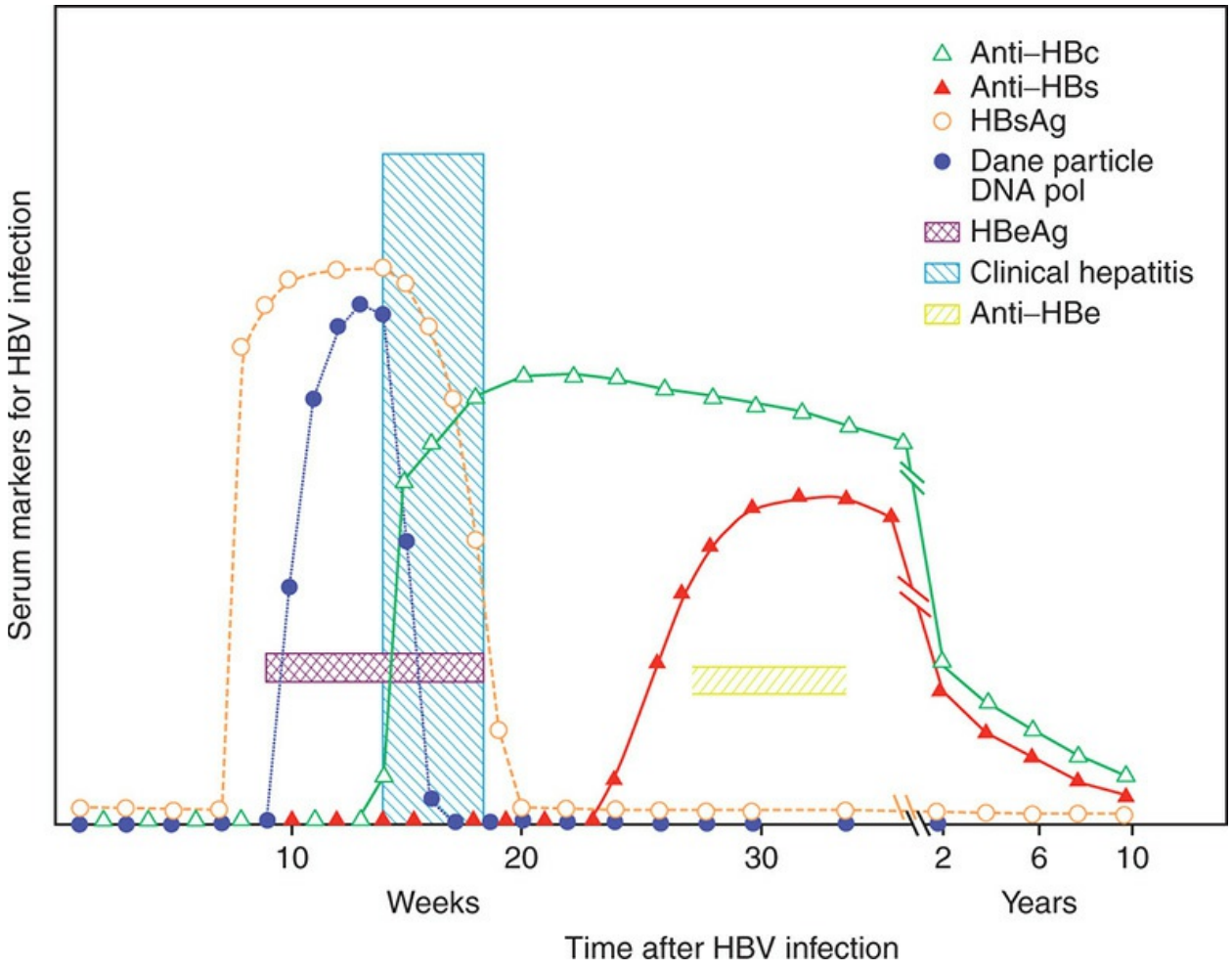

■ **FIGURE 23-19** Antigens and antibodies after self-limited infection with hepatitis B virus. The appearance of antigens and antibodies after self-limited infection with hepatitis B virus is an example of how knowledge of the biology of infection led to very effective diagnostic strategies, although the virus itself was difficult or impossible to culture. An assessment of both antigen and antibody is necessary to detect cases efficiently. Hepatitis B

surface antigen (HBsAg) appears first; after it is cleared from the blood, antibody to that antigen can be detected (anti-HBsAg). There may be a time wherein when antigen has been cleared but antibody is not detectable; measurement of antibody to the core antigen (anti-HBc) is necessary to detect patients in the "window" period. The presence of HBsAg does not equal acute, self-limited infection because some persons become chronic carriers. (Modified with permission from William S. Robinson, MD, Stanford University Medical Center, Stanford, CA.)

for sheep and horse red cells, hemolysins for beef red cells, and antibodies to the *Proteus* OX19 antigen of the Weil–Felix test are all produced. The heterophil antibodies that are very specific for infectious mononucleosis are absorbed by beef red cells but not by guinea pig kidney. The Paul-Bunnell–Davidsohn differential test for infectious mononucleosis is designed to characterize these heterophil antibodies; it is highly specific for EBV and confirmatory for making the laboratory diagnosis of infectious mononucleosis.[103] A variety of simplified tests for detecting the heterophil response in infectious mononucleosis are now available commercially.[145,232] Results that do not conform to clinical or hematologic data should be evaluated by performing the differential tube test or testing for EBV-specific antibodies. The agglutination tests for heterophil antibody are "simple" tests, which can be performed by "anyone" with no difficulty. In 1990, a pseudoepidemic of infectious mononucleosis was recognized in Puerto Rico.[55] Quality control and proficiency-testing results gave no hint of problems, but careful investigation revealed that two technicians with limited experience had been reporting examinations as positive based on "weak reactivity," a category that did not exist for the method used.

Ninety percent of adults with infectious mononucleosis will have heterophil antibodies. The frequency with which these antibodies develop in young children is much lower. Heterophil antibodies developed in only 3 of 11 infants less than 2 years of age after a primary EBV infection, whereas such antibodies developed in 16 of 21 children between the ages of 2 and 4 years.[230] Heterophil-negative infectious mononucleosis with circulating atypical lymphocytes is usually caused by EBV (i.e., demonstrable by specific antibodies to viral components) or by CMV.[231] Horwitz and colleagues[231] found that sera from 38.1% of patients with heterophil antibody-positive mononucleosis cross-reacted in an IgM-CMV test, but sera from patients with acute CMV infection did not react in the EBV VCA-IgM test. Cross-reacting antibodies to either the D or R component of the EA were detected in sera from 9 of 36 patients (25%) with mononucleosis caused by agents other than EBV (i.e., six cases of CMV infection, one of toxoplasmosis, one of probable hepatitis, and one of unknown cause).

The IgG antibody to EBV persists for long periods; other antibodies, such as those

against the EA of EBV, appear transiently and may serve as markers of acute infection.[388]

Hepatitis A Virus

Hepatitis A is the only primary hepatitis virus to have been cultured in vitro. The virus will grow in a variety of simian cells, most notably early passages of African green monkey kidney cells.[25] Culture is not a viable diagnostic option, however, because recovery from primary specimens is not reliable. Hepatitis A is diagnosed serologically by detection of specific IgM antibody.[383,431,448] Very early in the infection IgM antibody may not be detectable, so the test should be repeated after 2 weeks, if the clinical suspicion is high.[216]

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Hepatitis C Virus

The laboratory diagnosis of hepatitis C infections requires a combination of serologic and molecular tests. Early methods employed an EIA screen, with reflex testing to a specialized Western blot.[5]

At the time of this writing, there are several immunoassays that have been cleared by the FDA for diagnostic use. These include the Abbott HCV EIA 2.0 and the Abbott PRISM EIA (Abbott Laboratories), the Chiron RIBA HCV 3.0 Strip Immunoblot assay (Chiron Corp), the Ortho HCV version 3.0 Elisa (Ortho-Clinical Diagnostics, Raritan, NJ), and Vitros Anti-HCV Assay (an enhanced chemiluminescence assay; Ortho-Clinical Diagnostics). The use of HCV-encoded recombinant antigens has been important in the development of HCV screening assays.[6]

The current algorithm has retained the screening EIA, but reflexes positive specimen to quantitative RT-PCR for HCV rather than the immunoblot.

Parvovirus

Parvovirus infections in children are usually transient and do not require laboratory support for diagnosis. Concern is focused on immunosuppressed patients, patients with sickle cell disease, and pregnant women. Enzyme immunoassays, immunoblot tests, and radioimmunoassays have been developed.[41] Determination of IgG is sufficient to determine immune status in a pregnant woman who has been exposed to the virus; measurement of IgM is necessary for documenting acute infection. If hydrops fetalis is feared or suspected, a combination of serologic and virologic approaches is complementary.[507]

Herpes Simplex Virus

In the past, the serologic diagnosis of HSV infections was not useful because of the high prevalence of antibody to type 1 virus early in life and the inability of commercially available assays to distinguish between the two serotypes. However, it has been shown that the glycoprotein G antigen of herpes simplex elicits type-specific antibodies, for which there are now commercially available enzyme and immunoblot assays for IgG. Modern serologic assays for IgM and IgG provide a convenient way to document immune status and/or recent infection in pregnant women.[13,467] When measured against culture-documented genital infections, the type-specific serologic assays have good sensitivity and specificity.[339] Assays are also available for type 1 virus, although the diagnostic imperative is less compelling than for type 2 virus.[467]

Potentially the greatest use for these serologic assays is defining the risk of a primary herpes infection developing in the near term in pregnant women, when it may be transmitted to the neonate. The highest risk is in a woman who lacks antibody (i.e., not previously infected) and a partner who is seropositive. Avoidance of a primary infection is imperative in that situation. It should be noted that recurrent shedding of virus is not uncommon in seropositive women,[470] although the risk for the neonate is less in recurrent infection than in primary disease.

Varicella-Zoster Virus

An important use for serology in the diagnosis of VZV infections is for determination of immune status in patients or healthcare personnel. There have been a variety of methods developed including latex agglutination, a fluorescent test,[270,442] and these were found more sensitive than an enzyme immunoassay.[442] Others, however, have found that enzyme immunoassays yielded satisfactory results.[113]

Cytomegalovirus

Serology is useful in particular clinical settings and of secondary importance in others.[310] Infection with CMV, which is often asymptomatic or subclinical, may be accompanied by DNA in peripheral blood for many months after primary infection, even in the presence of a vigorous antibody response.[506] Recrudescence of this latent virus may be accompanied by an IgM response, so this usual marker of primary infection is flawed in this situation.[116] Challenges with the valid detection of IgM class antibodies complicate results interpretation. In one careful study of intrauterine CMV disease, only 10% of women with IgM antibody as determined by an EIA delivered a congenitally infected fetus.[287] When the presence of IgM antibody was confirmed with a specific immunoblot assay,[288] however, the risk was similar to documented

infection during the first trimester. The detection of primary CMV infection during pregnancy, however, remains an important use for CMV serology.

Serology also plays an important role in assessing risk of recipients of transplant for the risk of future CMV disease. This is based on the serologic status of both the donor and the recipient. The highest risk for serious CMV infection developing is transplantation of an organ from a seropositive donor into a seronegative recipient.[449] When possible, attempts are made to avoid this combination.

Once an immunocompromised individual is known to have contracted CMV, then serology is no longer useful. The detection and quantitation of CMV is accomplished by molecular methods, predominantly quantitative rapid-cycle PCR.

West Nile Virus and Chikungunya Virus

As with other flaviviruses, the West Nile virus does not grow readily in the cell cultures used in most virology laboratories. The diagnosis of this infection is made by serology and, to a lesser extent by molecular techniques. The clinical disease is primarily encephalitis, so the focus is on CSF. IgG and IgM antibody can be detected reliably in serum and CSF.[377] The presence of specific IgM antibody documents a recent infection. The initial outbreak was at first thought to be caused by the St. Louis encephalitis virus, because the two flaviviruses share common antigens. The recommendation for the early positive antibody tests was that positives should be confirmed with a different serologic approach.[306]

The chikungunya virus generates an antibody response close to the 1st week of illness that may be used for diagnosis.

Rubella

The traditional test for assessing immunity to rubella virus was hemagglutination-inhibition, but it has been replaced

by more convenient assays, particularly enzyme immunoassays.[181] These tests work well for determining immune status. For diagnosis of congenital infection, measurement of IgM antibodies with a rigorously validated method is necessary.

SARS and MERS Coronavirus

Antibody to the SARS coronavirus can be detected in >90% of patients with clinically evident disease, using an indirect immunofluorescence technique.[74] A transient IgM response occurs at approximately 20 days from the onset of disease, which is soon

replaced by an IgG response. A very small percentage of patients without clinically evident disease also had seroconversion. At present, serologic diagnosis appears to be the most sensitive method for diagnosing the infection.

Serologic tests for these coronaviruses are available at the CDC for prevalence and surveillance studies, but not for diagnostic purposes.

Miscellaneous Serologic Procedures

A wide variety of procedures can be used for serologic diagnosis of viral infections (Table 23-21). EIA and immunofluorescence tests are gaining in popularity in clinical laboratories; many of these tests are available commercially in kit form.

Diagnosis of Other Viral Infections

Diagnosis of some viral infections is beyond the scope of most hospital laboratories because the infections are rare or exotic, the etiologic agents are hazardous, or special diagnostic facilities are necessary. Tests for rabies, arbovirus infections, and viral hemorrhagic fevers are usually performed in a reference laboratory, such as those in state departments of health or at the CDC.

When the Ebola virus was imported to the United States through an infected individual, the CDC provided guidance regarding safe means in which to perform routine laboratory testing on potentially infected individuals, as well as directions for the packaging and shipping of specimens for testing (www.cdc.gov/vhf/ebola/healthcare-us/).[69] The guidelines were collated on the College of American Pathologist's website for ready access by all laboratorians. Although assays with "Emergency Use" clearance have been released, companion specimens from persons suspected to possibly have Ebola virus disease must also be sent to the authorized state public health laboratory for testing.

Antiviral Susceptibility Testing

Resistance to antiviral agents occurs more commonly in RNA viruses than DNA viruses, since RNA polymerase lacks the proofreading functions of DNA polymerase. The lack of proofreading causes a higher rate of errors in RNA replication, which translates into a large number of quasi-species, in addition to the replication of the parent RNA molecule. Although some of these mutations may produce dysfunctional viruses, others may produce viruses with some evolutionary advantage. One such advantage is resistance to an antiviral drug.

In some instances, the viral population acquires resistance so rapidly that susceptibility testing becomes unnecessary, since the majority of the population is resistant. This is what occurred during the H1N1 influenza epidemic. Initially, the isolates were uniformly susceptible to oseltamivir (i.e., Tamiflu), but soon resistance to

this agent became the rule.[207]

The area wherein antiviral resistance is routinely evaluated is in the care of individuals infected with HIV. Although phenotypic assays, which are expensive, time-consuming and complex, are available, the vast majority of this assessment is done via nucleic acid sequencing (i.e., HIV genotyping). This is commonly done to establish a baseline after the initial infection, which determines the resistance pattern of the virus with which the patient has been infected. HIV genotyping is also performed in patients who have rising viral loads and decreasing CD4 counts, despite compliance with therapy. In brief, the RNA from the virus is reverse-transcribed and select genes are amplified by RT-PCR. The products of amplification are submitted for DNA sequencing to determine if the wild-type sequence is present, or if resistance-inducing mutations are present.

Resistance in other viral agents, particularly the herpes group of viruses, is well recognized, but fortunately uncommon. Although extremely uncommon, occasionally ganciclovir-resistance may arise in patients on chronic suppressive therapy with this drug. Susceptibility testing of clinical isolates, which should be used judiciously, is available in selected reference laboratories.[450]

Infections with *Chlamydia* and *Chlamydophila* Species

Chlamydiae are obligate intracellular bacterial pathogens. Although genetic studies demonstrate that the three organisms considered below are closely related, there have been taxonomic changes.[165] Since the last edition of this text, two of the three species of *Chlamydia* have been reassigned to a new genus, *Chlamydophila.*[444] *Chlamydophila psittaci* causes an acute respiratory infection that is usually transmitted by infected birds.[410] *Chlamydophila pneumoniae* also causes respiratory infections, but is transmitted from person to person. This bacterium was originally referred to as the TWAR bacillus, using the initials of two patients from whom the bacterium was isolated. *Chlamydia trachomatis*, which includes 15 serologic variants (serovars), remains in the genus *Chlamydia.* Infections caused by *C. trachomatis* are common, given the sexual means of transmission and the and often cause asymptomatic infections in women.[407] *C. trachomatis* serovars L1, L2, and L3 produce another sexually transmitted disease, lymphogranuloma venereum. Finally, trachoma, a chronic conjunctivitis often complicated by blindness, is associated with serovars A, B1, B2, and C. Trachoma remains an important cause of blindness in resource-poor countries throughout the world.

All of the chlamydiae may be isolated in embryonated eggs and cell culture.

Recovery of *C. psittaci* in cell culture should not be attempted unless stringent isolation facilities are available for protection of laboratory personnel. Care to prevent laboratory-acquired infections should also be exercised if the serovars that cause lymphogranuloma venereum are suspected. It is rare to culture for these pathogens in modern laboratories, as infections are more commonly documented by molecular or serologic methods.

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TABLE 23-21 Serologic Diagnosis of Viral Infections

Virus	Tests Commonly Performed	Comments
RESPIRATORY INFECTIONS		
Influenza A and B	CF; HAI; EIA	HAI performed for strain-specific immunity
Parainfluenza	CF; HAI; EIA	
Respiratory syncytial	FA; EIA; IB	
Adenovirus	CF; HAI; EIA	Rhesus or rat RBC for HAI
Coronavirus	NT; EIA; FA; IB	Limited data for SARS and MERS virus
CENTRAL NERVOUS SYSTEM INFECTIONS		
Enteroviruses	NT	Not commonly performed
Mumps	CF; HAI; FA; EIA	
Herpes simplex	EIA; IB	Type-specific reagents required
Rabies	NT; EIA	For immune status
Hepatitis		
Hepatitis A	EIA	
Hepatitis B	EIA; RIA	See text
Hepatitis C	EIA; IB	Screening test followed by confirmatory assay

Hepatitis D	EIA; RIA	Rarely performed
Hepatitis E	EIA	
CUTANEOUS INFECTIONS		
Measles	HAI; EIA	
Varicella-zoster	EIA; ACIF; FAMA; LA	For immune status
GENITAL INFECTIONS		
Herpes simplex	EIA; IB	Type-specific reagents necessary
HIV	EIA; IB	Screening test followed by confirmatory test
OTHER INFECTIONS		
Cytomegalovirus	EIA; IFA; Passive latex agglutination	Primarily for immune status
Rubella	HAI; EIA; LA; IB	HAI reference method
Epstein–Barr	CF; FA;EIA	See discussion
Parvovirus	RIA; EIA	
Human herpes 6 and 7	NT; FA; EIA; IB	

ACIF, anti-complement immunofluorescence; CF, complement fixation; EIA, enzyme immunoassay; IFA, indirect immunofluorescent assay; FA, immunofluorescence; FAMA, fluorescent antibody staining of membrane antigens; HAI, hemagglutination-inhibition; IB, immunoblot or immunochromatograph; LA, latex agglutination; NT, neutralization test (the reference test for most viruses, although infrequently necessary); RIA, radioimmunoassay.

Chlamydia trachomatis

Clinical Features and Epidemiology. Inclusion conjunctivitis (an infection distinguished from trachoma by the absence of corneal scarring), pneumonia in neonates, and sexually transmitted infections in adults, all of which are caused by serovars D through K, are the chlamydial diseases of most importance for diagnostic laboratories.[254] The sexually transmitted infections include urethritis, mucopurulent cervicitis, and salpingitis. The sexually transmitted and neonatal infections are directly connected. Schachter and colleagues[408] followed 131 infants born to mothers from

whose cervix *C. trachomatis* was cultured. Culture-confirmed inclusion conjunctivitis developed in 18% of the infants, and 16% had neonatal chlamydial pneumonia.

Collection of Specimens. Cervical and ocular specimens are best collected by scraping the mucosa. Swabs are readily available and minimally traumatic. Wood-tipped swabs and

swabs made of calcium alginate should be avoided; some cotton swabs have been toxic to *Chlamydia*. Dacron or rayon material is preferred.[100] Urethral and nasopharyngeal specimens may be collected with a fine swab on a flexible wire. The swab should be inserted 3 to 5 cm into the urethra. Semen and purulent urethral discharge are not considered adequate specimens for culture.[88] *C. trachomatis* can be detected in first morning urine specimens with amplification techniques, as discussed later.

Cervical specimens are collected from the endocervix after carefully removing mucus. The isolation rate is increased if a urethral and cervical swab are placed in the same transport vial. Vaginal specimens are also acceptable for many of the amplification-based assays.

Isolation of *Chlamydia trachomatis* in Cell Culture. Although chlamydiae are bacteria, they are obligate intracellular pathogens. Methods for isolation in culture are similar to those used in the virology laboratory. It is essential, however, that the infective elementary bodies of chlamydiae be centrifuged onto the monolayer in a shell vial (Fig. 23-10). Isolation of the bacteria is optimized if a force of 3,000 *g* for 60 minutes can be achieved.[430] Cells that have been irradiated or treated with a metabolic inhibitor are used for culture. Cycloheximide-treated McCoy cells are the most commonly used cell line[502]; inclusion bodies of *C. trachomatis* contain glycogen, which may be stained with iodine. After incubation for 48 to 72 hours, the coverslips are removed and stained with iodine or, for somewhat greater sensitivity, with a fluoresceinated monoclonal antibody[438] (Image Plate 23-2C). Species-specific monoclonal antibodies directed against outer-membrane proteins and genus-specific antibodies directed against lipopolysaccharide are available. Chlamydial inclusions are well-demarcated, cytoplasmic structures.

Culture for chlamydia is not commonly performed in many laboratories, given the superior sensitivity of molecular methods for the detection of sexually transmitted chlamydial disease. Additional information regarding culture-based methods is present in previous editions of this textbook for those requiring additional information.

Direct Detection of *Chlamydia trachomatis* in Clinical Specimens

Morphologic Detection of Inclusions. If large numbers of chlamydial inclusion bodies are present, a provisional diagnosis may be established easily by staining smears by the Giemsa or Gimenez methods. The Gimenez stain, which contains carbolfuchsin, is preferred because the inclusions are colored well, but the Giemsa stain is more generally available. The inclusions, which are located in the cytoplasm of epithelial cells, often have a perinuclear location and must be distinguished from artifacts, such as fragmented nuclei (Image Plate 23-2A). The frequency with which inclusions are detected in smears is highest in neonatal conjunctivitis, lower in inclusion conjunctivitis of adults and in trachoma, and lowest in urethritis and cervicitis, where inclusions are rarely found. In practice, direct visualization is rarely attempted.

Detection of Antigen. The initial approaches to nonculture laboratory diagnosis were direct immunofluorescence (Image Plate 23-2B) and subsequently enzyme immunoassay. Although commercial products for some of these tests are still available, they have been supplanted by more sensitive molecular methods. Interested readers should consult the previous edition of this book.

Detection of Nucleic Acid. The first commercial molecular test applied to *C. trachomatis* was a DNA probe against rRNA (Pace 2 Systems Assays, Gen-Probe, San Diego, CA). The sensitivity of the test has ranged from approximately 80% to 95%, depending on the population of patients tested, the source of the specimen, the version of the test evaluated, and the "gold standard" applied.

The second-generation molecular tests were amplification tests of several kinds, many of which constitute the standard of practice today. Most comparative studies have evaluated either the hybridization assays (and/or antigen assays) versus culture, or amplification assays and culture. Numerous studies have been done comparing a variety of detection methods. The most valuable are those studies that compared all three approaches head to head[500]; amplification assays are more sensitive than previous technologies, and now are significantly more automated.

The most commonly used molecular tests at present use PCR (Amplicor CT/NG Test and Cobas Amplicor *Chlamydia trachomatis* [CT]/*Neisseria gonorrhoeae* [NG] Test; Roche Diagnostics),[296] strand-displacement amplification (BD ProbeTec CT and CT/GC assays; BD Diagnostic),[461] and TMA (Gen-Probe Aptima Combo 2 assay; Hologic).[166] There are hybrid capture methods (Hybrid Capture 2 CT/NG DNA test; Qiagen) still available, but these are used less frequently than nucleic acid amplification-based assays.[99] The various amplification methods appear to function in a similar fashion. All are more sensitive than other culture-based methods and immunoassays, but they are also more expensive.[20] In addition to sensitivity, the amplification methods also offer convenience. Some platforms list urine as a valid

specimen type, which increases the convenience of collection; users should refer to individual package inserts for approved specimen types. Research studies have documented the acceptability of self-collected vaginal swabs,[420] which may be an approved collection method in the future. In general, endocervical specimens are marginally more sensitive than urine in women, but urine is more sensitive than urethral swabs in asymptomatic men.[250]

It should be noted that most authorities consider all positive screening tests to be presumptive,[251] requiring confirmation unless the specificity is absolute (a criterion that only culture fulfills). The issue is more pressing for *N. gonorrhoeae* than for *C. trachomatis*, because the prevalence of the former is less. Most assays for *C. trachomatis* are performed in the absence of clinical symptoms and are, therefore, screening tests. Each laboratory director must make a determination. If the economics of repeat testing make universal adoption of confirmatory testing unlikely, it is certainly true that repeat testing is indicated should there be any question about the validity of the result from a clinical perspective. The complexity of the issue has been carefully reviewed by an expert panel.[264] For instance, if the test is repeated, should a different method be used? A second specimen? If the two results differ, which is correct? Although these assays are considered "screening" tests, they are commonly used as definitive tests, and follow-up, confirmatory tests are not usually performed.

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A challenge with any amplification-based assay is the presence of inhibitors of amplification in clinical specimens. This is addressed by the inclusion of an amplification control. Given the issues concerning laboratory-accreditation and competition among vendors, internal amplification controls are present in FDA-cleared, commercially available products. Some methods, such as TMA and helicase-dependent amplification, appear to be affected less by inhibitors.[83]

A common approach to endocervical cytology is the use of a liquid collection medium. This specimen type has been validated for the molecular detection of *C. trachomatis*, as well as HPV, *N. gonorrhoeae*, and in some instances, *Trichomonas vaginalis*.[24]

Serologic Diagnosis. Antibodies to *C. psittaci* and the lymphogranuloma venereum serovars of *C. trachomatis* are useful for establishing the diagnosis of disease caused by these organisms. A microimmunofluorescence test for antibodies to *C. trachomatis* has been found more sensitive for diagnosis of lymphogranuloma venereum infections and can also be used to document infections by other serovars.[153] This test is less widely available, and multiple antigens must be tested. It is most useful for

seroepidemiologic studies of populations at high risk for chlamydial infection.[264]

Other Methods for Diagnosis. Cytologic detection of *Chlamydia/Chlamydophila* has not generally been considered reliable. Kiviat and colleagues,[267] however, have reported a cytologic inflammatory pattern that strongly suggested the presence of chlamydial infection. If "transparent" lymphocytes and increased numbers of histiocytes were present, *C. trachomatis* was isolated in 53% of patients; in the absence of those findings *C. trachomatis* was isolated only from 4% of patients. If these cytologic features are noted in a cervical smear, specific tests for chlamydia should be considered.

Diagnosis of Sexual Abuse. The sexual abuse of children or members of other vulnerable populations may result in the contraction of sexually transmitted diseases, including *Chlamydia trachomatis*, among others.[100] The type of tests that are admissible in court varies from state to state, so local guidance is recommended regarding the use of nucleic acid amplification methods in this setting. Traditionally, the test of choice for investigation of suspected sexual abuse or in case of other legal considerations has been to obtain a culture.[251] This, however, may change in the future.

Chlamydophila psittaci

Chlamydophila psittaci is the causative agent of psittacosis or ornithosis. The source of infection is a variety of birds, especially psittacine species such as parakeets.[203] An outbreak of psittacosis occurred at a turkey-processing plant in North Carolina.[53]Many organ systems may be affected in the natural host. When this agent infects humans, the primary target is the lung.[410] The resulting pneumonia is usually subacute or chronic, but mild or inapparent infection[336] and acute, fulminating infection[45] can result. *C. psittaci* is also a rare cause of culture-negative endocarditis.[477]

The diagnosis of psittacosis is usually accomplished by serology. The traditional test was complement fixation, but the microimmunofluorescence test has become the reference serologic test.[153] In patients suspected of being infected by *Chlamydophila*, complement-fixing antibodies to *C. psittaci* were detected in 36 of 78 sera (46%) submitted for chlamydial serology.[492] An additional 12 sera (15%) were positive by microimmunofluorescence, and 9 more had antibodies to *C. pneumoniae* by microimmunofluorescence.

The organism can be isolated in cell culture using methods designed for *C. trachomatis*, but this agent has high potential for producing laboratory infections, and so this practice is discouraged. If culture is performed, then great care should be taken

with the specimen. Species-specific antisera are necessary for definitive identification of the isolate. Presumptive identification of *C. psittaci* directly in respiratory secretions has been reported by using antisera to chlamydial lipopolysaccharide.[355] The PCR has been used to identify the organism directly in clinical specimens[107] and after isolation in culture.[136]

Chlamydophila pneumoniae

Chlamydophila pneumoniae was originally isolated from patients with respiratory infections and was first called the TWAR agent after the source patients. It produces sporadic and epidemic lower respiratory disease characterized as atypical pneumonia.[185]It has been estimated that 10% of pneumonias worldwide are caused by *C. pneumoniae*,[264] but most infections do not require admission to the hospital. Asymptomatic infection occurs in 2% to 5% of individuals. *C. pneumoniae* was detected by culture or PCR in the nasopharynx of 2 of 104 (1.9%) asymptomatic adults.[242] The prevalence of antibody to *C. pneumoniae* in this population was 82%.

Primary infection occurs in children and young adults, but immunity is not protective and reinfections are possible.[264] The onset of pneumonia is often insidious, without purulent sputum or leukocytosis. The chest radiograph varies from normal to extensive involvement, and there are no diagnostic features. In primary infection, the most common pattern is unilateral alveolar infiltrates.[323] Chronic pulmonary infections occur, and patients may be symptomatic for weeks or months.[200]

Chlamydophila pneumoniae is a cause of pharyngitis; it may be the sole cause or may occur in combination with other agents.[241] It has also been reported as a cause of culture-negative endocarditis.[167] Multiple studies have documented an association between *C. pneumoniae* and atherosclerosis with or without coronary artery disease.[257] The organism has been demonstrated repeatedly in the pathologic lesions. The causality of the association, however, remains a matter for discussion and further investigation.[184]

Chlamydophila pneumoniae can be cultured in the laboratory using a variety of cell lines, but this is usually not attempted. Eagle's MEM or SPG with an added protein source, such as fetal calf serum, as used for viruses and *C. trachomatis*, serves as a useful transport medium.[299] Laboratory-adapted strains of *C. pneumoniae* are relatively stable, but wild-type strains are more labile and do not survive well for more than a few hours even at 4°C. If culture is to be attempted, samples should be frozen, preferably at −70°C, if they cannot be processed on the day of collection. Cultures

are incubated for 72 hours. Fluorescein-conjugated monoclonal antisera are available for detection and identification of isolates in culture.[332] The laboratory diagnosis of *C. pneumoniae* infections is complicated by the difficulty of culture and the absence of an alternative reliable gold standard. Most infections are diagnosed serologically. The serology may be more complicated than realized at present. Antigenic variation among isolates was sufficiently great in one study that some patients produced measurable antibody only against their own isolate.[26] The reference test is microimmunofluorescence. Enzyme immunoassays have been developed, but are variable in their performance.[213] Demonstration of seroconversion has been traditionally used, but this is being replaced by IgM- and IgG-specific assays.[366] Reproducibility of IgM determinations among collaborating laboratories was quite variable.[366]

Molecular amplification methods for direct detection of *C. pneumoniae* in respiratory specimens have been developed because of the difficulties inherent in cultural and serologic approaches. Lack of standardization limits the utility of these potentially important assays at present.[32] These tests are available through reference laboratories.

Infections with *Rickettsia*, *Coxiella*, *Ehrlichia*, and *Anaplasma*

The order Rickettsiales includes the families *Rickettsiaceae* and *Anaplasmataceae*.[121] The most numerous and important genus is *Rickettsia*. The former *Rickettsia tsutsugamushi* has been reclassified as *Orientia tsutsugamushi*. These organisms are found in the alpha-1 subgroup of *Proteobacteria*. *Coxiella*, once classified in the order Rickettsiales, is actually more closely related to *Legionella* and *Francisella*, members of the gamma subgroup of Proteobacteria.[321] Laboratory diagnosis of rickettsial infections and identification of *Rickettsia* in ticks have been reviewed by La Scola and Raoult.[276] A century after the recognition of these infectious agents, Dr. David Walker has described the origins of the field of rickettsiology and the life of Howard Ricketts, who ultimately died of typhus in Mexico City.[474]

Rickettsia *and* Coxiella

Clinical Features and Epidemiology. *Rickettsia* species infect vascular endothelial cells. The clinical manifestations of infection are consequently protean and may reflect damage to any organ system. The skin, lung, and brain are most frequently involved

symptomatically. *Rickettsia* can be divided into the typhus, scrub typhus, and spotted fever groups (Table 23-22). The agent of Q fever, *Coxiella burnetii*, is also an obligate intracellular pathogen. The primary means of transmission of rickettsial infections is by the bite of an infected arthropod, with the exception of *C. burnetii*, which is most commonly transmitted through infected milk or by aerosol.

Epidemic typhus has been one of the great scourges of humankind, appearing during periods of crowding and starvation. *R. prowazekii* has been demonstrated in flying squirrels in the eastern United States,[493] and a small number of associated human infections have occurred. A patient with epidemic typhus has also been recorded in the Southwest.[317]

Although epidemic (louse-borne) typhus is not a serious problem in North America, as recently as 1996 to 1997, this classical disease killed 6,000 people in Burundi. In the United States, endemic (flea-borne or murine) typhus has received renewed attention. A focus of murine typhus was identified in Southern California and in South Texas in the 1990s. It was found to be transmitted by the cat flea and maintained in cats and opossums.[435] The etiologic agent is the classical *R. typhi*[435] and additionally a newly recognized *Rickettsia*, initially named the El Labs (ELB) agent.[416,490] It is difficult to distinguish the two rickettsiae serologically, so molecular analysis is necessary.[416] The ELB *Rickettsia* has now been classified as *R. felis*.[215] By molecular analysis it belongs in the spotted fever group, rather than the typhus group, of *Rickettsia*.[35]

In the United States, the most important rickettsial infection is Rocky Mountain spotted fever, caused by *R. rickettsii*.[471] This multisystemic febrile disease is usually accompanied by a skin rash that begins on the extremities.[472] The infection is widely distributed throughout the country, wherever the tick vectors exist (see Chapter 22; Appendix I). Paradoxically, it is more common in the midwestern and south central states than in the western and mountain states.[206] Similar infections, caused by other rickettsial species, are widely distributed around the world (Table 23-22).[385s]

Scrub typhus, caused by *Orientia tsutsugamushi*, is not endemic in the United States. Scrub typhus is often accompanied by a primary eschar, which is seen only rarely in Rocky Mountain spotted fever. Primary eschars are also seen in rickettsialpox, which is caused by *Rickettsia akari*. The infection was first documented in New York City and associated with the bites of blood-sucking mites. Rickettsialpox is a mild disease and is not commonly recognized, but occurs worldwide. It remains endemic in New York City.[262]

Q fever usually causes asymptomatic infection, undifferentiated febrile disease, granulomatous hepatitis or atypical pneumonia.[321,386] It may be transmitted through milk, products of conception, or excreta of infected cattle, sheep, and goats. The

organism is easily transmitted to susceptible humans, and epidemics have occurred in laboratories where sheep were used for research purposes.[198] A chronic form of the infection may result in subacute bacterial endocarditis. Rash is rare in Q fever.

Collection of Specimens. Culture of *Rickettsia*, which is dangerous, is performed only in reference, public health or laboratories with enhanced precautions. Blood or biopsy tissue from a lesion should be frozen at −70°C. Direct immunofluorescence may be performed on a frozen or formalin-fixed biopsy or autopsy specimen. Rickettsial infections are usually diagnosed serologically, using acute and convalescent sera.

Isolation of *Rickettsia* and *Coxiella* in Culture. *Rickettsia* and *Coxiella* may be isolated in small laboratory animals or in embryonated eggs.[475] *Coxiella burnetii* has been isolated in shell vial cultures of human embryonic lung fibroblasts from blood of 17% of untreated patients

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TABLE 23-22 Rickettsial Infections

Disease	Species	Insect Vector
TYPHUS GROUP		
Epidemic typhus[a]	*Rickettsia prowazekii*	Human body louse
Relapsing typhus (Brill–Zinsser disease)[a]	*R. prowazekii*	Flea
Murine typhus[a]	*R. typhi*	
SPOTTED FEVER GROUP		
Rocky Mountain spotted fever[a]	*R. rickettsia*	*Dermacentor* spp.
Mediterranean spotted fever (Boutonneuse fever)	*R. conorii*	*Haemaphysalis* spp.
Siberian tick typhus	*R. sibirica*	*Haemaphysalis* spp., *Dermacentor* spp.
Queensland tick typhus	*R. australis*	*Ixodes holocyclus*

Queensland tick typhus	*R. australis*	*Ixodes tasmanii*
Japanese spotted fever	*R. japonica*	*Haemaphysalis longicornis; Dermacentor taiwanensis*
Flinders Island spotted fever	*R. honei*	*Aponomma hydrosauri*
African tick bite fever	*R. africae*	*Amblyomma* spp.
Rickettsialpox[a]	*R. akari*	*Allodermanyssus sanguineus* (mite)
Flea-borne spotted fever	*R. felis*	*Ctenocephalides felis* (cat fleas)
SCRUB TYPHUS GROUP		
Scrub typhus	*Orientia tsutsugamushi*	*Leptotrombidium* chiggers (larval mites)
Q FEVER GROUP		
Q fever[a]	*Coxiella burnetii*	Parturient animals, unpasteurized dairy foods

[a] Found in the United States.
Adapted from reference 410.

with acute Q fever and 53% of untreated patients with chronic disease.[347] All of these bacteria are highly infectious when they are aerosolized. They have been responsible for many laboratory-acquired infections, some of which were fatal.[356] Isolation of the agents should be attempted only in laboratories where adequate containment of infection is possible. Whole blood or biopsied tissues may be cultured.

Direct Detection of Antigen and Nucleic Acid in Clinical Specimens. Antigens of *Rickettsia rickettsii*, the cause of Rocky Mountain spotted fever, can be detected directly by staining sections of infected skin lesions using the direct or indirect immunofluorescence or immunoenzymatic techniques.[378,475] The sensitivity of the procedure is approximately 70%, and the specificity approaches 100%. Usually, petechial skin lesions are biopsied. The rickettsiae are most likely to be in the blood vessel at the center of the lesion, so it is important to ensure that such vessels are included in the tissue section. Antigens can also be demonstrated in paraffin-embedded, formalin-fixed specimens if the section is treated first with proteases to

early diagnosis of Rocky Mountain spotted fever, it is available in very few laboratories.

Patients with fatal disease may die before a serologic response develops. Identification of rickettsial antigens in tissue is the only method available for making a specific etiologic diagnosis in that situation.[358]

Molecular amplification assays for documentation of *Rickettsia* in clinical specimens have been described, but are not widely available.[131,276]

Serologic Diagnosis. Most diagnoses of rickettsial infections are made by serology.[118] The microimmunofluorescence assay has become the reference test.[372] This procedure appears to be the most sensitive and specific method for the diagnosis of rickettsial infections.[260] The degree of cross-reaction among rickettsial species varies from patient to patient.[372] Cross-reactions are strongest within the rickettsial subgroups. It may be difficult to distinguish between epidemic and endemic typhus or between Rocky Mountain spotted fever and rickettsialpox.[475] Antibodies first appear 7 to 10 days after infection. A fourfold rise in serum antibody is desired for diagnosis, but a single titer of more than 1:64 is highly suggestive of infection.

The microimmunofluorescence test requires highly trained personnel and a fluorescence microscope. Latex agglutination tests, which are now commercially available for Rocky Mountain spotted fever, are in more general use. The

latex tests appear to produce positive reactions only during an acute infection, so a single positive test is diagnostic.[210] The sensitivity has ranged from 70% to 95%.[210,260] After infection by *Coxiella burnetii*, antibodies may be produced to two biologic phases of the organism. Antibodies to phase II are produced first. Antibodies to phase I, which appear weeks or months later, may reach high levels in patients with Q fever endocarditis. If antibodies to both phases of *Coxiella* are high in a patient with culture-negative endocarditis, a diagnosis of Q fever is likely.[265,437]

Historically, the most common diagnostic test for rickettsial disease has been the Weil–Felix test, in which anti-rickettsial antibodies that cross-react with strains of *Proteus* species are detected. The test originated from the chance observation that sera from patients with typhus agglutinated strains of *Proteus vulgaris*. As experience with immunologically specific serologic tests has been accumulated, it has become apparent that the Weil–Felix test is both insensitive and nonspecific.[211,260,475] In some populations, a positive Weil–Felix test result has been more common in those who did not have Rocky Mountain spotted fever than in those who were infected.[475] This test,

not have Rocky Mountain spotted fever than in those who were infected.[475] This test, therefore, has been appropriately relegated to the archives of laboratory medicine history.

Ehrlichia *and* Anaplasma *Species*

The family *Anaplasmataceae* constitutes Rickettsia-like bacteria that can infect humans and a variety of animal species, producing diseases such as tropical canine pancytopenia and Potomac horse fever (Table 23-23).[398] These organisms are obligate intracellular parasites and, like rickettsiae, grow in the cytosol of infected cells.

Human ehrlichial infection, which is a recently recognized phenomenon, is caused by several distinct species, of which two are most important: *Ehrlichia chaffeensis*, which infects human monocytic cells,[6,104] and *Anaplasma phagocytophilum* that infects granulocytic cells.[77] Both species are transmitted to humans by ticks and produce a febrile illness with leukopenia, thrombocytopenia, and elevated serum aminotransferase.[120]

TABLE 23-23 Some *Ehrlichia* and Related Organisms with Associated Diseases[a]

Organism	Disease	Host
Ehrlichia chaffeensis	Human monocytic	Humans
Ehrlichia ewingii	Canine granulocytic ehrlichiosis	Dogs
	E. ewingii ehrlichiosis	Humans
Ehrlichia canis	Canine monocytic ehrlichiosis	Dogs
Anaplasma phagocytophilum	Human granulocytic anaplasmosis	Humans
	Canine granulocytic anaplasmosis	Dogs
	Equine granulocytic anaplasmosis	Horses
Neorickettsia sennetsu	Sennetsus fever	Humans
Neorickettsia risticii	Potomac horse fever	Horses
Neorickettsia		

[a] With courtesy of input from J. Stephen Dumler, M.D.

Human Monocytic Ehrlichiosis. The monocytic variety of ehrlichiosis, caused by *Ehrlichia chaffeensis*, was recognized first. It is distributed widely through the Southeast, Midwest, and Far West of the United States. Monocytic ehrlichiae have been documented worldwide. In areas where tick vectors are prevalent, seroconversions to *Rickettsia rickettsii* occurred in 2.5% and to *E. chaffeensis* in 1.3% of military personnel.[501] In southern Georgia, human monocytic ehrlichiosis was seven to eight times more prevalent than Rocky Mountain spotted fever.[144] Contact with ticks is a risk factor for both monocytic ehrlichiosis and Rocky Mountain spotted fever.[7] An outbreak of monocytic ehrlichiosis occurred in a golf-oriented retirement community (community A) that abutted a natural wildlife area.[440] Cases were not recognized at a nearby community and golf course (community B) that were not surrounded by a natural area. The prevalence of antibody to *E. chaffeensis* in community A was 12.5%, whereas in community B the prevalence was 3.3%. Thousands of *A. americanum* ticks were found in community A, but only three ticks were detected in community B. The risk factors for ehrlichiosis included tick bites, contact with wildlife, failure to use insect repellent, and golfing. Among golfers, those at greatest risk were the hookers and slicers, who were constantly retrieving their balls from the rough. The moral is that if you golf in a rural area, you should wear insect repellent and hit the ball straight.

The clinical symptoms of monocytic ehrlichiosis range from nonspecific febrile disease to severe systemic infection with multiorgan failure.[120,143] In one study of 237 infected patients, 60.8% were hospitalized.[156] Severe monocytic ehrlichiosis with failure of multiorgan systems has been mistaken for thrombotic thrombocytopenic purpura[315] and toxic-shock syndrome.[141] Life-threatening disease may occur in individuals who are infected with HIV.[357]

Masses of ehrlichiae (morula) may be seen within the cytoplasm of infected monocytes (Image Plate 23-2G), but these diagnostic structures are infrequently seen with *E. chaffeensis* infection.[120,122] The diagnosis is made serologically, less commonly by culture of the agent,[78] and more commonly by molecular methods.[8,137]

Human Granulocytic Anaplasmosis. The second major pathogenic organism is the agent of human granulocytic anaplasmosis (HGA), which was first recognized in Minnesota and Wisconsin by an alert infectious disease physician, who recognized the similarity of the intracellular granulocytic inclusions to those described in *E. chaffeensis* infection.[15,87] The agent of HGA has been classified as *Anaplasma*

similarity of the intracellular granulocytic inclusions to those described in *E. chaffeensis* infection.[15,87] The agent of HGA has been classified as *Anaplasma phagocytophilum*; it is genetically related to the human monocytic agent, *E.* chaffeensis, and *E. equi*, but sufficiently distinct to warrant a new genus designation.[121] Although variants have been described,[318] *A. phagocytophilum* strains in the United States show a high degree of genetic uniformity.[119]

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The most important vector in the United States is *Ixodes scapularis* (Chapters 20 and 22 and Appendix I), the same tick that transmits the microbes that cause Lyme disease and babesiosis.[360] The duration of attachment required for transmission of *A. phagocytophilum* appears to be somewhat less than that required for infection with *Borrelia burgdorferi*,[110,263] but the exact dynamics are not yet clear. Patients in the North Central United States appear from serologic studies to have been infected with all three pathogens.[328] Not surprisingly, the distribution of HGA is expanding to cover the range of the host tick. Infections have been reported from multiple areas in the North Central and Eastern United States. HGA has also been documented in California,[147] as well as in Europe and Asia. In some parts of the country, rickettsial and ehrlichial infections must all be included in the differential diagnosis of febrile disease.[49] Dual infections with ehrlichia and *Rickettsia* have been described.[419] It may be important, therefore, to include antigens of all possible pathogens when attempting serologic diagnosis; demonstration of seroconversion assumes even greater diagnostic importance.[92]

The clinical disease appears to be similar to that produced by *E. chaffeensis*. Thrombocytopenia is more common than leukopenia. Leukocytosis is accompanied by relative and absolute lymphopenia.[14] Instances of interstitial pneumonitis[393] and facial diplegia[289] also occur. Reinfection has been reported.[229]

Laboratory diagnosis favors the use of serology and molecular diagnostics.[2] Intracellular inclusions (morula) in granulocytes (Image Plate 23-2H) appear to be more common than are inclusions in monocytic ehrlichiosis,[15] but there may be an ascertainment bias without readily available laboratory diagnostic support. The morphologic detection of morulae in either of these infections is an insensitive method of detection, but if positive is highly specific. Culture of the organism in vitro has been reported,[180] but confirmation of most cases is by serologic techniques, which are available in reference laboratories.[473] The most common technique is indirect immunofluorescence. Antibodies reach their peak within a month after infection and are still detectable a year later in approximately half of patients.[2] The vast majority of

ehrlichiosis/anaplasmosis, the major outer-membrane proteins do not share antigenic determinants.[460] Western blot analysis may help to resolve confusing serologic results.

Molecular amplification assays for these agents have been described, and reagents are available commercially.[319] The validated assays are available through reference laboratories.

Other Ehrlichial Infections. *Ehrlichia ewingii* and *E. canis* are both agents of canine granulocytic ehrlichiosis that can also cause disease in humans.[43,143,303] Additional agents within this group, termed *Neorickettsia,* have also been described and cause disease in humans (*N. sennetsu*), horses (*N. risticii*), and salmon (*N. helminthoeca*).[398] Other agents that will likely be classified as *Ehrlichia, Anaplasma,* or *Neorickettsia* species are under investigation.

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APPENDIX I

Ectoparasites and Other Invertebrates in the Clinical Laboratory: A Brief Guide

thePoint For additional ancillary materials related to this chapter, please visit the point.

Submission of Specimens to the Laboratory

***Arachnida*: Ticks, Mites, and Spiders**

Ixodidae

Biology of Hard Ticks
Relation to Disease
Geographic Distribution
Identification
Summary of Identification

Ornithodoros Species

Relation to Disease
Identification
Geographic Distribution

Mites

Sarcoptes scabiei (Scabies)

Demodex folliculorum (Hair Follicles) and *Demodex brevis* (Sebaceous Glands)
Spiders
Loxosceles reclusa
Latrodectus mactans
Tegenaria agrestis
Insecta: Flies, Bugs, Fleas, and Lice
Myiasis-Producing Fly Larvae
Dermatobia hominis (Human Bot Fly)
Relation to Disease
Identification
Geographic Distribution
Cordylobia anthropophaga (Tumbu Fly)
Relation to Disease
Identification
Geographic Distribution
Other Flies
Mosquitoes
Relation to Disease
Identification
Geographic Distribution
True Bugs or *Hemiptera*
Cimex lectularius
Kissing Bugs: *Panstrongylus*, *Rhodnius*, and *Triatoma* Species
Fleas
Relation to Disease
Identification
Geographic Distribution
Lice
Pediculus humanus subsp. *humanus*
Pediculus humanus subsp. *capitis*
Phthirus pubis

Ectoparasites are organisms that live in or on the skin of a host, from which they receive nourishment. The relation is, therefore, parasitic or possibly symbiotic. Intimacy of contact varies for the time required for a blood meal from days, weeks, or even months of association. Organisms that contact a host casually (e.g., houseflies) or

without deriving benefit (e.g., scorpions, bees, and spiders) are not, strictly speaking, ectoparasites, but are of medical importance.[64]

Arthropods are invertebrates with jointed appendages (hence the name) and a chitinous exoskeleton. They have long been recognized as direct or indirect causes of human disease. In addition to causing direct injury to the human host, they may serve as the vectors for transmission of infectious agents and facilitate entry of the agent into the human host. In some instances, the arthropod serves as a mere carrier, depositing the infectious agent in the proximate environment, where it can contact the potential victim. An example of such a scenario is the transmission of bacterial pathogens, such as *Shigella,* from one site to another on the bodies of flies or cockroaches.[2,45] A much more intimate relationship occurs when the arthropod is the direct mediator of cellular damage or when a biting insect transmits an infectious agent into the tissue of the victim. Most biting insects ingest the infectious agent when they take a blood meal from an infected human or from an infected nonhuman mammal, which is referred to as a reservoir of infection. When an insect bites an animal or human, it often injects salivary secretions that contain enzymes and anticoagulants designed to permit the ingestion of a blood meal. If the infectious agent has moved from the gut of the insect into the hemolymph and subsequently made its way to the salivary glands, the infection is transmitted in the salivary secretions at the time of the bite.[43] If the infectious agent is present in the gut—with or without infectious material in the saliva—the microbial agent may be excreted in the feces when the arthropod defecates after ingestion of the blood meal. Entry of the agent

from the feces into the injection site is facilitated when the host scratches the site of irritation.

The list of medically significant arthropods continues to expand, as does the list of diseases they can produce. At the same time, clinical laboratories are receiving increasing numbers of specimens for identification. Many arthropods that are very important in human infectious disease are rarely, if ever, seen in clinical microbiology laboratories because the insects were not seen, were exterminated, or were not collected for examination. Other important insects, such as scorpions, are seen in localized geographic distributions. Conversely, clinical microbiologists may be called on to identify insects that caused alarm at their presence even though they have done no harm.

This section is designed to assist the reader in understanding the medical significance and laboratory identification of some arthropods that may be encountered in the clinical laboratory. An abbreviated classification schema is detailed in Table A-1, and select definitions and related taxonomy of the ectoparasites of importance to

humans are listed in Box A-1. The reader is referred to several excellent reference textbooks for more detailed information.[17–20,40,49,54,56]

Submission of Specimens to the Laboratory

Because of the diversity of specimens encountered in medical entomology, it is difficult to recommend a single method for submission of specimens. In general, 70% ethanol will work well as a preservative for most specimens, also preventing escape or infection. It should be added to the specimen in a clean container so that the arthropod is completely submersed. Specimens can darken after extended periods of preservation, negating the utility of color differences for identification after long periods of fixation. We have observed no degradation of color in specimens preserved in 70% ethanol over a period of days, which is the usual time from collection to processing.

Formalin and sterile saline may be satisfactory but generally have more disadvantages when compared with ethanol. Flying insects should be killed using gases, such as chloroform, and then preserved as a dry mount.[18] Garcia lists a recipe for Berelese's medium, which she suggests is a good solution to use for most specimens.[18] Killing in hot water has been suggested for examination of fly larvae.[17] If in doubt, check with the facility that will be handling the specimen for specific recommendations.

The preferred method for removal of ticks is to grasp the tick with fine tweezers as close to the site of penetration as possible without pinching the skin. Steady outward pressure, while lifting the tick upward and forward, usually removes the specimen intact.[36] The capitulum of hard ticks is an important identifying feature. Specimens that are degraded because of improper removal are more difficult to identify.

The materials and techniques required for examination of arthropods are simple. Low magnification can be obtained with a hand lens or optimally with a stereomicroscope. Low- to moderate-power magnification can also be obtained with a low-power objective (e.g., 2× or 4×) and a compound microscope. A good light source is essential for observation. Fiberoptic lights provide excellent illumination without heating and excessive drying of the specimen. Lights with flexible arms allow maximal control of the illumination angle. The specimen can be manipulated best with a pair of dissecting needles.

TABLE A-1 Classification of Arthropods of Medical Importance

Class	Order or Subclass	Examples	Direct Injury or Infections Transmitted
Diplopoda		Millipedes	Direct
Chilopoda		Centipedes	Direct
Hexapoda (Insecta)	*Hemiptera*	Bedbugs Kissing bugs	Direct
Hexapoda (Insecta)	*Siphonaptera*	Fleas	Direct
Hexapoda (Insecta)	*Anoplura*	Sucking lice	Direct; typhus; trench fever; relapsing fever
Hexapoda (Insecta)	*Dictyoptera*	Cockroaches	Sanitation problems; allergic reactions
Hexapoda (Insecta)	*Hymenoptera*	Ants, wasps, bees	Direct
Hexapoda (Insecta)	*Coleoptera*	Beetles	Direct
Hexapoda (Insecta)	*Diptera*	Flies, mosquitoes, midges	Direct; arboviruses; parasitic infection; *Bartonella; Francisella*
Hexapoda (Insecta)	*Lepidoptera*	Moths, butterflies, caterpillars	Direct
Pentastomida		Tongue worms	Direct
Arachnida	Subclass: *Scorpiones*	Scorpions	Direct
Arachnida	Subclass: *Araneae*	Spiders	Direct
Arachnida	Subclass: *Acari*	Ticks, mites, chiggers	Direct; *Borrelia*; arboviruses; *Ehrlichia; Anaplasma; Rickettsia; Francisella*

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BOX A-1

Ectoparasites: Definitions and Taxonomy

Phylum *Arthropoda*: Organisms within this phylum have a hard, jointed exoskeleton and symmetrical paired, jointed legs. Of the five classes of arthropods, only the *Arachnida* and the *Insecta* serve as vectors of human disease.

Class *Arachnida* (ticks, mites, and spiders): This group of arthropods are characterized by the absence of wings, presence of four pairs of legs, and the fusion of the head and thorax into a cephalothorax.

Ticks are larger than mites, have a leathery body that is either hairless or covered with short hairs, an exposed hypostome, and a pair of spiracles near the coxae of the fourth pair of legs. They are divided into the argasid, or soft ticks (*Ornithodoros* species being of human importance), and the ixodid, or hard ticks, including the genera *Dermacentor, Amblyomma,* and *Ixodes,* which are responsible for transmitting the agents of several rickettsial, viral, bacterial, and spirochetal diseases.

Mites are smaller than ticks, do not have a leathery covering, and have a hypostome that may be unarmed. Of importance to humans are the trombiculid mites, causing chiggers, and the mange mites of the family *Sarcoptidae,* which include *S. scabiei,* the agent of scabies, and *Demodex folliculorum,* which infest the hair follicles and sebaceous glands.

Spiders are unsegmented, and have a hairy abdomen to which are attached four pairs of legs through a slender constriction. They also possess a pair of poisonous jaws through which venom flows from a pair of glands in the cephalothorax. The brown recluse, black widow, and hobo spiders most commonly cause painful and toxic bites in humans.

Class *Insecta* (flies, mosquitoes, bugs, fleas, and lice): This group of arthropods include organisms with a body divided into three parts, head, thorax, and abdomen, and possess three pairs of legs. The order *Hemiptera* includes True Bugs, which include winged or wingless bugs and lice that have mouth parts adapted for piercing and sucking; the order *Diptera* (two-winged) includes flies, gnats, and mosquitoes, and the order *Siphonaptera* includes the fleas, which are wingless and have mouth parts adapted for sucking blood.

Flies are two-winged insects that serve as vectors for the transmission of several agents of human disease. Included are the *Phlebotomus* sandflies (leishmaniasis),*Simulium* black fly (onchocerciasis),*Chrysops* deer fly (*Loa loa*), and the *Glossina* or tsetse fly (trypanosomiasis). Mosquitoes transmit a variety of viral, protozoan, and helminthic diseases, most notably malaria and filariasis. Humans may also be infested with the larval forms of flies, a condition known as myiasis.

The biting and blood-sucking True Bugs of importance in human disease include *Cimex lectularius,* the common bedbug, and the "kissing bugs" of the family *Reduviidae,* which transmit the causative agent of South American trypanosomiasis (Chagas' disease).

Fleas are small, brown, wingless, laterally compressed, blood-sucking insects most notably cited as vectors for transmission of the agents of bubonic plague and murine typhus. Fleas are also involved in the transmission of parasitic diseases, including the dog tapeworm,*Dipylidium caninum,* and the rat tapeworm,*Hymenolepis diminuta*.

Lice are dorsoventrally flattened, wingless insects that include three species of importance to humans: *Pediculus humanus* subspecies *capitis* (head louse),*Pediculus humanus* subspecies *corporis* (body louse), and *Phthirus pubis* (crab louse). The order includes both biting and sucking lice; however, only the latter, in the suborder *Anoplura,* are ectoparasitic for humans. Lice are important vectors for epidemic typhus and relapsing fever.

In the exceptional circumstance that culture of the potential pathogen from the vector is under consideration, the insect must be unfixed. If the specimen was fixed before submission to the laboratory, modern molecular methods allow specific identification of infectious agents by amplification of nucleic acids.

Arachnida: Ticks, Mites, and Spiders

Ticks are arthropods in the suborder *Ixodida*, which is divided into three families, two of which, *Ixodidae* (hard ticks) and *Argasidae* (soft ticks), have been established as vectors for human disease.[36,51] In *Ixodidae*, the anterior portion of the body is sclerotized and includes a scutum ("dorsal shield") and an anterior-facing capitulum ("head"), thus the term "hard tick."[51] The genera most commonly submitted to microbiology laboratories are *Ixodes*, *Dermacentor,* and *Amblyomma*. *Argasidae* have a leatherlike outer surface with no scutum and have ventrally located mouthparts.[51] Increasingly in past decades, hard ticks have been recognized as important vectors for a wide variety of human diseases, the diversity of which is greater than for any other group of arthropods.[51] In the United States, hard ticks are more prevalent than soft ticks and are more important vectors of human pathogens. Identification of a submitted tick should include description of the species, life cycle stage, gender for adults, level of engorgement, and status of mouthparts.

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Ixodidae

Biology of Hard Ticks. Hard ticks have a life cycle that consists of four stages (instars): egg, larva, nymph, and the sexually dimorphic adult. Larval ticks have six legs, a key identifying feature (Image Plate A-1A), whereas nymphs and adults have eight. Immature stages lack a genital aperture but otherwise resemble adult females. Both have a scutum that covers one-third to one-half of the dorsum in female ticks and allows the prolific intake of blood and tissue fluids (Image Plate A-1B). Feeding is necessary for females to achieve fertility, and their body weight can increase as much as 100 times while feeding.[36,51] Males have a scutum that covers almost the entire dorsum (Image Plate A-1B) as well as sclerotized ventral plates that limit engorgement in most species, if the male tick feeds at all.

The important vectors of human disease in the United States are three-host ticks, meaning each instar feeds once on a host, after which the tick leaves the host to molt into the next instar in a suitable microenvironment, such as leaf litter or a nest.[51] The process proceeds until adult mating takes place. Mating can occur on the host or off, depending on the species. After mating, the female finds a suitable environment, lays hundreds to thousands of eggs depending on the species,[36,51] and then dies. The duration of a life cycle, averaging 1 to 2 years, varies with the presence or absence of suitable environmental conditions and availability of a suitable host.[51] In adverse conditions, ticks are capable of long periods of diapause, a torpid phase in which the

tick secretes a hygroscopic saliva that allows them to absorb moisture, enhancing survival until host-seeking conditions improve.

Most hard ticks are burrow or nest dwellers, living out their lives in a closed environment, feeding on a readily available host. In these species, human contact is rare or nonexistent.[51] *Ixodes spinipalpus* and wood rats perpetuate an enzootic cycle of *B. burgdorferi* infection in Colorado.[31] The absence of human infection can be attributed to the lack of contact between *I. spinipalpus* and humans.

In only few instances does host specificity place infected ticks in an environment that promotes human contact, which makes them more competent as vectors for disease. The preferred host of *I. scapularis* in early life stages is *Peromyscus leucopus*, the white-footed mouse, known to be the reservoir for *Babesia microti* as well as *Borrelia burgdorferi* in these locations. Although the reservoir for *Anaplasma* (*Ehrlichia*) *phagocytophila* remains unclear, *I. scapularis* is the documented vector. The preferred host of adult ticks is the white-tailed deer (*Odocoileus virginianus*), a far-ranging animal with a distribution that is expanding to include areas of dense human population. With the ability to harbor and transmit multiple pathogens and the willingness to feed on humans, *I. scapularis* is a competent vector. In contrast, *I. scapularis* in the Southeast and *I. pacificus* in the Northwest are less competent as vectors of Lyme borreliosis. Host selectivity appears to be the reason. The preferred hosts in both cases are lizards, in which the spirochetes multiply poorly. This biologic phenomenon could account for fewer cases reported in the West and the virtual absence of reported cases in the Southeast.[31,55]

Ticks seek their host by questing, a passive ambush style of host-seeking, in which they climb low grasses and herbaceous vegetation, waiting to attach themselves to a passing host.[51] A few species, on the other hand, actively seek their prey. *Amblyomma americanum* is the only species in the group of ticks discussed here that actively seeks a host.[36] The species that commonly seek human hosts appear to have anatomic preferences for their attachment.[14] *Dermacentor variabilis* prefers the head and neck, whereas *Amblyomma americanum* is more likely to attach to the buttocks, groin, or legs. *Ixodes scapularis* is ubiquitous, attaching to all parts of the body without any obvious preference for a particular site.

The geographic ranges of ticks seem to be expanding. The explosion in the white-tailed deer population into areas of dense human population may be one reason. Another explanation could be that current lifestyles in the United States promote outdoor activities that place people in close proximity with ticks in their natural environment.

Within the life cycle of a tick, a pathogen may be acquired in one of two ways: (1) transstadial (horizontal; i.e., larva to nymph, nymph to adult) or (2) transovarial (vertical; i.e., gravid female to egg).[51] The larval forms of *Ixodes scapularis* are

unable to transmit Lyme disease because *B. burgdorferi* is not transmitted vertically, but must be acquired from a reservoir host.[36,51,54] The preferred host for *I. scapularis* larvae and nymphs is the white-footed mouse (*Peromyscus leucosis*), a principal reservoir for *B. burgdorferi*, *B. microti*, and *A. phagocytophilum* in endemic areas. A parallel cycle for *A. phagocytophilum* has been documented between cottontail rabbits and *Ixodes dentatus*,[21] although this species feeds on humans much less frequently than does *I. scapularis*. Ticks acquire *B. burgdorferi* or *A. phagocytophilum* while feeding. In contrast, *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever, is transmitted vertically from infected *Dermacentor variabilis* females to their progeny,[36,51] making larvae potential vectors.

The level of engorgement gives a rough estimate of feeding time. *B. burgdorferi* dwells in the midgut of infected *I. scapularis*. When the tick feeds, the spirochete multiplies and begins to migrate via the hemolymph. It requires 2 to 3 days for the spirochete to reach the salivary glands of the tick,[13,41,52] where in the process of shedding excess water to make room for the blood meal the tick regurgitates into the feeding site, thus inoculating and infecting the host with the spirochete.[50] In experimental studies, *I. scapularis* nymphs and females were allowed to feed on rodent hosts for defined periods. Given the fact that body length increases while the size of the rigid scutum remains constant, regression equations based on a ratio of body length to scutum width, termed the "scutal index," were developed. The scutal index is considered a more accurate predictor of feeding time than subjective visual estimates.[13,52] In the case of Lyme borreliosis, the identification of an unengorged specimen of *I. scapularis* with intact mouthparts would suggest brief host contact and would make transmission doubtful even if the patient had traveled to an endemic area. *Anaplasma* (*Ehrlichia*) *phagocytophilum* appears to be transmitted somewhat more quickly than is *B. burgdorferi*, so the degree of engorgement and scutal index may be less useful for assessing the likelihood that the pathogen has been inoculated into the patient.[10,26] More studies are required to elucidate this issue.

Nymphs are considered the most likely instar to transmit disease because their small size often allows them to complete feeding undetected (Image Plate A-1B). A high percentage of tick-related diseases present in late spring and the summer months because of increased nymph activity.

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TABLE A-2 Hard Ticks and Associated Disease with Geographic Distribution

Tick Species	Disease	Geographic Range

Ixodes scapularis	Lyme disease Human granulocytic anaplasmosis Babesiosis	Eastern and upper midwestern United States
Ixodes pacificus	Lyme disease	Northwest United States
Dermacentor variabilis	Rocky Mountain spotted fever Tularemia Tick paralysis	Eastern two-thirds of the United States, West coast of the United States
Dermacentor andersoni	Rocky Mountain spotted fever Colorado tick fever Tick paralysis	Rocky Mountain states
Amblyomma americanum	Human monocytic ehrlichiosis	South Central United States, southeastern United States, Mid-Atlantic states as far north as southern New York State
Ixodes cookei	Tularemia Powassan virus	Northeastern United States and southeastern Canada
Ixodes marxi	Powassan virus	Northeastern United States and southeastern Canada

Relation to Disease. The history of *Ixodidae* is long in veterinary medicine and more recently intense in human disease. *Ixodidae* (hard ticks) are vectors for a wide variety of pathogens associated with human disease,[51] including spirochetes, *Rickettsia*, gram-negative bacilli, viruses, protozoa, and neurotoxins. The species described here are associated with one or a combination of these agents (Table A-2).

Infections transmitted by ticks may present with similar vague early symptoms of fever, fatigue, and other flu-like symptoms that make a quick accurate diagnosis problematic,[11,32,36,54] considering that antibiotic therapy differs depending on the agent involved. In most cases, tick-transmitted diseases are mild and resolve with an early accurate diagnosis, when followed by proper treatment. Misdiagnosis can lead to increased morbidity, chronic infection, and in rare cases, death.

Coinfection after tick bites seems likely.[32,39] Although not proof of coinfection as opposed to sequential infection, serologic studies have shown that, in overlapping endemic areas, patients with clinical Lyme disease have antibodies to *Babesia* or *Ehrlichia* as well.[32] At one point, *Babesia* was considered the agent of Lyme disease because patients diagnosed as having babesiosis presented with erythema migrans,

which is now known to be characteristic of *B. burgdorferi* infection.

Certain species are the vectors for specific pathogens. For instance, Lyme borreliosis is well documented worldwide. *Ixodes scapularis* is defined as the vector for *Borrelia burgdorferi*, the Lyme disease spirochete, in the northeastern and upper Midwestern sections of the United States. Elsewhere, *I. ricinus* (Europe), *I. persulcatus* (Asia), and *I. pacificus* (northwestern United States) are vectors. Currently, other *Ixodes* species are not considered competent vectors. In the eastern United States, identification of an *Ixodes* sp. other than *I. scapularis* would all but rule out Lyme disease, although if the clinical presentation were classical (erythema migrans present)[35,50,54] the presence of a second unnoticed tick would need to be considered. In the southern United States, a Lyme-like borreliosis caused by *Borrelia lonestari*, similar to *B. theileri* (the agent of bovine borreliosis), has been attributed to exposure to *Amblyomma americanum* (the Lonestar tick).[24]

Tick paralysis is a fascinating disease of unknown pathogenesis.[12,15,46] The acute flaccid paralysis can mimic botulism and Guillain–Barré syndrome; removal of the tick produces a rapid and dramatic reversal of the paralysis. There are no sequelae. Although over 60 species of ticks can produce tick paralysis in animals, only *Dermacentor andersoni*, *Dermacentor variabilis,* and *Ixodes holocyclus* (the Australian marsupial tick) bite humans. In North America, the disease occurs most commonly in the southeastern and northwestern states of the United States and in British Columbia.

Geographic Distribution. The geographic distribution of the hard ticks is summarized in Table A-2; this is also available online from the Centers for Disease Control and Prevention at http://www.cdc.gov/ticks/geographic_distribution.html, and elsewhere.

Ixodes scapularis. Once referred to as *Ixodes dammini* in the Northeast and *Ixodes scapularis* elsewhere, the two are now considered by most authorities to be a northern and southern variety of the same species because of their ability to mate successfully.[34,54] The name *Ixodes scapularis* has taxonomic precedence. *I. scapularis* occupies a wide range east of the Rocky Mountains, although infectious populations appear to be focal in the upper Midwest and in the Northeast from Maryland to southern New England.

Ixodes cookei. The range of *Ixodes cookei* (the woodchuck tick) overlaps that of *I. scapularis*; it is found primarily in the northeastern United States and southeastern Canada.

Ixodes marxi. *Ixodes marxi* (the squirrel tick) also has a range that overlaps *I. scapularis* in the northeastern United States and southeastern Canada.

***Dermacentor* spp.** *Dermacentor variabilis* (the dog tick) is widely distributed throughout the United States east of the Rocky Mountains. The wide range and host-

seeking habits of *D. variabilis* increase the incidence of contact with humans when compared with other *Dermacentor* spp.

Dermacentor andersoni is distributed in the Rocky Mountain states and is the tick that was described by Ricketts as the vector of Rocky Mountain spotted fever in the Bitter Root Valley of Idaho.[60]

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Amblyomma americanum. *Amblyomma americanum* (the Lonestar tick) is widely distributed in the South (east of the Rocky Mountains) and in the East to southern New York State. There are indications that the range may be extending into New England. *A. americanum* is unique among the tick species described here in that it actively seeks a host.[36,51]

Identification. Acari (ticks and mites) are distinguished from other arachnids by the absence of obvious segmentation. An anterior prosoma (appendages) is fused with the opisthosoma (abdomen). A gnathosoma (capitulum) bearing the mouthparts is unique to Acari.[51] Figures A-1 and A-2 represent the dorsal and ventral view, respectively, of a generic hard tick. The drawings were made to place as many key features in view as possible. A glossary of identifying features depicted in these figures follows. All features are useful for identification of ticks; size, placement, color, and presence or absence of each characteristic may be important.

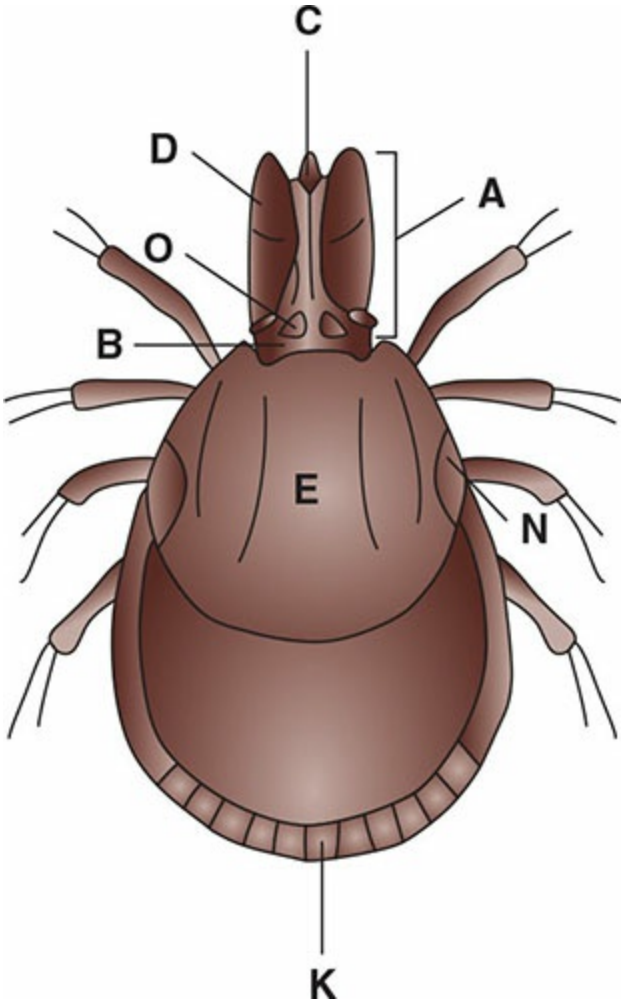


FIGURE A-1 Generic diagram of the dorsum of a hard tick.

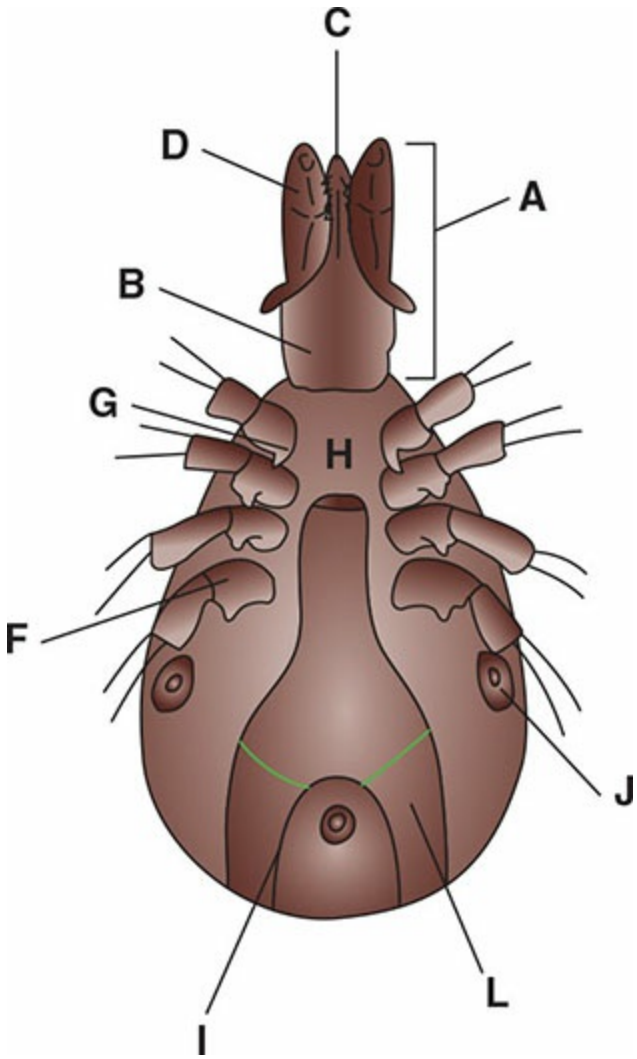


FIGURE A-2 Generic diagram of the ventral aspect of a hard tick.

A. Capitulum: The anterior portion of the body, including the basis capituli, hypostome, chelicerae, and palpi.
B. Basis capituli: The base of the capitulum to which the mouthparts are attached. (The length of the capitulum in proportion to the basis capituli is useful in identification.)
C. Hypostome: The median structure of the mouthparts parallel to and between the palpi. The hypostome holds teethlike structures called denticles. Denticles appear on the hypostome in a specific arrangement called the "dentition formula" (ratio of left denticles to right denticles). *Ixodes scapularis*, for instance, has a dentition formula of 3/3, becoming 2/2 as it progresses posteriorly.
D. Palpi: Paired movable appendages lying parallel to the hypostome.
E. Scutum: The sclerotized dorsal plate posterior to the capitulum.
F. Coxa: The basal segment of the legs. Coxa are numbered I through IV, with coxa I

located closest to the capitulum.

G. Coxal spurs: Projection on the posterior margin of the coxa. Spurs on the median side are called internal spurs while those on the lateral side are external spurs. There is little or no variation in spur size or configuration from specimen to specimen of the same species.

H. Genital aperture: External opening of the genital organs, located ventrally on the median axis between the coxae.

I. Anal groove: Semicircular groove curving around the anus of some species. In *Ixodes* spp., the curve is anterior to the anus. In other genera, the curve is posterior or absent.

J. Spiracular plates: External opening of the respiratory system, located ventrally, posterior to coxa IV on the lateral margins. These are useful in differentiating male *I. scapularis* from male *I. cookei* and male *I. marxi*.

K. Festoons: Rectangular areas separated by distinct grooves located on the posterior margin of some hard ticks. Festoons are absent in *Ixodes* spp.

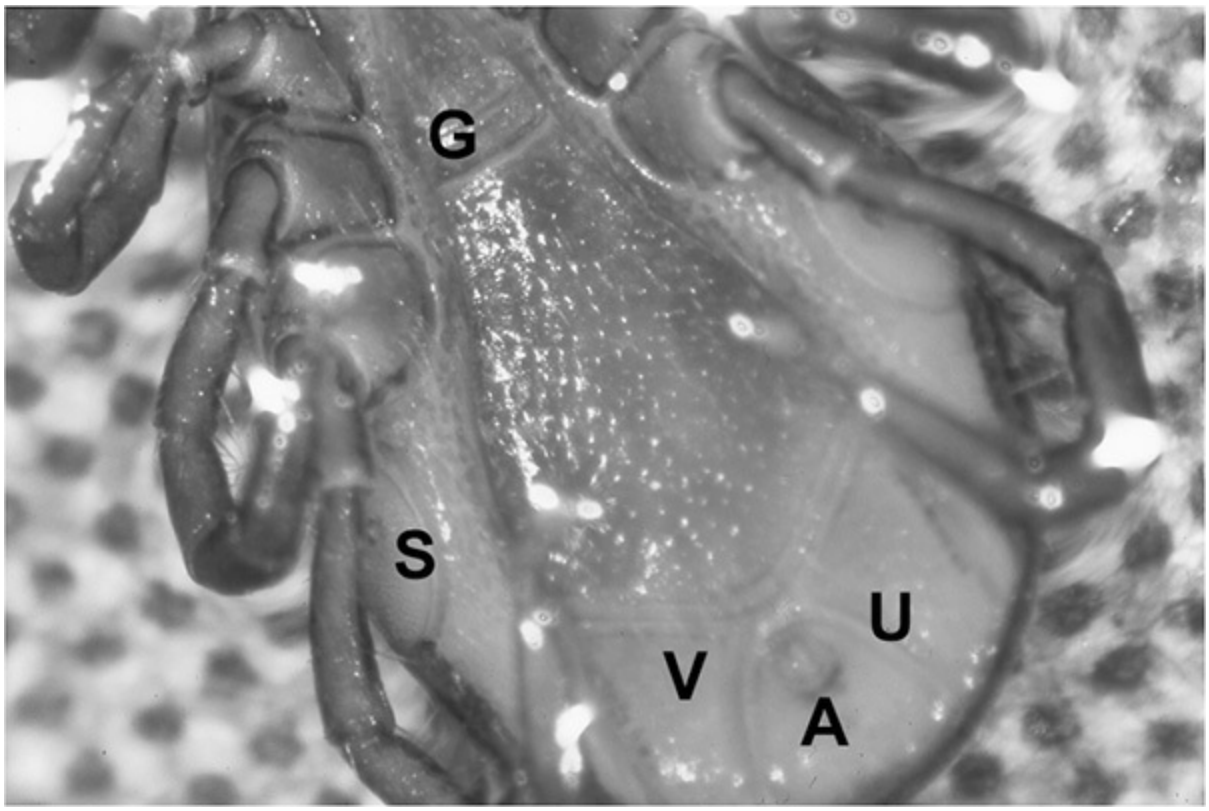
L. Ventral plates: A group of plates of definite shape and size on the ventral surface of adult males. We will specifically refer to the anal plate when describing *Ixodes* spp.

M. Ornamentation: Some ticks are referred to as ornate ticks based on the presence of colorful markings distinct to certain species. *D. variabilis* and *A. americanum* are ornate ticks.

N. Eyes: Ornate ovoid markings on the lateral margins of the scutum of some ticks.

O. Porose areas: Areas resembling "eyes" found on the dorsal basis capituli of adult ticks.

Ixodes scapularis. An important feature of all *Ixodes* spp. is an anterior-facing U-shaped groove that surrounds the anus and terminates at the posterior margin of the ventral surface (Fig. A-3 and Image Plate A-1C). Other genera of hard ticks lack this feature.[51] *I. scapularis* nymphs and adult females have an oval-shaped scutum (Image Plate A-1B) that is approximately half the size of the dorsum in unengorged specimens. The scutum of adult males covers essentially the

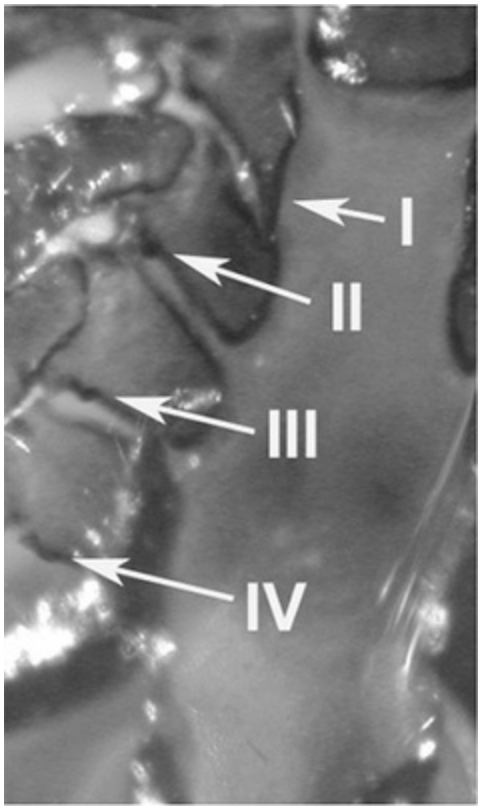


■ **FIGURE A-3** Ventral aspect of adult male *Ixodes scapularis*, demonstrating the anus (A), U-shaped anal groove (U), genital pore (G), ventral plates (V), and spiracular plates (S).

entire dorsum (Image Plate A-1B), a characteristic of all hard ticks that will not be mentioned in further descriptions. The presence of coarse hairs over the entire scutum is one of the characteristics that distinguish *I. scapularis* males from *I. cookei* and *I. marxi* specimens, in which they are fine or absent. When viewed ventrally (Image Plate A-1C), mouthparts appear twice as long as the basis capituli. The hypostome of nymphs and adult females has a dentition formula of 3/3, progressing to 2/2 anterior to posterior. Adult males have a more "businesslike" hypostome than most other *Ixodes* spp., with severe lateral denticles that get blunt to crenulated as they approach the median.

Color can help in distinguishing *I. scapularis* from other *Ixodes* spp. The sclerotic anterior portion of nymphs and adult females (includes the capitulum, legs, and scutum) is dark brown, in sharp contrast to the abdomen, which appears reddish-brown in unengorged specimens (Image Plate A-1B) to gray-tan in engorged specimens, and thus the name *black-legged tick.* Adult males appear entirely dark brown (Image Plate A-1B). Coxal spurs (Image Plate A-1D and Fig. A-4) are present, with coxa I having a severe internal spur that overlaps coxa II. Coxae I through IV have blunt external spurs. The internal spur on coxa I appears less severe in nymphs. The spiracular plates of nymphs and females are round (Image Plate A-1C); in males,

they are elongated anterior to posterior (Fig. A-3). The spiracular plates in males of *I. cookei* and *I. marxi* appear round. The anal plate in males is short anterior to posterior in comparison to *I. cookei* and *I. marxi*.



■ **FIGURE A-4** Detail of Image Plate A-1D. Coxal spurs of adult *Ixodes scapularis*. The spur on coxa I is long, pointed, and oriented internally. The spurs on coxae II through IV are short, blunt, and oriented externally.

Ixodes cookei. The scutum of nymphs and female adults is angular from the posterior apex to the midlateral margins (Image Plate A-1E). In adult females, the scutum appears about as wide to the lateral margins as it is anterior to posterior, whereas in nymphs the scutum appears wider laterally than anteriorly to posteriorly. The mouthparts of all life stages appear proportional in length to the basis capituli (Image Plate A-1E). When compared to *I. scapularis*, the hypostome of females and nymphs is similar, with a dentition formula of 3/3, progressing to 2/2, although in *I. cookei* females the apex is more rounded. The hypostome of nymphs is more rounded still. The hypostome in males is short, round, and crenulated. An interesting feature, most pronounced in the nymph of this species, is the projection of the first segment of the palpi laterally from the posterior margin. These projections have a spurlike appearance and are often referred to as palpal spurs. In all stages, the color is a lightly-

creamed-coffee brown. Color is consistent over the entire tick. The arrangement of coxal spurs is similar to that of *I. scapularis,* with a pronounced internal spur on coxa I and blunt external spurs on coxae I through IV. Spiracular plates in nymphs and females are similar to *I. scapularis*. In males they appear more round, not elongated, as is the case in *I. scapularis* males. The anal plate in males of this species appears twice as long anterior to posterior in proportion to lateral width, which helps distinguish this species from *I. scapularis*.

Ixodes marxi. The scutum of nymphs and females of this species is similar to *I. cookei* in that it is angular from the midlateral margin to the posterior apex (Image Plate A-1F). This fact can confuse the identification of these species. In females, there are differences in length and width. The scutum of *I. marxi* females is noticeably longer anterior to posterior in relation to lateral width. In *I. cookei,* length and width are equally proportional. In *I. marxi* nymphs, length and width are basically equal, whereas in *I. cookei*, the scutum appears laterally wider than it is long anterior to posterior. Given the similarity in scutal proportion of an *I. marxi* nymph and an *I. cookei* adult female, identification should be made by observing the presence or absence of a genital aperture. The male scutum appears to be absent of hair.

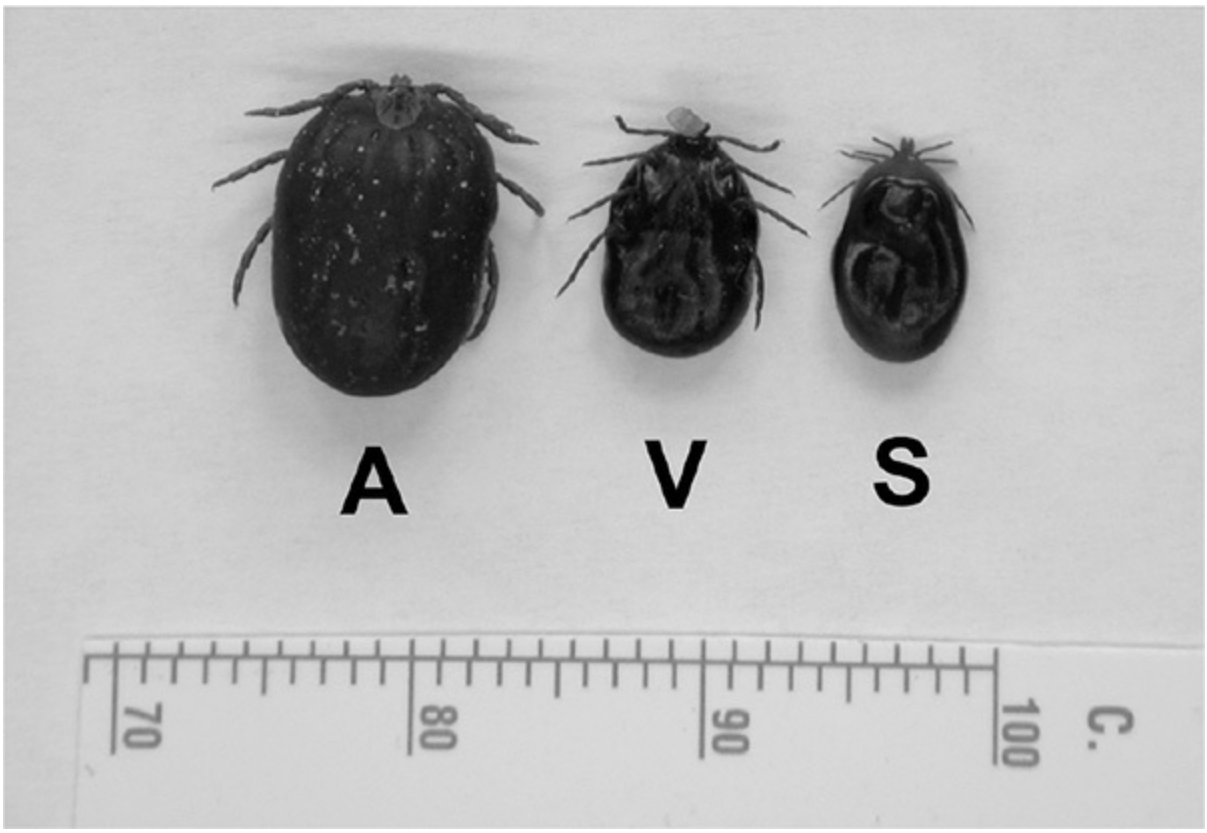
When viewed ventrally, the mouthparts of all three stages appear proportional in length to the basis capituli. The hypostome of nymphs and females is similar to *I. scapularis*, with a dentition formula of 3/3 at the apex, and then 2/2 as it approaches the posterior end. The apex in females is severely pointed. The male hypostome appears short, rounded at the apex, and crenulated. Color in this species is similar to *I. cookei*, in which the entire body of all three stages appears a lightly-creamed-coffee brown. The arrangement of coxal spurs is a key characteristic in the identification of *I. marxi*. Nymphs and females have a blunt internal spur and the hint of an external spur on coxa I. The notable absence of spurs on coxae II through IV distinguishes the females and

nymphs of this species from the other *Ixodes* described here. The coxal spur arrangement of males is similar to that of *I. cookei,* with the exceptions of a less severe internal spur on coxa I and the notable absence of spurs on coxa IV. Round spiracular plates in all stages help distinguish males of this species from *I. scapularis*, in which they appear elongated. The anal plate in male *I. marxi* is longer anteriorly to posteriorly in proportion to lateral width than in *I. scapularis*, in which proportions appear equal.

Dermacentor variabilis. *D. variabilis* is an ornate tick, meaning the tick is quite colorful, with contrasting markings. Coloration in ornate ticks is described as the base color (the predominant overall color of the tick) and the pattern color (contrasting

markings) (Image Plates A-1G and A-1H).[8] Pattern color is present on the scutum as well as the legs of *D. variabilis*. Eleven festoons are arranged in a semicircle around the posterior margin (Image Plate A-1G). They can be observed in both the dorsal and ventral view of all stages. The scutum of females is angular and appears roughly equal in length anteriorly to posteriorly as side to side. Pattern color is predominantly pearl-white to gray and appears in stark contrast to the rest of the tick, in which the base color is chestnut brown. Brown lines and spots in random patterns, not consistent from specimen to specimen, are present. The presence of "eyes" can be observed at the midlateral margins (Image Plate A-1H). Color and contrast are similar in males and females. Ornamental markings cover the entire dorsum because of the nature of the male scutum. Base color areas appear larger as they approach the midlateral and posterior margins. Markings on each side of the median axis are roughly mirror-imaged. The scutum of nymphs is long and does not appear as angular as it is in the female; from the midlateral margins it is rounded to the posterior median. Pattern color appears to be more prominent. "Eyes" are present at the angles of the lateral margins, as they proceed to the posterior apex. Nymphs are not commonly seen in the laboratory and are known to have infrequent human contact.[8]

The basis capituli of all stages appears wider than it is deep (Image Plate A-1H). The basis capituli of nymphs in the dorsal view have pronounced posterior lateral spurlike projections that make the basis look triangular. The hypostome in all stages is short and rounded. Palpi appear short and thick in adults, but narrow in nymphs. Coxal spurs are similar in adults. Coxa I has large internal and external spurs that converge in the median, conveying a notched appearance. Coxae II through IV have smaller external spurs. Nymphs have a predominant external spur and less dominant internal spur on coxa I, with unremarkable external spurs on coxae II and III and no spurs on coxa IV. In the Northeast, *D. variabilis* must not be confused with *Dermacentor albipictus* (i.e., the moose tick, also called the winter tick). Just as a moose is bigger than a dog or a deer, *D. albipictus* is appreciably larger than *D. variabilis* or *I. scapularis* (Fig. A-5). A mean of 2 mm divides unengorged specimens of these species.[8] A fed *D. variabilis* can achieve impressive dimensions (Image Plate A-1G), but a fully engorged *D. albipictus* female can achieve the size of an adult's fourth fingernail. In optimal environmental conditions, *D. albipictus* has been known to exsanguinate a moose because of the number of ticks feeding simultaneously. Fortunately, human contact with *D. albipictus* is minimal to absent.



■ **FIGURE A-5** Size comparison of engorged ticks. An engorged moose tick, *Dermacentor albopictus* (A), dwarfs an engorged American dog tick, *Dermacentor variabilis* (V), which in turn is larger than the enlarged deer tick, *Ixodes scapularis* (S). All are adult females.

Amblyomma americanum. *A. americanum* is another ornate tick (Image Plate A-2A). Eleven festoons are present. Base color is more predominant than in *D. variabilis,* and pattern colors are more consistent. The scutum in females is angular and about as long as wide. Approaching the lateral margins it becomes darker than the base color, which is red-tan. "Eyes" are present at the lateral apices (Image Plate A-2B). The remarkable scutal pattern in females is an iridescent patch at the posterior apex (Image Plate A-2A). Depending on the angle of the light source, this marking appears blue-green to pearl-pink and brings to mind gasoline on water. The scutum of nymphs appears wider than long, with "eyes" at the lateral apices. The iridescent patch found at the posterior apex of female nymphs is missing. The male scutum is most remarkable for the presence of two, inverted, pattern-colored "horse shoes" at the posterior margin (Image Plate A-2C). "Eyes" are present at the lateral margins. Pattern color markings proceed below the "eyes" toward the median and give the appearance of a pseudoscutum resembling that of the female. Male and female specimens may be confused by an inexperienced observer. Random pattern color

markings follow the posterior lateral margins as well.

The mouthparts on all stages are long in proportion to the basis capituli. The hypostome appears bulbous in that it is narrow posteriorly and is wide and rounded anteriorly. Coxal spur arrangement is similar in all stages. There is a severe internal spur and a short, rounded external spur on coxa I. A short, pointed spur on coxae II through IV appears closer to the median, though is most likely an internal spur. Males have a long severe internal spur on coxa IV (Image Plate A-2D). All stages have an anal groove. The groove appears as a semicircle posterior to the anus. From the median of the semicircle, a single groove descends toward the posterior margin. The overall appearance is that of a wineglass or chalice (Image Plate A-2D).

Summary of Identification. The following sequence of observations has been found useful for tick identification.

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When a specimen is presented with an anterior-facing U-shaped groove, *Ixodes scapularis* must be identified or ruled out. When distinguishing *I. cookei* from *I. marxi*, coxal spur arrangement and scutal configuration are key. When the tick is ornate, distinctions are made based on scutal configuration, pattern color markings versus base color, configuration of pattern color markings, the length of the mouthparts in relation to the basis capituli, and coxal spur arrangement. The diagnostic features are summarized in Table A-3.

Good reference books make these descriptions more comprehensive.[8,9,27,37] Appropriate tick identification is important as emergent disease, as well as established disease, is now being associated with tick transmission. An accurate identification of a recovered tick from a patient who presents with vague flu-like symptoms can and should influence early therapy.

Ornithodoros *Species*

Relation to Disease. *Ornithodoros* spp. transmit the *Borrelia* spp. that cause endemic relapsing fever.

Identification. *Ornithodoros* spp. are soft ticks. By definition, their mouthparts are not visible from above, and they do not have a scutum. They have a prominent hypostome covered with teeth (Image Plate A-2E).

Geographic Distribution. It is difficult to differentiate among the several species that may transmit relapsing fever. In the northwestern United States and western Canada, *O. hermsi* is the primary vector. This species feeds on small rodents as well as

humans and typically inhabits cracks and crevices in shacks and cabins. A famous outbreak in a Boy Scout troop was concentrated in senior scouts and scoutmasters, who commandeered an abandoned cabin, leaving the younger scouts to spend their night unbitten under the stars.[58]

In the Southwest and southern Midwest, the common vector is *O. turicata*, which is found on a variety of small and large mammals, rodents, birds, and reptiles. This species is often found in animal burrows.

Mites

Numerous species of mites exist in nature. Species of the family *Trombiculidae* (chigger mites) produce intense itching or dermatitis as they feed transiently. Members of the genus *Leptotrombidium* transmit scrub typhus in Southeast Asia and Australia.[65]House-dust mites in the genus *Dermatophagoides* serve as allergens for certain individuals. The mouse mite, *Liponyssoides* (*Allodermanyssus*) *sanguineus*, transmits *Rickettsia akari*, the agent of rickettsialpox. Rickettsialpox was first described in New York City and continues to be a problem there.[25]

The itch mite, *Sarcoptes scabiei*, and the follicle mite, *Demodex folliculorum*, are the two mites that have an intimate and prolonged parasitic association with humans.

Sarcoptes scabiei (Scabies)

Relation to Disease. *Sarcoptes scabiei* is known as the "itch mite." The mite causes mange by burrowing into the skin and laying eggs in the burrows. Resulting larval and nymph stages of the mite live in the burrows and adjacent hair follicles. Fecal deposits left by the mites provoke intense itching, leading to significant irritation and hair loss. Recurrence of disease is usually by reinfestation from mites on inadequately treated contacts, but relapse may also occur even after a standard course of treatment.[61]

Identification. Microscopic examination of skin scrapings reveals the characteristic mite, which measures 0.2 to 0.4 mm in length, eggs, or fecal pellets (scybala) (Image Plate A-2F). Specimens are collected after placing a drop of mineral oil on a sterile scalpel blade and allowing some of the oil to cover the papule. Scrape the papule vigorously six or seven times to remove the top; then transfer the scraped material, which should contain some flecks of blood in the oil, to a glass slide, using an applicator stick if necessary. After adding a couple of drops of mineral oil to the preparation and mixing, place a glass coverslip on top.[18]

Geographic Distribution. Itch mites are distributed worldwide.

Demodex folliculorum (Hair Follicles) and *Demodex brevis* (Sebaceous Glands)

Relation to Disease. Infections are usually found on the face. Organisms can cause pruritus and local tissue reaction as they burrow into the skin. They are also found, however, in asymptomatic individuals.

Identification. Microscopic examination of specimens from skin reveals the characteristic mite, which measures 0.1 to 0.4 mm in length.

Geographic Distribution. Follicle mites are distributed worldwide.

Spiders

Thousands of species of spiders exist in nature. Most possess potent toxins, but only a small number are capable of penetrating human skin when biting.[17]

Loxosceles reclusa

Relation to Disease. *Loxosceles reclusa*, known commonly as the brown recluse spider, prefers to exist in stored clothes and sleeping bags, basements, closets, and other sites with reduced human activity.[17,18] Their venom is injected through biting, and can cause serious desquamation and necrosis of skin. Large doses of venom are associated with dissemination and significant mortality. The bite of the brown recluse spider does not produce a diagnostic lesion; several reports suggest that dermonecrotic lesions are incorrectly attributed to spiders on a regular basis.[59]

Identification. The brown recluse spider is 1 to 2 cm in size and is characterized by its yellow to brown coloration and a violin- or fiddle-shaped marking on the back of the cephalothorax (Image Plate A-2G).[17,18] There are several related species, which may require expert help for correct identification.

Geographic Distribution. The brown recluse spider is found in most of the continental United States except for the extreme western sections.

Latrodectus mactans

Relation to Disease. Several species of widow spiders exist. *Latrodectus mactans*, the most common black widow spider, may cause a serious systemic reaction, even death, after

TABLE A-3 Diagnostic Features of Hard Ticks

Species	Stage	Scutum	Anal Groove	Coxal Spurs	Mouthparts/Basis Capituli	Eyes	Festoons	Color
Ixodes scapularis	Nymph	Oval; covers one-third of the dorsum and is dark in contrast to the abdomen	Anterior-facing U-shaped; surrounding the anus	Prominent internal spur on coxa I; blunt external spurs on coxae I through IV	Mouthparts twice as long as basis capituli when viewed ventrally	No	Absent	Capituli, legs, and scutum are black-coffee brown with contrasting red-tan abdomen
	Adult female	Covers half the dorsum, otherwise resembles nymph	Same as nymph	Same as nymph	Same as nymph	No	Absent	Same as nymph
	Adult male	Covers entire dorsum; hairy	Same as nymph	Same as nymph	Same as nymph; more "businesslike" hypostome than other *Ixodes* spp.	No	Absent	Overall black-coffee brown
Dermacentor variabilis	Nymph	Covers half the dorsum; predominant pattern color	Absent	Prominent external spur on coxa I; less blunted external spurs on coxae II and III	Dorsal basis capituli looks triangular; palpi are narrow	Yes	Present	Pattern colors more predominant
	Adult female	Angular; as long as wide; pattern-colored with random base color markings	Absent	Prominent internal and external spurs on coxa I, giving a notched appearance	Thick palpi; basis capituli wider than mouthparts are long	Yes	Present	Base color is chestnut brown; pattern color is gray-white
	Adult male	Base color and pattern color roughly equal	Chalice-shaped	Same as female	Same as female	Yes	Present	Same as female
Amblyomma americanum	Nymph	Angular; wider than long; predominant base color; "eyes" at lateral apices	Chalice-shaped	Spurs appear at the posterior median of all four coxae; spur on coxa I is more pronounced	Mouthparts appear much longer than the basis capituli; hypostome is bulbous	Yes	Present	Predominant caramel base color; pattern color is iridescent blue-green to yellow
	Adult female	Angular; about as wide as long; predominant base color with "eyes"; pattern-colored patch (Lone-star) at posterior apex	Chalice-shaped	Coxa I has a prominent median spur; spurs appear absent on coxae II through IV	Same as nymph	Yes	Present	Same as nymph
	Adult male	Predominant base color; inverted pattern color "horseshoes" at posterior margin	Chalice-shaped	Similar to the female with a severe internal spur on coxa IV	Same as nymph	Yes	Present	Same as nymph

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Ixodes cookei	Nymph	Angular; wider than long	Anterior-facing U-shaped; surrounding the anus	Similar to *I. scapularis*	Mouthparts equal in length to the basis capituli; hypostome similar to *I. scapularis*, though more rounded at the apex; palpal spurs present	No	Absent	Lightly-creamed-coffee brown
	Adult female	Angular; as wide as long	Same as nymph	Same as nymph	Same as nymph	No	Absent	Same as nymph
	Adult male	Less hairy in comparison with *I. scapularis*	Same as nymph	Same as nymph	Proportional to nymph; hypostome rounded and crenulated	No	Absent	Same as nymph
Ixodes marxi	Nymph	Angular; as wide as long	Anterior-facing U-shaped; surrounding the anus	Blunt internal spur and presence of external spur on coxa I; absence of spurs on coxae II through IV	Mouthparts equal in length to the basis capituli	No	Absent	Lightly-creamed-coffee brown
	Adult female	Angular; longer than wide	Same as nymph	Same as nymph	Same as nymph; hypostome is severely pointed	No	Absent	Same as nymph
	Adult male	Appears hairless	Same as nymph	Coxa I has prominent internal spur and blunt external spur; coxae II through IV have blunt external spurs; spurs absent on coxa IV	Proportional to nymph; hypostome rounded and crenulated	No	Absent	Same as nymph

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the injection of venom. Fortunately, most patients do not become gravely ill and recover quickly.[17,18] Most bites occur on the hands.[56] Male spiders produce mild or no symptoms after their bite.[20]

Identification. The black widow female is 3 cm long (including legs), glossy black, and displays a characteristic red hourglass marking on the abdomen (Image Plate A-2H).[17,18,56] Differentiation among related species is difficult and best left to experts.

Geographic Distribution. Different species of widow spiders exist throughout the

entire continental United States. *Latrodectus mactans* occurs from southern New England south to Florida and west to California and Oregon; it is more common in the southern part of this range. The northern black widow spider, *L. variolus*, is present in New England and southern Canada south to Florida and west to eastern Texas, Oklahoma, and Kansas; it is more common in the northern part of this range.

Tegenaria agrestis

Relation to Disease. There have been increasing reports of toxic reactions to the bite of the hobo spider, *T. agrestis*. Many bites are not followed by serious reactions, but some bites may produce a dermonecrotic lesion similar to that of the brown recluse spider. Erythema is followed by blistering and then ulceration. An eschar forms and subsequently sloughs, with or without formation of a scar. Systemic effects are usually mild but may be severe. The male spider is more venomous than the female.[7]

Identification. Hobo spiders are brown with gray markings. They are moderately large, measuring 7 to 14 mm in body length and 27 to 45 mm in leg span.

Geographic Distribution. *Tegenaria agrestis* is distributed throughout the Pacific Northwest, southwestern Canada, and the Alaskan panhandle. The distribution of this spider does not overlap that of *Loxosceles reclusa*. The hobo spider builds funnel-shaped webs in damp, moist places, such as wood piles and crawl spaces. It is rarely found above foundation or ground level.

Insecta: Flies, Bugs, Fleas, and Lice

Myiasis-Producing Fly Larvae

Myiasis occurs when fly larvae infect humans either accidentally or by penetrating skin directly to form subcutaneous skin lesions, for the purpose of feeding on tissue. Imported cases of myiasis are increasing because of more frequent travel to endemic areas.[4,62] The two most common genera that parasitize humans are *Dermatobia* and *Cordylobia*. Larvae of flies in the genera *Wohlfahrtia*, *Cochliomyia*, *Hypoderma,* and *Oestrus* affect humans much less commonly and will not be discussed here.[17,20,49]

Dermatobia hominis *(Human Bot Fly)*

Relation to Disease. The fascinating life cycle of this organism requires some discussion to understand infection. The female attaches her eggs to another insect vector, often a mosquito. While the insect is biting a host, the eggs release larvae, which immediately penetrate the skin. Larvae mature in the skin for approximately 6 to 12 weeks, after which they leave the subcutaneous tissue for the soil, where they require another 3 weeks of maturation before an adult fly emerges.[4,49,62]

While in the skin, the larvae produce a lesion that resembles a "boil," which is often quite pruritic and occasionally painful. Complete excision of the larvae eliminates the disease and allows confirmation of the diagnosis. Secondary bacterial infections of the lesions occur occasionally.

Identification. Larvae range in size from several millimeters to 18 to 25 mm, depending on maturity at the time of excision. Rows of spines that are easily visible with a dissecting microscope encircle the larva (Image Plate A-3A). Larval stages may have a distinct "flask" shape, with a long tapered neck. Microscopic examination of the spiracles (stigmal plates) is useful when making an identification.[17,18]

Geographic Distribution. *Dermatobia hominis* is common in Central and South America.[4,17,47]

Cordylobia anthropophaga *(Tumbu Fly)*

Relation to Disease. Larvae emerge from eggs that have contaminated bed linen, clothing, or other materials and penetrate the host's skin shortly after contact.[4,28] Development in the subdermal layer occurs much faster than with *Dermatobia hominis*, usually within 2 weeks,[4] reducing the relative number of imported infections.[28] Patients are generally exposed to a larger number of eggs, so numerous lesions are more likely with *Cordylobia* infection than with *Dermatobia*.[4]

Identification. Larvae reach 7 to 12 mm at maturation. Their more conical shape readily distinguishes them from *Dermatobia*.[28] Spiracles appear at the widest end.

Geographic Distribution. *Cordylobia* is found primarily in sub-Saharan Africa.

Other Flies

Several other medically important flies warrant brief discussion, although specimens are rarely submitted to clinical laboratories.

Sandflies belonging to the genera *Lutzomyia* and *Phlebotomus* are responsible for transmission of leishmaniasis. These flies are hairy, small, and tend to have wings held in an erect V shape. *Chrysops* spp., commonly known as deer flies, are involved in transmission of *Loa loa*, the African eye worm. Black flies (*Simulium* spp.) are the vectors for the microfilariae causing onchocerciasis. Different *Simulium* species exist in distinct geographic regions, but all reproduce near fast-flowing waterways near which is where the largest number of bites occur. Tsetse flies (*Glossina* spp.) exist only in tropical Africa and transmit African trypanosomiasis (sleeping sickness). These

elongated flies possess a powerful proboscis capable of inflicting painful bites.[56]

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Mosquitoes

Mosquitoes are responsible for transmitting diseases causing greater morbidity and mortality than any other arthropod.[17,18,20,56]

Relation to Disease. Mosquitoes produce pruritic skin lesions that appear shortly after they take a blood meal. They are also capable of transmitting serious infections. *Anopheles* spp. are the arthropod vectors responsible for transmitting malaria. Filariasis is spread by some *Aedes* spp., *Mansonia* spp., and *Anopheles* spp. A variety of arthropod-borne viruses (arboviruses) use *Aedes* spp., *Culex* spp., *Culiseta* spp., *Haemagogus* spp., and *Sabethes* spp. as vectors of serious infections, such as equine encephalitis, West Nile fever, yellow fever, dengue and, now, Zika virus.[5,42,56] Both male and female mosquitoes feed on nectar and pollinate flowers. The female mosquito must take a blood meal to complete development of eggs.[20]

Mosquitoes use olfactory clues to find their human targets. A specific component of human sweat has been identified as an important compound for *Anopheles gambiae*, an important vector for *Plasmodium falciparum*.[22] A variety of insect repellents are available to discourage mosquitoes from biting. Products based on botanicals are attractive options for those who wish to avoid chemical deterrents; unfortunately, these options are not as effective as products that contain *N,N*-diethyl-3-methylbenzamide (DEET).[16] Perhaps in the future, compounds that block relevant components in sweat will make effective deterrents.

Identification. Mosquitoes are dainty-appearing, flying insects easily recognized by most. Identification to genus and species level requires significant experience and should be left to the experts. Mosquito bites are sufficiently common that patients rarely bring specimens to the clinical laboratory for identification.

Geographic Distribution. Mosquitoes are found worldwide, but individual species have well-defined environmental niches. For instance, classical yellow fever was an urban disease because the primary vector, *Aedes aegypti*, was limited to standing water in the vicinity of human dwellings. It was hoped that the disease could be completely eliminated by controlling the mosquito vector. Unfortunately, control of urban yellow fever revealed a sylvatic cycle among monkeys and several forest mosquito species, and it soon became clear that the virus could be controlled but never eliminated.[56]

The range of certain mosquitoes has been expanded secondary to global commerce. *Aedes albopictus*, also known as the "tiger mosquito," is a resident of Southeast Asia, but it hitched a ride to the United States by laying eggs in accumulated water in automobile tires.[33] This aggressive mosquito species is a competent vector for important human pathogens. Eastern equine encephalitis virus, Cache Valley fever virus and Zika virus are transmitted by this mosquito species.

True Bugs or Hemiptera

Bedbugs (*Cimex* spp.) and reduviid (family *Reduviidae*) bugs suck the blood of vertebrates, including humans, and the latter acts as an important vector in transmission of disease.[17,56] They are nocturnal, feeding at night and hiding and resting during the day. The common bedbug (*C. lectularius*) has been submitted to microbiology laboratories for identification more recently secondary to the rise in bedbug infestations.

Cimix lectularius

Relation to Disease. The common bedbug can cause numerous small lesions on the skin wherever a blood meal is taken. The severity of the lesions is related to the sensitivity of the individual. Bedbugs feed primarily at night, otherwise hiding in the bed linen, loose wallpaper, and mattresses.[17] They do not act as vectors of infectious agents.

Identification. Bedbugs are light to orange-brown, and 5 mm long by 3 mm wide.[17,18,20] Adults have small, nonfunctional wing appendages (Image Plate A-3B).

Geographic Distribution. *Cimex lectularius* is distributed worldwide, although more common in temperate zones, whereas *C. hemipterus* is more common in tropical climates.[56]

Kissing Bugs: *Panstrongylus*, *Rhodnius*, and *Triatoma* Species

Relation to Disease. The family *Reduviidae* has several popular names. These bugs are sometimes known as "cone-nose bugs" because of the elongated shape of the head. The majority of the group are known as assassin bugs from their lethal aggression against other insects. Assassin bugs may also bite humans and cause painful lesions.[20]

Medically, the most important group are the "kissing bugs," so-named because they may take their blood meals from the skin around the lips. Kissing bugs transmit the causative agent of Chagas' disease, *Trypanosoma cruzi*. They are unable to bite through clothing, so lesions are most common on exposed parts of the body.[20] Feces containing *T. cruzi* are deposited on the host while the bugs feed. Subsequent scratching of pruritic skin at the bite site introduces organisms into the wound.

Transmission also occurs when fecally-contaminated fingers are rubbed in the eyes, nose, or mouth.[56]

Identification. Unlike bedbugs, reduviid (triatomid) bugs possess functioning wings. They range in size from 1 to 4 cm, are more elongated than bedbugs, and have relatively long, thin heads (Image Plate A-3C).[17,18,20,56]

Geographic Distribution. Kissing bugs occur in southern portions of the United States and in Central and South America.

Fleas

Relation to Disease. Fleas may cause irritating, itchy bites after sucking blood from a host. The species that transmit the most serious human infections most commonly are considered here. The Oriental rat flea, *Xenopsylla cheopis*, is the classical vector of plague[30] and murine typhus.[20] *Tunga penetrans*, the "jigger" flea found in Africa and in Central and South America, penetrates the skin and lays eggs while inside the host (Image Plate A-3D).

The cat flea, *Ctenocephalides felis*, and the dog flea, *Ctenocephalides canis*, can infect either species of domestic animal (Image Plate A-3E). In North America, the dog flea is uncommon, and most human disease is caused by the cat

flea.[20] Either species can bite humans. The dog tapeworm, *Dipylidium caninum*, may occasionally infect humans when a flea containing the cysticercoid stage of the tapeworm is accidentally ingested.[17,18] Several species of fleas, in addition to other insects, may transmit the larvae of the rat tapeworm, *Hymenolepis diminuta,* to humans. It has been recognized that endemic plague may be associated with exposure to cats, and endemic typhus, caused by *Rickettsia typhi*, may be transmitted by the cat flea.[53] A *Rickettsia*, known as the ELB agent,[48,63] also infects the cat flea and causes an illness that resembles murine typhus. This rickettsial organism can be maintained by transovarial transmission in cat fleas.[1] The ELB agent has now been classified as *Rickettsia felis*.[23] In developed countries, cats may be more important than rats in transmission of these infections.

Identification. Recognition of fleas is relatively easy. They are wingless, and have long, muscular legs[17,18,56] hanging down from a body that looks "hunched over" (Image Plates A-3D and E). Species level differentiation of fleas is very difficult, usually requiring considerable expertise.

Geographic Distribution. Fleas are found worldwide, following the distribution of

the mammalian host. *Xenopsylla cheopis* is associated primarily with brown rats (*Rattus norvegicus*, also known as Norwegian sewer rats) and black rats (*Rattus rattus*, also known as house or roof rats). *Ctenocephalides* spp. are found on cats and dogs.

Lice

Lice are a considerable public health concern, producing diseases that range from relatively benign to life-threatening. As agents of direct injury, they cause minor, yet irritating, skin and hair infestations. As arthropod vectors, they are capable of transmitting important bacterial pathogens. Individual subspecies of *Pediculus humanus* are associated with different parts of the body, and some overlap does occur, whereas *Phthirus pubis* resides in the pelvic region.

Pediculus humanus subsp. *humanus*

Relation to Disease. *Pediculus humanus* subspecies *humanus* is also known of as the human body louse. It parasitizes most parts of the human body other than the head. Body lice are primarily found in the hairier regions, where they migrate back and forth from clothing to skin to take blood meals.[17] They may be found in the cuffs and folds of the clothing of infested individuals. Feeding causes irritating, pruritic bite wounds, a general characteristic of infestations with lice. Most eggs, called nits, are laid on clothing.

Body lice transmit *Borrelia recurrentis*, one of the less common causes of relapsing fever,[3,57] and *Rickettsia prowazekii* and *Bartonella quintana*,[29] which cause epidemic typhus and trench fever, respectively.[17,56] In relapsing fever, crushed lice release organisms onto the skin that then enter through abrasions, likely secondary to biting. Typhus and trench fever agents, which are present in louse feces, enter when scratched into the skin by the host.[56] The mortality rate in untreated epidemic typhus may be as high as 60%,[56] perhaps because of the presence of other complicating factors, but antimicrobial therapy effectively controls the infection.[38]

Body lice are spread by fairly close contact with infected individuals. Shared clothing, bedding, and close communal habitation are associated with transmission.[17] Not surprisingly, louse-borne infections occur under conditions of extreme privation, such as war and famine.

Identification. Body lice reach 2 to 4 mm in length, with a relatively long, slender abdomen. They have three pairs of legs, with clawlike hooks on the ends that aid in securing the louse to its host (Image Plate A-3F).

Geographic Distribution. Body lice occur worldwide.

Pediculus humanus subsp. *capitis*

Relation to Disease. Head lice produce irritating infections of the head, hair, and neck regions.[44] They infect only humans; pets and other animals are not susceptible. Unlike the body louse, head lice do not serve as vectors for transmission of bacteria, although secondary bacterial infection of the bite wound may occur.[6] The 1-mm-sized nits (eggs) are most often glued to a hair shaft near the scalp (Image Plate A-3G).

Infestations are most common in children.[6] They occur through close contact and sharing of fomites such as combs, hats, and scarves.[17]

Identification. Head lice closely resemble body lice. Although size overlap does occur, they may be slightly shorter than body lice, averaging 1 to 3 mm.[17,18,20] The elongated abdomen and the three pairs of legs with hooks are virtually identical to those found on the body louse. Nits are easily confused with dandruff, seborrheic scales, hair spray, or fungal infection (Image Plate A-3G).[6]

Geographic Distribution. The head louse is found throughout the world.

Phthirus pubis

Relation to Disease. *Phthirus pubis*, also known as the crab or pubic louse, causes an irritating infection of the pubic area. It may be found less frequently in the armpits, chest, thighs, and short facial hairs of mustaches, eyebrows, and eyelashes.[17,18,20]The lice leave a pruritic bite wound on the skin after taking a blood meal. Secondary infection of the bite wound may occur, but is uncommon. Nits are laid on the lower portion of hair shafts as with head lice. Pubic lice do not transmit infectious agents. Transmission occurs primarily through intimate contact.

Identification. Pubic lice are 1 to 2 mm long and have a short, round abdomen. Their shape has been described as "turtlelike." Pubic lice possess three pairs of legs, with claws that appear larger and thicker than those of the body or head louse (Image Plate A-3H).

Geographic Distribution. Pubic lice can be found worldwide.

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APPENDIX II

Free-Living Amebae

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Introduction

Ecology, Epidemiology, Pathogenesis, and Disease

- Primary Amebic Meningoencephalitis
- Granulomatous Amebic Encephalitis
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Laboratory Investigation

- Collection and Transport of Specimens
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Introduction

Small, free-living water and soil amebae of the genera *Naegleria, Acanthamoeba,* and *Balamuthia* are capable of producing opportunistic infections in humans. The major diseases produced by this group are forms of devastating, usually fatal meningoencephalitis. *Acanthamoeba* species may also cause keratitis, an ulcerative, vision-threatening infection involving the cornea of the eye; this disease is not caused by *Naegleria* or *Balamuthia*.[30] In rare instances, skin infections may be caused by *Acanthamoeba* or *Balamuthia.*[9,45] The free-living amebae are not related to the intestinal amebae discussed previously and do not inhabit the intestines of humans or other mammals.

Ecology, Epidemiology, Pathogenesis, and Disease

Naegleria, Acanthamoeba, and *Balamuthia,* along with many other genera of free-living amebae, have a worldwide distribution in various freshwater habitats, including rivers, lakes, ponds, hot springs and spas, domestic water systems, air-conditioning systems, humidifiers, and cooling towers.[19,20] They are believed to play a role in a hypersensitivity pneumonitis known as humidifier fever.[41] They are ubiquitous in soils, dust, air, and composts; have been found on the surfaces of vegetables; and have been isolated from sewage sludge and water polluted by domestic or industrial waste. *Acanthamoeba* and *Balamuthia* species have been isolated from soil, sea water, especially associated with discharges of inadequately treated sewage from hotels, and municipal sewage effluents (D. A. Munson, personal communication, 1991).[20,42] *Naegleria* and *Acanthamoeba* have also been found associated with thermally polluted discharges in ponds and lakes connected with electric power plants, nuclear power plants, or thermal polluting factories.[19,20]

In nature, free-living amebae feed on bacteria, including *Legionella* species,[3,37,41,46] and the amebae form cysts that are resistant to adverse environmental conditions, such as drying in soil. De Jonckheere and Van de Voorde reported that the cysts of some strains of *Acanthamoeba* are highly resistant to chlorine and are not killed by the usual concentrations of chlorine maintained in municipal or domestic water supplies or in chlorinated swimming pools.[21] Conversely, the cysts of *Naegleria fowleri* were more sensitive to chlorine; these authors concluded that viable *N. fowleri* is not likely to be found in clean water where low concentrations of chlorine (e.g., 0.5 mg free available chlorine per milliliter) are maintained.[21] Rowbotham was the first to show that *L. pneumophila* can grow within *Acanthamoeba* species, thus providing an explanation of

how nutritionally fastidious legionellae can maintain viability in pristine, low-nutrient waters.[41] *Legionella* species also proliferate within various other free-living amebae.[46] It has been speculated that legionellae could be aerosolized through the air to humans in droplets of water while residing within amebae.[41] This seems feasible, especially if amebic cysts could protect legionellae from chlorine in domestic water supplies (Chapter 10).

In addition, free-living amebae, including *Naegleria fowleri, Hartmanella* species, and various *Acanthamoeba* species, have been isolated from the throats and nasal passages of humans[1,6,28,40,46,47]—probably as cysts that were inhaled with dust in the air, in aerosols, or were present in ingested food or water.[17]Trophozoites of *Naegleria* or *Acanthamoeba* have not been demonstrated in the human nasopharynx in the absence of CNS disease.[17]

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Primary Amebic Meningoencephalitis

Prior to 1958, none of the free-living water and soil amebae were recognized as pathogenic. Then Culbertson and associates observed an ameba contaminant in tissue culture cells.[18] This strain, now called *Acanthamoeba culbertsoni* strain A-1, when instilled intranasally into young mice or various other animals, produced infection within the nasal mucosa and submucosa that spread from there along the olfactory nerve through the cribriform plate of the skull to produce invasion of the brain and a rapidly fatal meningoencephalitis.[13,14,18] Based on his experimental work with animals, Culbertson predicted that similar disease could occur in humans.[13,16,18] In 1965, the first human case was reported in a child from Australia by Fowler and Carter.[23] The following year, three additional infected patients were reported by Butt from Florida, who called the disease "primary amebic meningoencephalitis."[10] Thus, conceptually, primary amebic meningoencephalitis differed from amebic disease of the CNS involving *Entamoeba histolytica* because primary amebic meningoencephalitis was not "secondary" to amebic disease of the colon.

It is now known that the portal of entry for *Naegleria* is the nose and the true "primary" site of the infection is the upper respiratory tract.[11,14,17] Most patients with *Naegleria* CNS infections have been children or young adults who were swimming in lakes or rivers or otherwise had an opportunity for water, which is believed to contain the *Naegleria*, to enter the nose.[3,4,31,33] After infecting the nasal mucosa, *N. fowleri* then spreads along the olfactory nerve, through the cribriform plate into the brain via the route originally observed in animals by Culbertson and colleagues.[13,18] Pathologic findings in humans with acute meningoencephalitis caused by *N. fowleri* have been

remarkably similar to those seen originally in the experimental animal models by Culbertson and associates with *Acanthamoeba culbertsoni* strain A-1, which is the most virulent of the *Acanthamoeba* species.[10,11,14–17,25,30,32] Other *Acanthamoeba* species are not as virulent and tend to produce subacute or chronic brain abscesses, with or without granulomas.

N. fowleri produces a diffuse and fulminant meningoencephalitis characterized by large areas of hemorrhagic necrosis, especially involving the olfactory bulbs, frontal lobes, base of the brain, proximal spinal cord, and/or temporal lobes of the brain. The parietal lobes and occipital cortex may also be involved. Acute purulent exudate is found in the leptomeninges and within the cortex. Amebic trophozoites are scattered or may be seen focally in clusters within necrotic cortex. They especially show a predilection for blood vessels; small arteries, arterioles, veins, venules, and capillaries may be surrounded by 10- to 15-µm *Naegleria* trophozoites containing a large prominent karyosome.[14,16,33] Importantly, cysts are not formed in the tissue by *Naegleria,* which is a differentiating feature between this organism and both *Acanthamoeba* spp. and *Balamuthia mandrillaris.*

Granulomatous Amebic Encephalitis

In humans, a more indolent CNS infection may be caused by two genera of amebae. *Acanthamoeba* species have long been recognized as a cause of meningoencephalitis, with a presentation distinct from that of *N. fowleri*. This is a rarely encountered disease and is not a reportable infection, so the incidence is not known; it is, however, well reported in the medical literature.[30] In 1986, *Balamuthia mandrillaris* was also shown to be a cause of chronic granulomatous amebic meningoencephalitis. Most patients with granulomatous amebic encephalitis (GAE) have been immunocompromised and, unlike PAM, have not had a history of exposure to water.[17,30,32,33] Even more rarely, infections have been reported in otherwise healthy individuals.

The cysts of these organisms are ubiquitous and may be present in soil or dust; it is possible that the organisms could have been inhaled into the nasal cavity or even the lower respiratory tract.[17] It is conceivable that in humans, *Acanthamoeba* species might spread either from the nasal cavity to the brain by way of the olfactory nerve or intravascularly from the lungs or possibly from skin lesions (reported rarely) to the brain.[17,33]

Within the brain, *Acanthamoeba* species produce acute inflammation in early stages of the disease in animals. However, species less virulent than *A. culbertsoni* strain A-1 tend to produce focal or multifocal chronic abscesses, with or without granulomas. Trophozoites and cysts may be focal or scattered in small numbers throughout involved areas but may be located in perivascular spaces and can be seen within the

walls of small vessels in association with perivascular hemorrhage and intravascular thrombosis. Amebic cysts are seen within tissue in *Acanthamoeba* and *Balamuthia* infections but have not been observed in tissues infected with *Naegleria*.[14,16,33] The cysts and trophozoites of *Acanthamoeba* and *Balamuthia* are morphologically indistinguishable. Molecular techniques must be used to differentiate these amebae. PCR-based assays are available at the Centers for Disease Control and Prevention (CDC).

Acanthamoeba *Keratitis*

Acanthamoeba keratitis is a serious, often devastating infection of the cornea involving certain species of *Acanthamoeba* (e.g., *A. polyphaga, A. castellanii, A. rhysodes, A. culbertsoni,* and *A. hatchetti*).[5,30,35] Since the first infected patient was described in 1975,[26] more than 200 cases have been reported worldwide; this number is likely significantly lower than the true frequency of infection, since this is not a publicly reportable disease.[30] Until about 1984, the majority of infections were associated with trauma to the cornea and exposure to contaminated water. Although this is still a means of infection, many infections are now associated with the use of contact lenses, especially daily-wear or extended-wear soft contact lenses.[5,12,35,44] Other factors involved in *Acanthamoeba* keratitis include trauma to the cornea and the use of contaminated homemade saline or tap water rinses to clean lenses, the failure to disinfect lenses properly or as often as recommended by manufacturers, and wearing lenses while swimming.[8,30,44] Acanthamoebae may be present in contact lens cases and contact lens wash solutions, and they are capable of adhering to contact lens surfaces.[8,29,35,44,48] *Acanthamoeba* keratitis presents as an ophthalmologic emergency. Most age groups have been affected, from children to the elderly.[44] The infection progresses relentlessly with corneal ulceration and may result in blindness. It is a disease that is difficult to diagnose and treat. The disease has often

been misdiagnosed as herpes simplex keratitis, fungal keratitis, or bacterial or mycobacterial infection, sometimes resulting in delays of weeks or months before a diagnosis has been made and proper treatment is begun. Detailed clinical findings have been published elsewhere in several excellent reviews.[29,35,39,44,48]

Laboratory Investigation

Collection and Transport of Specimens

When amebic disease is suspected, fresh cerebrospinal fluid or tissue (e.g., corneal biopsy, corneal scrapings, CNS tissue) should be collected aseptically and submitted for examination. Specimens intended for culture must never be frozen or refrigerated and should be maintained at 20°C to 30°C during transport to the laboratory.[3,15] If immediate examination is not possible or not practical, amebae have survived in sterile fluid or otherwise sterile tissue for several days at room temperature prior to processing, but extended transport times are not recommended. If a transport medium is needed for transport of tissue biopsy samples or corneal scrapings, the minimal essential medium used in virology laboratories is preferred (Chapter 23) over Page's ameba saline (D. Place, F. Curtis, E. Powell, and A. Newsome, personal communication, 1991).

Direct Microscopic Examination

Small drops of cerebrospinal fluid, other body fluids, or tissue suspensions should be examined in wet mounts (on slides under coverslips) by light microscopy under reduced light, or by phase microscopy for motile trophozoites (using a 40× objective). Keeping the microscope stage warmed to 30°C to 37°C may enhance motility, especially of *Acanthamoeba* species.[15] *Naegleria* may be actively motile at 22°C to 25°C, but the motility of *Acanthamoeba* species at room temperature is very sluggish. These amebae tend to be more actively motile than leukocytes, and the nuclei and nucleoli of *Naegleria* and *Acanthamoeba* species differ from those of granulocytes or macrophages.

Material to be stained on slides should first be fixed in 10% neutral buffered formalin. After the slide is air-dried, it can then be stained with trichrome, the Papanicolaou stain, or hematoxylin and eosin.[3,15] Tissue sections should be stained with hematoxylin and eosin. Other stains preferred for direct examination preparations by some include lactophenol cotton blue, Giemsa, acridine orange, and calcofluor white.[30] Specific detection methods, such as indirect fluorescent antibody procedures, immunohistochemistry, and PCR-based assays, are available through CDC (http://www.cdc.gov/dpdx/dxassistance.html) or select reference laboratories.[3,30]

Culture of Free-Living Amebae

Culbertson's method for culturing amebae with *Escherichia coli* on salt-free, water agar,[15] a slight modification of Singh's original procedure, has proven to be useful for culturing body fluids, suspensions of eye tissue, and other specimens. Agar plates are prepared with 20 to 25 mL of 1.5% agar in distilled water without nutrients or salt added. A loopful or one drop of a heavy suspension of *E. coli*, from a 24- to 48-hour

blood agar plate culture is either placed in the center of the plate in a 1 cm diameter area or is used to make a lawn on the plate.

The inoculum (a drop of cell suspension, sediment, or tissue fragments) is placed in the center of the bacterial lawn and the plate is sealed with tape. Duplicate plates are incubated at 30°C and 37°C, or a single plate is incubated at 34°C to 35°C. After 18 to 24 hours of incubation, the plate is kept sealed and examined agar side up with a 10× objective. Alternatively, an inverted microscope can be used. If no amebae are seen, the plate is reincubated and examined daily for 10 days. The amebae may be in clusters of cells or will appear as small refractile bodies that migrate from the inoculum, often leaving irregular trails or tortuous tracks behind them where they have moved through the bacteria.[15] Individual amebae can be transferred from the agar to a slide to view as a wet mount or stained preparation. Subculture can be made by cutting out a plug of agar at the "front" containing amebae (using a sterile scalpel) from outside the zone of bacteria, where the amebae are more likely to be free of bacteria, and then transferring the amebae to another water agar plate containing *E. coli* (the addition of amphotericin B in final concentration of 2.5 mg/mL in the agar aids in minimizing fungal contaminants) (E. Powell, personal communication, 1991). From there, the amebae are transferred to a broth medium (e.g., trypticase-soy broth) containing antibiotics (e.g., penicillin 1,000 µg/mL and streptomycin 0.1 mg/mL final concentration).

After the amebae are free of bacteria (axenic), they can be transferred to trypticase-soy broth without antibiotics to be used as a growth medium.[3,15] *Naegleria* will not grow in trypticase-soy broth unless it contains 10% sterile fetal calf serum. The growth of amebae in trypticase-soy broth is optimized by using plastic tissue culture tubes so that the amebae will attach to the tubes.[3,15] The cultures are incubated at 35°C almost horizontally at a 15 to 20 degree angle, analogous to what is often done with cell cultures in virology laboratories. Not all amebae grow in trypticase-soy broth. Alternative media include Plate Count Broth (BD, Sparks, MD) and broth media recommended in the American Type Culture Collection Catalogue (www.atcc.org), including *Acanthamoeba* medium, PYNFH medium, and Fresh Water Ameba medium (E. Powell, personal communication, 1991). Neroad and co-workers[36] have also described a chemically defined medium for cultivating pathogenic and high temperature tolerant species of *Naegleria*.

Tissue Culture

The more virulent free-living amebae (e.g., *A. culbertsoni* strain A-1) grow in mammalian cell cultures, including monkey kidney, McCoy cells, and others, and may produce cytopathogenic effects (CPE) resembling those seen with viruses.[18,47] Less virulent amebae may grow poorly in tissue (cell) culture without producing CPE.

Amebae can be extremely difficult to see when growing with tissue culture cells; fluid should be removed from a tissue culture tube or flask, centrifuged at 500 to 800 *g* (×5 minutes), or can be prepared using a Cytospin; then, the sediment is used to prepare a wet mount that is examined by phase or light microscopy using low illumination. Alternatively the slide preparation can be dried, fixed in formalin, and stained with H&E to aid

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in observing amebic features.[3] Once isolated in tissue culture, isolates can be transferred to a broth medium and/or to water agar with *E. coli*., as described in the preceding section.

Morphologic Features

When rounded up, the trophozoites of *Naegleria* are 8 to 15 µm and usually smaller than those of *Acanthamoeba* species, which are usually 10 to 25 µm in diameter.[2,4] The nuclei of both genera are similar and contain large, prominent karyosome. *Naegleria* have one or more smooth, lobate pseudopodia and are actively motile at room temperature or 35°C to 37°C. *Acanthamoeba* species are very sluggish at 22°C to 25°C but may become more actively motile at 35°C and 37°C. Acanthamoebae have numerous, spiny pseudopodia on their surface called acanthopodia.*Naegleria* species are characterized by a temporary ameboflagellate stage; when *Naegleria* trophozoites are put in distilled water, they may develop pear-shaped, actively motile forms containing two or more flagella (within 1 to 3 hours). The cysts of acanthamoebae and *Balamuthia* are more thick-walled than those of *Naegleria* and have distinctive appearances that aid in identification.[38] In addition, *Naegleria* species produce a distinctive form of mitosis (promitosis) in which the nuclear membrane remains intact.[38] In contrast, acanthamoebae produce a more classical type of mitosis (metamitosis) in which the nucleolus and nuclear membrane disappear and the mitotic spindle appears with the chromosomes in metaphase.[15,38] For more detailed descriptive information, readers are referred to references.[3,15,17,27,30,38]

Identification of *Naegleria*, *Acanthamoeba,* and *Balamuthia* beyond the genus level is now usually achieved by nucleic acid sequencing. In the past, a detailed morphologic study but also the use of other procedures, including tests with species-specific immunologic reagents (e.g., immunofluorescent antibody tests, coagglutination tests) and tests for pathogenicity (in mice) were used more commonly.[22] Identification of these organisms to species level may be useful in an epidemiologic investigation or research projects, but its practical value for the clinical laboratory to assist clinicians in the management of patients has not been demonstrated.

Interpretation of the Significance of Free-Living Amebae Isolated in Culture

Because free-living amebae are ubiquitous in water, soil, dust, and the air, it is possible that cysts, carried about in the air, could contaminate the surface of a corneal scraping, tissue biopsy, or fluid specimen during the time of specimen collection, processing, during the inspection of primary plates for growth, or following prolonged incubation. Amebae could also be on the skin, lid margin, or in the conjunctival sac of an eye without causing keratitis, although this has not been demonstrated. As with any positive culture, it should be correlated with the findings of the direct microscopic examination of the specimen, histopathologic examination, and clinical findings.[3]

Treatment

The treatment for primary amebic meningoencephalitis and GAE has not been standardized. At least two patients with *Naegleria* meningoencephalitis have survived. Each received amphotericin B. One of the patients, in addition to receiving amphotericin B, also received miconazole, rifampin, and sulfasoxazole.[3,7,39,43]

The treatment of choice for *Acanthamoeba* CNS disease has not been established. It is clear that therapy should be promptly administered and that a multidrug regimen should be considered. Agents with activity include azoles, such as fluconazole, itraconazole, and related drugs, pentamidine, sulfadiazine and trimethoprim–sulfamethoxazole, paromomycin, and others.[3,17,24]

The treatment of *Acanthamoeba* keratitis has been successful in several patients using an intensive treatment protocol involving topical propamidine isethionate (Brolene drops) and neomycin sulfate–polymyxin B sulfate–gramicidin drops (Neosporin Ophthalmic Solution).[34,35,39,49]

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IP-1

Image Plates

IP-2

IMAGE PLATE 1-1

Gram's Stain Evaluation of Sputum Smears

The quality of sputum samples can be evaluated by counting the relative numbers of squamous epithelial cells and segmented neutrophils per low-power field in a Gram-stained smear. The presence of squamous epithelial cells indicates contamination with oropharyngeal secretions. In contrast, bacterial pneumonia will produce large numbers of neutrophils that can be detected in sputum samples. The following are photomicrographs of Gram-stained smears of sputum samples illustrating the relative presence of the various cellular components.

A. Low-power view of Gram-stained sputum smear, revealing full field of squamous epithelial cells and absence of segmented neutrophils. This specimen represents saliva, would give a low quality score, and is not acceptable for culture.

B. The squamous epithelial cell shown here is heavily colonized with gram-positive bacteria. The overgrowth of bacterial cells in sputum specimens generally indicates that the specimen has been delayed in transit to the laboratory and that any semiquantitative results will be of little value.

C. This Gram-stained sputum smear shows a mixture of squamous cells and segmented neutrophils. The presence of segmented neutrophils indicates that a nidus of infection exists somewhere in the respiratory tract; however, the squamous epithelial cells indicate contamination with upper respiratory tract secretions. This type of sputum smear is difficult to interpret because upper respiratory tract infections cannot be distinguished from lower respiratory tract infections (i.e., the predictive value of the result is significantly decreased).

D. Full field of neutrophils and virtual absence of squamous epithelial cells. This is a

high-quality sputum sample that provides a good chance for producing relevant results.

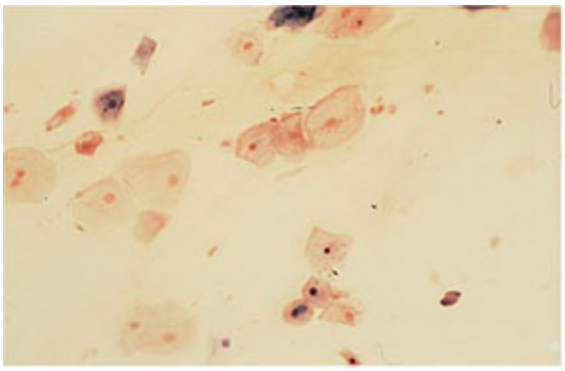
E. Higher-power view of sample in **D**, revealing scattered segmented neutrophils against a background of pink-staining mucus threads.

F. This smear of an induced sputum sample, stained with Diff-Quik, illustrates several ciliated, columnar epithelial cells. These cells have their origin in the lower respiratory tract and, when present in a sputum smear such as that seen here, indicate a high-quality specimen from which relevant results can potentially be obtained.

G. High-power view of a Gram-stained sputum specimen illustrates the morphology of a ciliated columnar epithelial cell. Note the distinct cilia atop the flared terminal end of this eosinophilic staining columnar epithelial cell.

H. Toluidine blue stain of an induced sputum specimen revealing mononuclear inflammatory cells consistent with alveolar macrophages. As with columnar epithelial cells, alveolar macrophages have their origin deep within the lower respiratory tract, and their presence in sputum samples increases the significance of any bacterial species recovered.

IP-3



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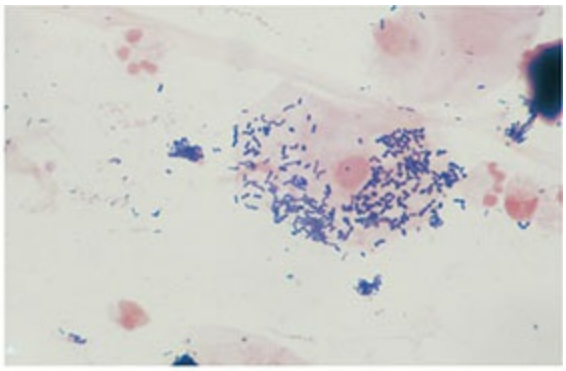

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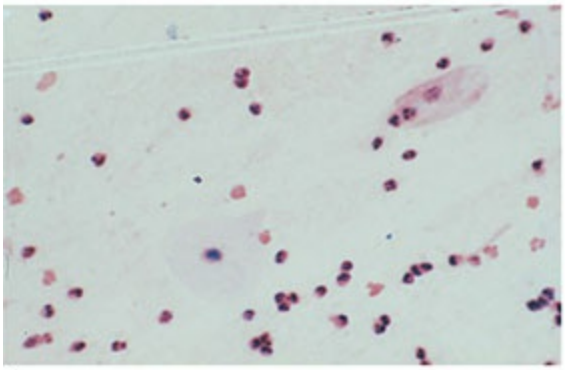

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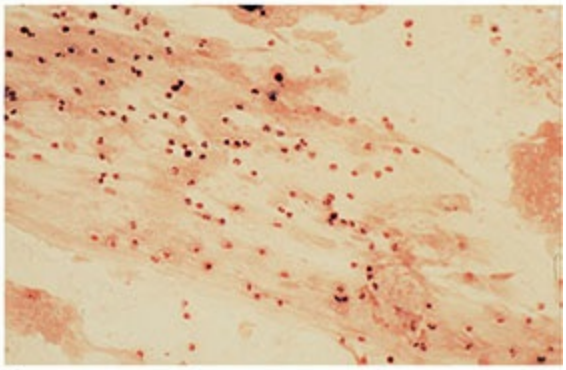

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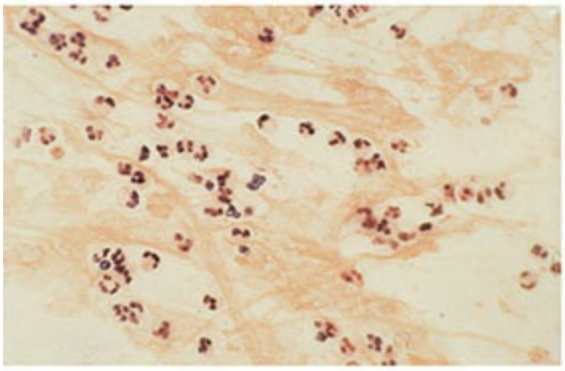

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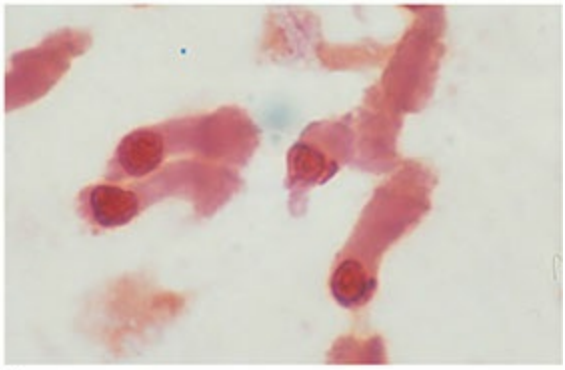

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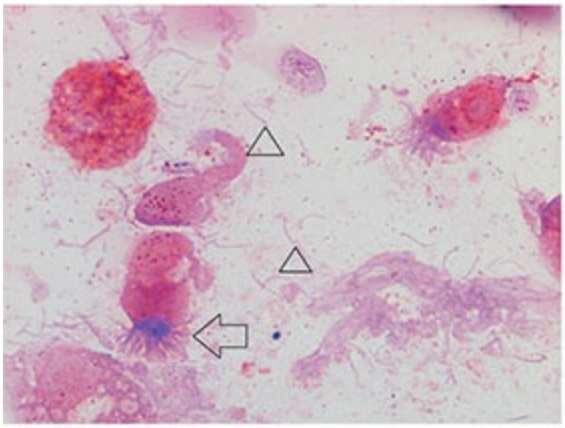

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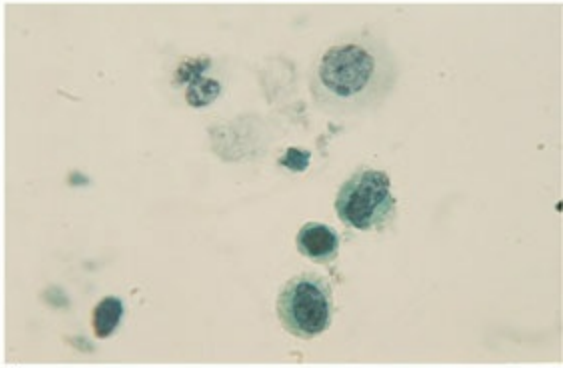


H

IMAGE PLATE 1-2

Miscellaneous Stains Used in Microbiology

Stains are needed to make possible the detection and study of many of the microbes of medical importance, since they have refractive indices near that of water. A variety of stains have been developed over the years, each designed to highlight specific components of the cell wall or specific aspects of the cell (e.g., flagella or spores). The following are illustrations of selected stains and some of their applications.

A. Gram's stain. The most common stain used in the microbiology laboratory is designed to differentiate bacteria that can retain crystal violet dye and appear deep blue-black after decolorization (i.e., gram-positive) from those that cannot and stain red (i.e., gram-negative). Illustrated here is a Gram-stained preparation revealing red-staining, gram-negative bacilli.

B. Acid-fast. This stain and a modification thereof (i.e., the partial or modified acid-fast stain) are commonly used to demonstrate a variety of "acid-fast" organisms, including *Nocardia* species in sputum specimens and *Cryptosporidium* oocysts in fecal samples. Shown here are clusters of red-staining, acid-fast bacilli in a liver biopsy specimen from a patient with AIDS. The organisms stain red with the acid-fast stain, and those shown here were identified as belonging to the *Mycobacterium avium/M. intracellulare* complex.

C. Direct fluorescence. Fluorescent dyes (rhodamine and auramine) react directly with the cell wall of mycobacteria. Fluorescein-conjugated antibodies are also available to demonstrate a variety of microbial agents using the direct fluorescent antibody test. Shown here are yellow-green, fluorescing mycobacteria in a sputum concentrate from a patient with pulmonary tuberculosis.

D. Acridine orange. This rapid fluorescent stain is used to demonstrate bacterial forms in direct smears and mounts of biologic fluids. It is particularly useful in detecting gram-negative bacteria in positive blood culture broths, which may be missed in Gram's stains because of the deep red-staining debris in the background. Shown here is a long, rod-shaped bacterium, with a characteristic orange glow, when microscopically observed with a fluorescence microscope.

E. Methylene blue. This nonspecific, rapid stain is occasionally used to demonstrate bacteria and other microbes in direct smears. Illustrated here are bacilli that have taken the blue stain. One important application is in the detection of bacteria in

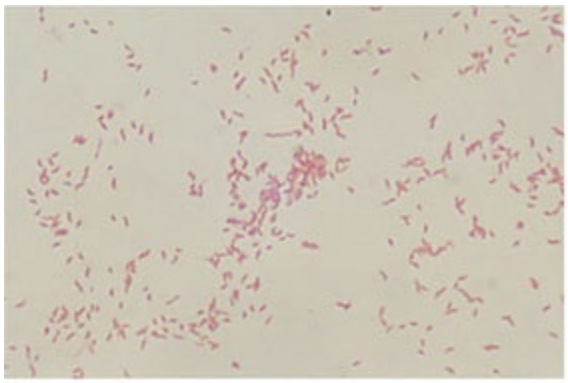
direct smears of cerebrospinal fluid in specimens from patient with suspected acute meningitis, particularly gram-negative species, which may be obscured by the red background staining of the proteinaceous material.

F. Calcofluor white. This autofluorescent whitening agent has the property of coupling with carbohydrate moieties in the cell walls of fungi. Shown here are brilliant yellow-green staining hyphae in a direct calcofluor white-stained mount as observed with a fluorescence microscope.

G. Wright–Giemsa. This stain is commonly used to demonstrate the cellular elements in peripheral blood and bone marrow smears. In microbiology, the stain is most commonly used in parasitology to detect intraerythrocytic (plasmodia, babesiae) and exoerythrocytic (trypanosomes, microfilaria) parasites. Chlamydial inclusions are highlighted by this stain, as well as the intracellular yeast forms of *Histoplasma capsulatum* (Illustrated here).

H. Periodic acid-Schiff (PAS). This general stain is used to demonstrate polysaccharide-rich cell walls. It has specific applications in detecting fungi in tissue sections and smears. Recognized here is the fruiting body of an *Aspergillus* species in an aspirate of a fungus ball lesion of the lung surrounded by red-staining *Aspergillus* conidia.

IP-5



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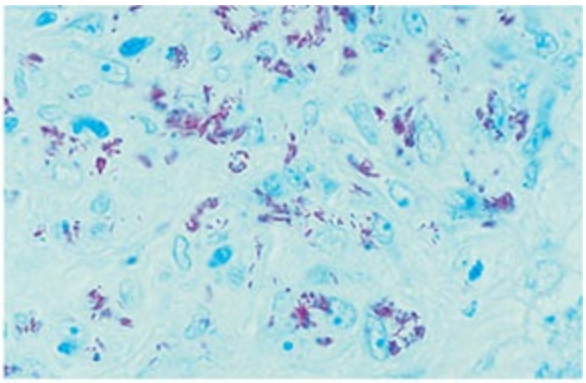

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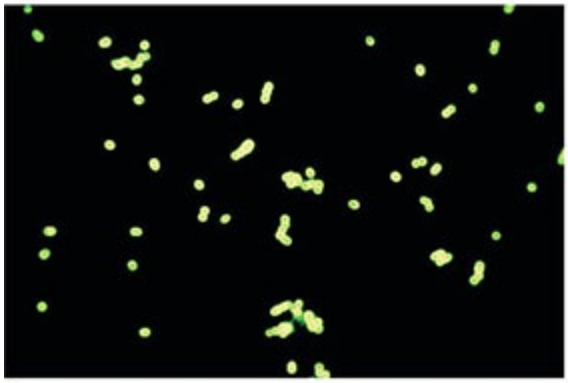

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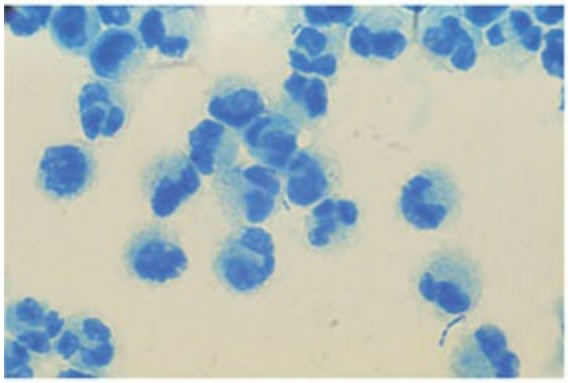

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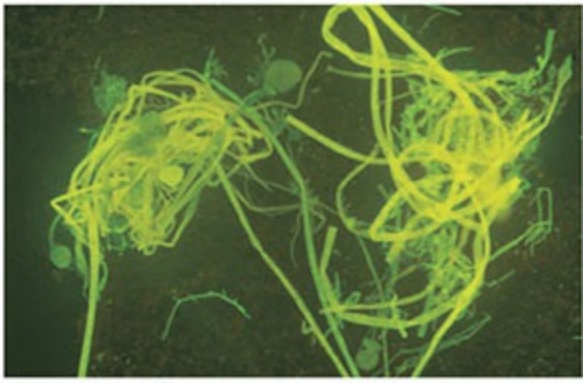

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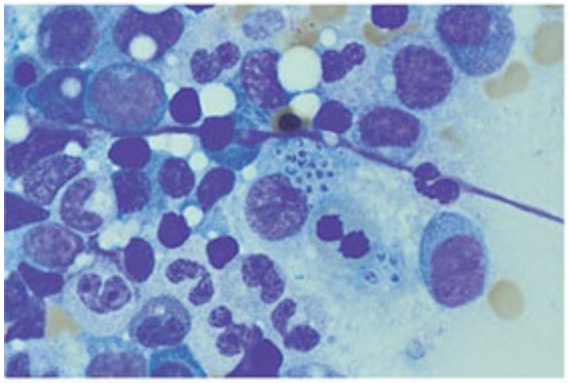

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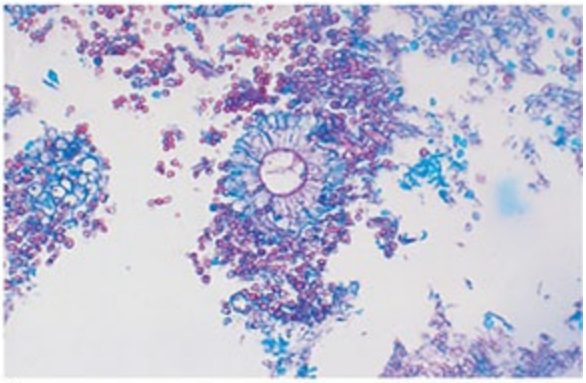

H

IMAGE PLATE 1-3

Presumptive Bacterial Identification Based on Observing Microscopic Cellular Morphology in Stained Smear Preparations

The Gram's stain of bacteria, in addition to other staining techniques, is one of the more important determinations in the presumptive identification of microorganisms. The morphology of the bacterial cells, their arrangement, and their staining characteristics are often distinctive enough to allow a presumptive identification in a Gram-stained smear. The microscopic characteristics suggestive of several groups of bacteria are included in this plate.

A. Relatively slender, gram-positive bacilli arranged in a "Chinese letter" pattern, suggests one of the coryneform (diphtheroid) bacteria.

B. Gram-positive, spore-forming bacilli. The aerobic spore formers belong to the genus *Bacillus*; the anaerobic spore formers belong to the genus *Clostridium*. Illustrated here are spore-forming cells of *Bacillus sphaericus*.

C. Direct Gram's stain of purulent exudates illustrating gram-positive cocci arranged in small clusters, characteristic of a *Staphylococcus* species.

D. Direct smear of necrotic exudate from a patient with myonecrosis. The tiny, gram-positive cocci seen in this photomicrograph are streptococci.

E. This direct Gram's stain of a smear of purulent sputum demonstrating gram-positive diplococci, characteristic of *Streptococcus pneumoniae*.

F. This purulent exudate was from the chest wall of a patient with acute, suppurative empyema. The branching, club-shaped gram-positive bacilli are suggestive of a *Bifidobacterium* species, which was what was recovered in anaerobic culture. Some of the *Actinomyces* species may have a similar morphology.

G. Gram-stained preparation of a direct smear of urine sediment from a patient with acute cystitis. Note the several gram-negative bacilli amid the background segmented neutrophils. *Escherichia coli* was recovered in pure culture.

H. The Gram-stained preparation of a direct smear prepared from purulent urethral exudates of a sexually active male, illustrates the intracellular, gram-negative, diplococci characteristic of *Neisseria gonorrhoeae*. The bacterial cells in this photograph are paler staining than normally seen by direct microscopic

examination.

I. This photomicrograph of a sputum smear illustrates a few segmented neutrophils in the background and a diffuse infiltration with many short, gram-negative bacilli some of which appear to be surrounded by a halo. *Klebsiella pneumoniae* grew out in culture.

J. This photomicrograph of delicate, branching gram-positive filaments is suggestive of *Propionibacterium acnes*, which is what grew out in culture. Some of the *Actinomyces* species may have a similar morphology in Gram's stain.

IP-7

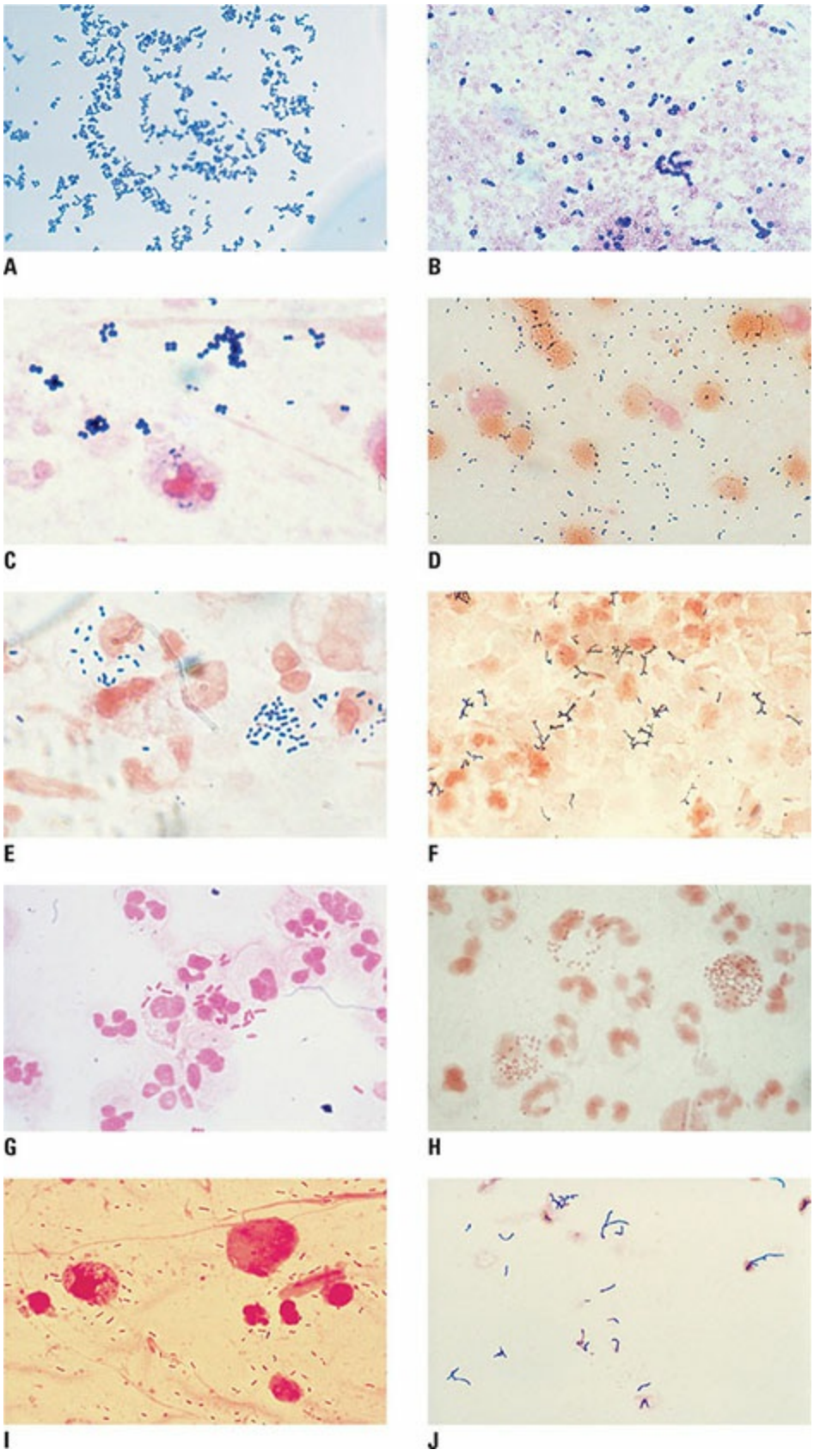
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IMAGE PLATE 1-4

Pitfalls and Artifacts in Gram's Stain

A. *Acinetobacter* sp. in a blood culture. These organisms are coccobacillary and tend to retain the crystal violet. Thus, a Gram-stained smear may be misinterpreted as gram-positive cocci *(arrows)*, or gram-positive bacilli. The microscopist must evaluate the pattern of all the bacterial cells in the smear. The presence of cells that stain magenta is one clue that the true reaction may be gram-negative. Original magnification, 1000×.

B. Overdecolorized staphylococci in a genital specimen misinterpreted as gram-negative cocci. Although both gonococci and staphylococci may appear as diplococci, with a rare exception *(arrow)* the "kidney bean" shape of gonococci is not present here, and the bacterial cells are not intracellular. It is important not to imply the presence of *Neisseria gonorrhoeae* unless the morphology is characteristic. Original magnification, 1000×.

C. Elongated cells of *Streptococcus pneumoniae (arrows)* in a sputum smear may be misinterpreted as gram-positive bacilli, and their importance as a major respiratory pathogen may be missed. Other cells in the smear had the characteristic "lancet-shaped" appearance of pneumococci. The microscopist must assess the overall pattern and not be misled by occasional outliers. Original magnification. 1000×.

D. *Clostridium perfringens* decolorizes easily and may appear gram-negative. Clues to the true nature of the bacterial cells are provided by the occasional gram-positive cells *(arrow)* and the large "boxcar" appearance that does not resemble a typical enteric bacillus. *Bacillus* spp. may also decolorize and appear gram-negative. Original magnification, 1000×.

E. Gram's stain debris masquerading as gram-positive cocci. The true nature of the material is suggested by the small size and the marked variation in size. Artifactual material often appears refractile when the focus is varied. Original magnification, 1000×.

F. Retinal rods and cones masquerading as gram-positive cocci and bacilli in a vitreous specimen. The true nature of the structures is suggested by the anatomical site and their refractile appearance. Original magnification, 1000×.

G. Conidia of *Aspergillus fumigatus* mimicking gram-positive cocci in a sputum

specimen. The true nature of the structures is suggested by the enlarged size and by roughened edges of the conidia if present. A search of the smear may reveal septate hyphae. Original magnification, 1000×.

H. The exfoliated cilia from a respiratory cell *(arrow)* resemble gram-negative bacilli. A pale-staining bacillus such as *Fusobacterium nucleatum* may be suggested. On closer inspection, however, the structures do not have the morphologic detail of a bacterium. Further search will usually reveal intact cells with their attached cilia. Original magnification, 1000×. (Image enhanced.)

IP-9

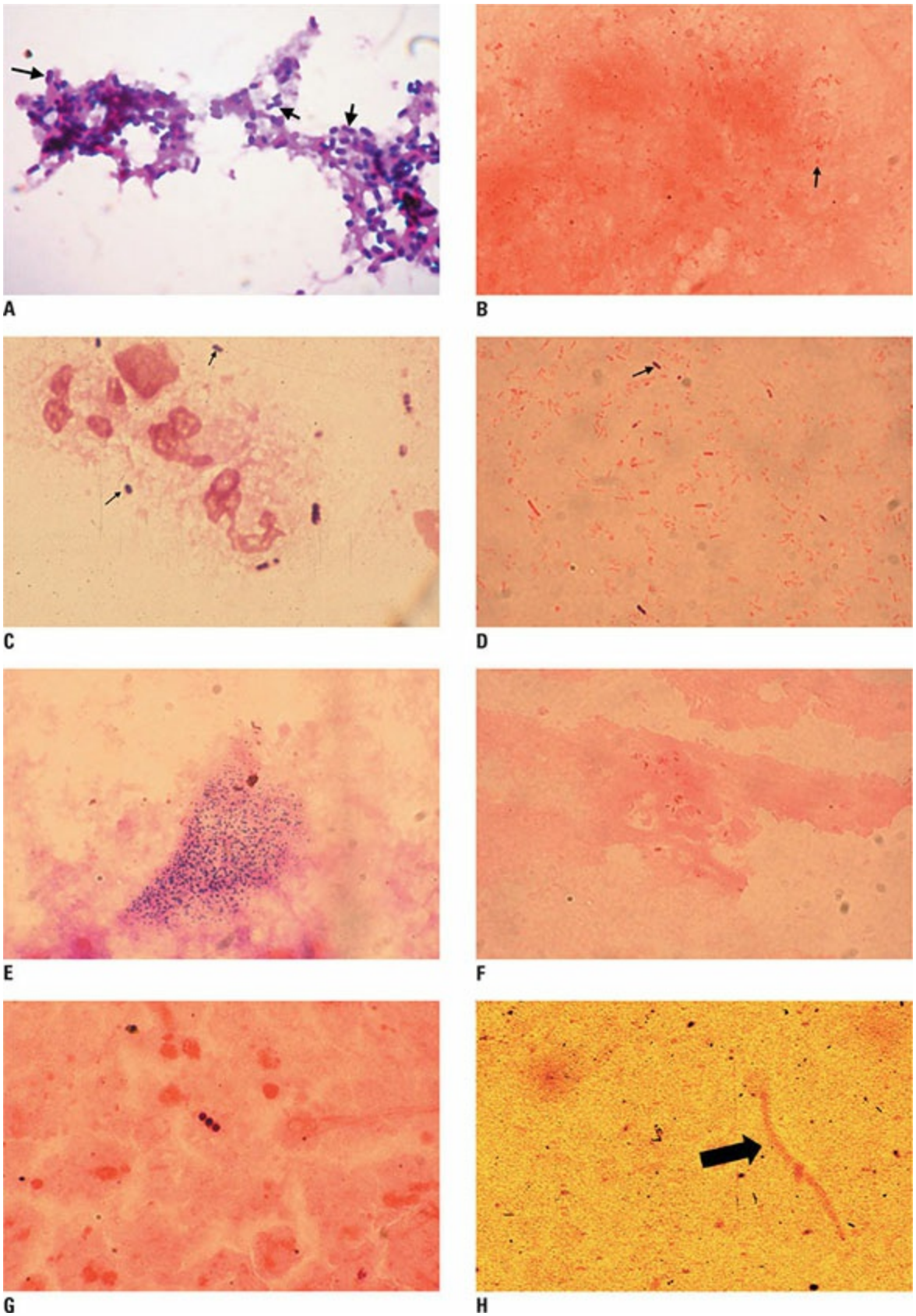
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IMAGE PLATE 1-5

Presumptive Bacterial Identification Based on Observing Colonial Morphology

Microbiologists use various characteristics of bacterial colonies that grow on the surface of agar culture media to make a presumptive identification of the group or genus and as a guide in selecting differential tests to make the final species identification. Size, shape, consistency, color, and pigment production by the colonies, as well as the presence of hemolytic reactions on blood agar, are the criteria commonly used.

A. Blood agar plate on which are growing round, yellow-white, entire, convex, nonhemolytic colonies of coagulase-negative *Staphylococcus* species.

B. Colonies growing on blood agar illustrating distinct zones of β-hemolysis around the colonies. The relation between the size of the colonies and the zones of hemolysis is used to differentiate between certain species. Shown here are *Streptococcus* species, which commonly have a lower ratio of colony size to hemolytic zones, in contrast with *Staphylococcus* species, in which the relative size of the colonies is generally larger.

C. These off-white colonies of *Listeria monocytogenes* are weakly β-hemolytic upon transillumination (i.e., the colony color, but not the hemolysis is appreciable in this photograph). This organism was cultured from a CSF specimen from a patient with meningitis.

D. Opaque, dull gray, somewhat moist colonies growing on blood agar (right) and MacConkey agar (left), suggestive of one of the members of the family *Enterobacteriaceae*. The growth on MacConkey agar shows no red pigmentation, indicating that the organism is a nonlactose fermenter. The isolate shown here is one of the *Salmonella* species.

E. Dry, wrinkled colonies of a *Bacillus* species. Similar-appearing, wrinkled colonies are also seen with *Pseudomonas stutzeri*.

F. Yellow-pigmented colonies on blood agar. Pigment production is an important feature in the differential identification of many bacterial species, particularly those belonging to several groups of nonfermentative bacilli. The colonies shown here are those of *Flavobacterium* species after 48 hours of incubation, with the last 24

hours of incubation being at room temperature. The intensity of pigment is often accentuated after additional incubation at room temperature.

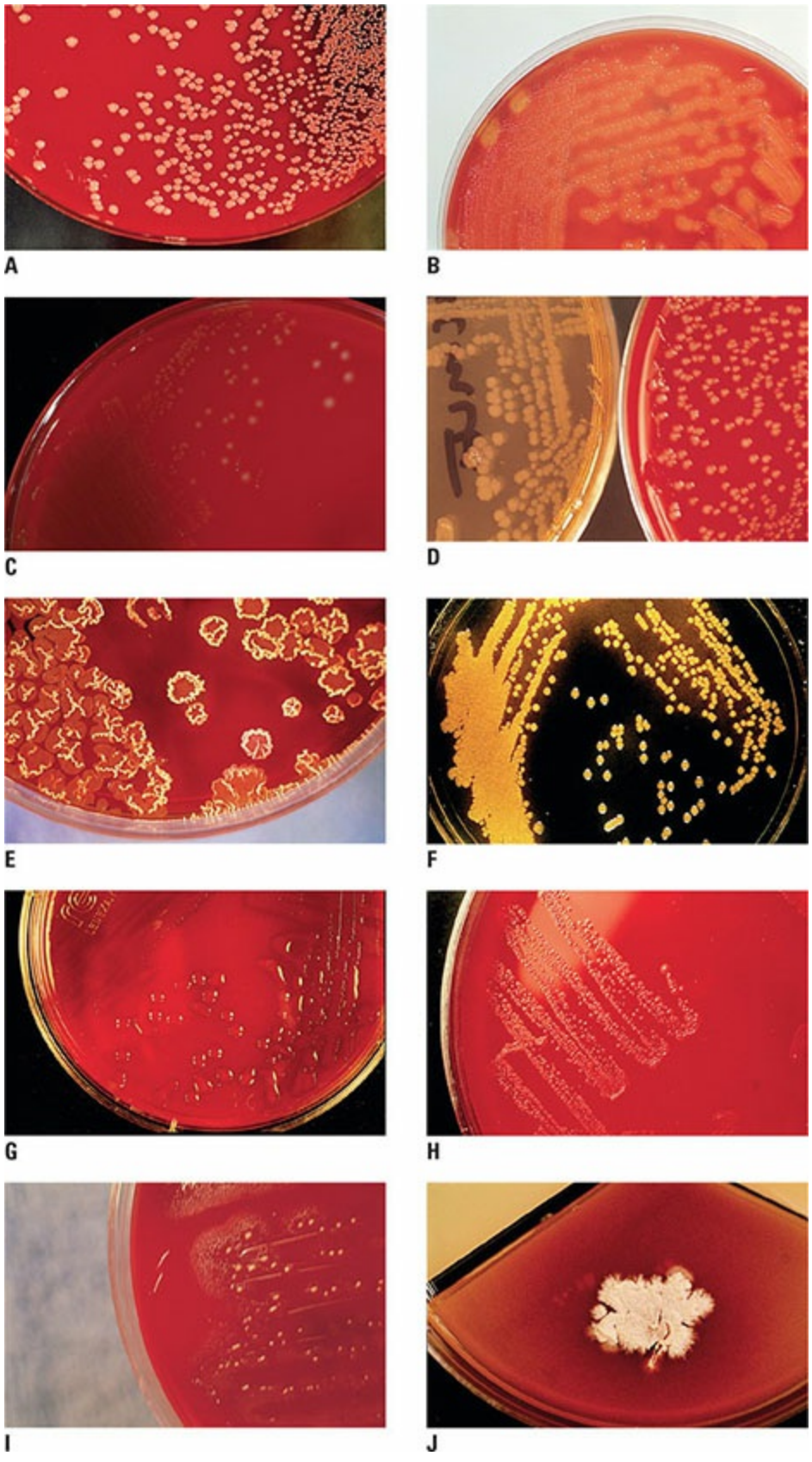
G. Distinctly mucoid colonies growing on blood agar. The mucoid consistency of colonies is sometimes secondary to the production of capsules, a protective mechanism used by several bacterial species in defense against phagocytosis. *Pseudomonas* species, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Cryptococcus neoformans* are among the more commonly recovered microbial species producing mucoid colonies. The colony shown here is *Streptococcus pneumoniae*.

H. Gray semi-translucent colonies of *Eikenella corrodens* growing on blood agar. Note the halo around the colonies, illustrating the "pitting" characteristic of the species.

I. Gray, smooth, semitransparent colonies of *Capnocytophaga ochracea*. Note the mist-like extension of the colonies, illustrating the gliding motility characteristic of *Capnocytophaga* species.

J. White, dry, chalky colony of *Nocardia* species. *Streptomyces* species produce similar-appearing colonies. These presumptive identifications can be confirmed if the musty basement odor characteristic of these species can be detected.

IP-11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IP-12

IMAGE PLATE 6-1

PRESUMPTIVE IDENTIFICATION OF THE ENTEROBACTERIACEAE

Presumptive identification of the *Enterobacteriaceae* is based on the appearance of colonies growing on primary isolation media and on an assessment of certain biochemical reactions. By definition, for an organism to be classified within the *Enterobacteriaceae*, it must ferment glucose, producing acid or acid and gas; reduce nitrates to nitrites; and exhibit no cytochrome oxidase activity.

A. A Gram's stain of a sputum smear showing characteristic short, plump, gram-negative rods that are typical for members of the *Enterobacteriaceae*. Also depicted are two polymorphonuclear leukocytes, suggesting an ongoing infectious process in this patient. In this instance, the culture confirmed the organism to be *Klebsiella pneumoniae*.

B. Mixed culture showing 24-hour growth on blood agar of two morphotypes of large gram-negative rods and a third morphotype of a smaller organism that can be suspected to be a gram-positive species. One morphotype can be seen as large, shiny, white colonies, while the other appears as large, gray colonies with irregular edges. Both of these colony types can be suspected to be gram-negative rods typical for members of the *Enterobacteriaceae*. Also present are many small, white, entire colonies typical of one of the gram-positive cocci.

C. Large, mucoid, glistening, pink colonies on MacConkey agar typical of many *Klebsiella* and *Enterobacter* species. By their appearance alone, these colonies can be suspected to be one of the species of *Enterobacteriaceae*.

D. Swarming pattern of a motile strain of *Proteus* species on chocolate agar plate.

E. Series of Kligler's iron agar (KIA) slants illustrating several reaction patterns. The tube on the far left illustrates an acid (yellow) slant and acid (yellow) butt, indicating both glucose and lactose fermentation. Also note the space at the bottom of the tube and the split in the agar in the middle of the tube, which indicates gas (CO_2) production by the organism. Copious amounts of CO_2, such as seen here, are usually produced only by organisms belonging to Tribe V (*Klebsielleae*). The second tube from the left shows an acid–acid reaction, but without the presence of gas, typical of the type of reaction seen with *E. coli*. The third tube from the left illustrates an alkaline (red) slant/acid butt characteristic of a nonlactose fermenter. The fourth tube illustrates a red slant/black butt, indicating hydrogen sulfide (H_2S)

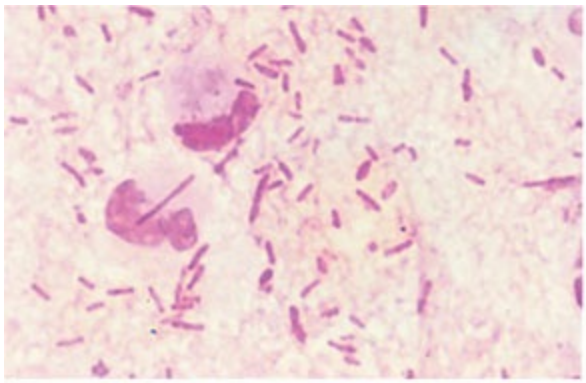
production. When this type of reaction is seen with an oxidase-negative organism, the assumption is made that the butt portion of the tube is acid (yellow), indicating glucose fermentation even though the yellow color is masked due to the H_2S production. The reaction in the fifth tube (far right; red/red) is typical of nonfermenting gram-negative bacilli that ferment neither lactose nor glucose.

F. Three tubes of purple broth media containing Durham tubes to demonstrate gas formation. The two tubes on the right illustrate acid from glucose (yellow), compared with the negative control on the left. The tube on the far right shows the collection of gas within the Durham tube, characteristic of an organism that produces both acid and gas from glucose.

G. Cytochrome oxidase test revealing a positive purple color reaction (left) compared with a negative reaction (right; no blue color within 10 seconds—see Chart 1-5). Any organism giving a positive reaction can be excluded from the family *Enterobacteriaceae*.

H. Nitrate test media containing Durham tubes to demonstrate nitrogen gas formation. The tube on the left shows a positive (red) reaction after addition of α-naphthylamine and sulfanilic acid. The test organism had reduced the nitrates in the medium to nitrites, which reacted with the reagents to form the red pigment *p*-sulfobenzene-azo-naphthylamine (Chart 6-2). The tube in the middle also depicts a positive nitrate reaction, but in this case all the nitrate was first reduced to nitrite and then further reduced to nitrogen gas, indicated by the collection of gas within the Durham tube. Since there is no nitrite in the tube, there is no red color when the reagents are added. The tube on the far right shows no red color and no gas and depicts a negative test for nitrate reduction.

IP-13



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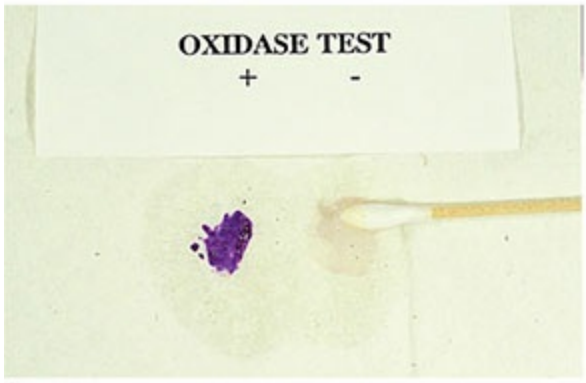

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IMAGE PLATE 6-2

APPEARANCE OF THE *ENTEROBACTERIACAE* COLONIES ON MACCONKEY AND EMB AGARS

MacConkey and eosin methylene blue (EMB) agars are two commonly used selective primary isolation media for the presumptive differentiation of lactose-fermenting from non–lactose-fermenting members of the *Enterobacteriaceae*. On MacConkey agar, lactose-fermenting colonies appear red because of the acid conversion of the indicator, neutral red. On EMB, a green metallic sheen is produced by avid lactose fermenters when the production of acid is sufficient to lower the pH to approximately 4.5 or below.

A. Surface of MacConkey agar with 24-hour growth of purple, lactose-fermenting colonies. The diffuse purple color in the agar surrounding the colonies is produced by organisms that avidly ferment lactose, producing large quantities of mixed acids, and cause precipitation of the bile salts in the medium surrounding the colonies (e.g., *Escherichia coli*).

B. Surface of MacConkey agar illustrating both purple, lactose-fermenting colonies and smaller, clear non–lactose-fermenting colonies.

C and **D.** Surface of EMB agar plates illustrating the green sheen produced by avid lactose (or sucrose) fermenting members of the *Enterobacteriaceae*. Most strains of *E. coli* produce colonies with this appearance of EMB agar, and since *E. coli* is among the most frequent isolates from clinical specimens, the appearance of such colonies can often serve as presumptive identification of *E. coli*. However, characteristics other than the production of a green sheen on EMB must be assessed before an organism can be definitely identified as *E. coli*, since other lactose-fermenting *Enterobacteriaceae* can have a similar appearance.

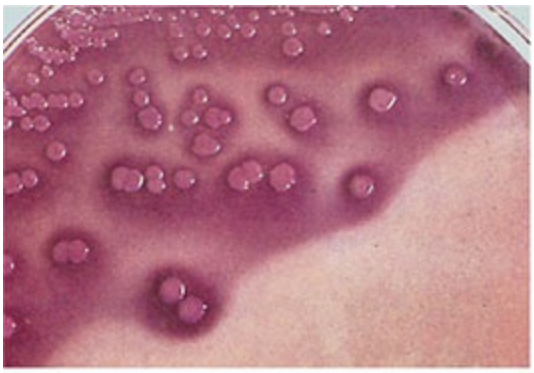
E and **F.** Surface of EMB agar plates illustrating a mixed culture of *E. coli* (green sheen colonies) and *Shigella* species. Most *Shigella* species do not ferment lactose, and thus produce nonpigmented, semitranslucent colonies on EMB agar. Other species incapable of fermenting lactose produce colonies that appear similar to those illustrated in these photographs.

G. Surface of MacConkey agar illustrating growth of red-pigmented colonies of *Serratia marcescens*. Note that this species is non–lactose-fermenting and that the colonies appear red due to pigment production, which must be differentiated from

the purple appearance of lactose-fermenting colonies as seen in Image Plate 6-2 A and B

H. Hypermucoviscous colonies of *K. pneumoniae* on BAP showing positive string test. String test is positive when an inoculation loop or needle is able to generate a viscous string >5 mm in length by touching the bacterial colony and raising the loop above the rim of the agar plate creating a string of bacteria.

IP-15



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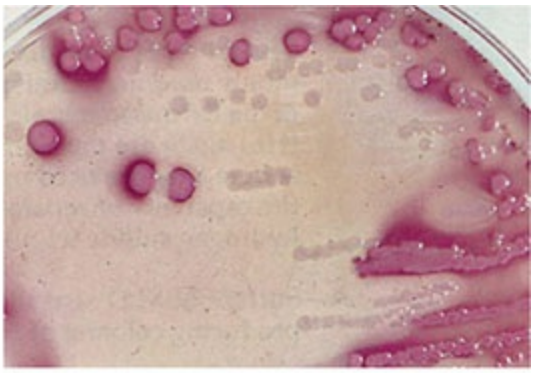

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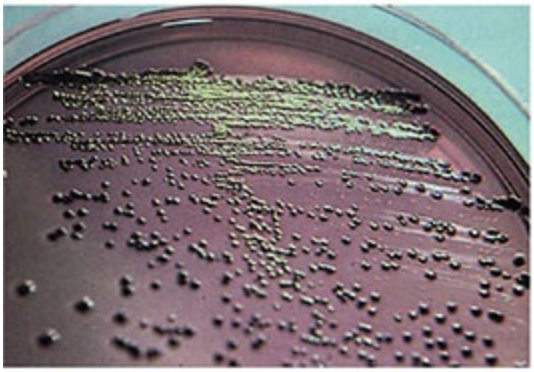

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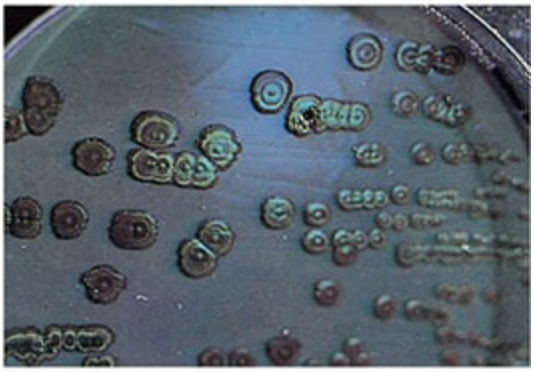

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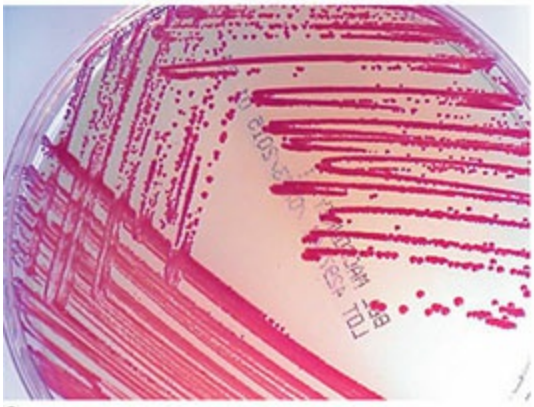

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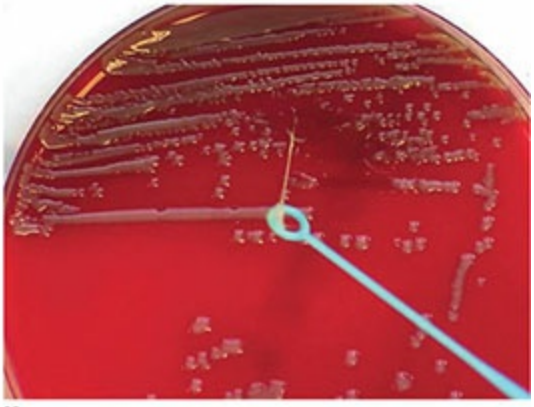

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IP-16

IMAGE PLATE 6-3

APPEARANCE OF THE *ENTEROBACTERIACEAE* ON XLD AND HE AGAR PLATES

Several types of media more selective than MacConkey or EMB agars are commonly used in clinical microbiology laboratories for recovering select members of the *Enterobacteriaceae*. Xylose-lysine-deoxycholate (XLD) and Hektoen enteric (HE) agars are most commonly used; highly selective media such as bismuth sulfate agar are used only for special applications. These media not only have the capability of separating lactose fermenters from nonlactose fermenters but can detect hydrogen sulfide (H_2S)-producing microorganisms as well.

A. Surface of XLD agar illustrating yellow conversion of the medium from acid-producing colonies of *E. coli*.

B. Non–lactose-fermenting colonies (no acid conversion of the medium) of *Salmonella* species growing on the surface of XLD agar. Note the black pigmentation of some of the colonies, indicating H_2S production.

C. Photograph illustrating an XLD agar plate inoculated with a 50/50 mixture of *E. coli* and *Salmonella* species. Note the preponderant growth of the *Salmonella* species (red colonies) compared with the few yellow, lactose-fermenting colonies of *E. coli* that have been effectively inhibited. The distinct pink halo around the *Salmonella* colonies indicates the decarboxylation of lysine, a helpful feature in differentiating *Salmonella* species (positive) from H_2S-producing colonies of *Proteus* species.

D. XLD agar plate inoculated with an H_2S-producing strain of a *Proteus* species. Note the lack of a light pink halo around the colonies, indicating the lack of lysine decarboxylation (compare with the colonies shown in panel **C**).

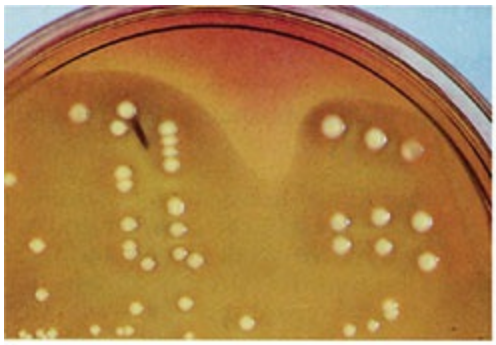
E. Surface of HE agar illustrating yellow acid production by colonies of *E. coli*.

F. Surface of HE agar illustrating the faint green (colorless) colonies of non–lactose-fermenting members of the *Enterobacteriaceae*.

G. Thermo Scientific Remel Salmonella Shigella Agar (SS Agar) illustrating black colored colonies typical of H_2S-producing *Salmonella* species. The sodium thiosulfate and ferric citrate in the medium enable the detection of hydrogen sulfide production as evidenced by colonies with black centers.

H. Thermo Scientific Remel Salmonella Shigella Agar (SS Agar) illustrating growth of lactose-fermenting coliform bacteria. Organisms that ferment lactose produce acid, which in the presence of the neutral red indicator, results in the formation of red colonies.

IP-17



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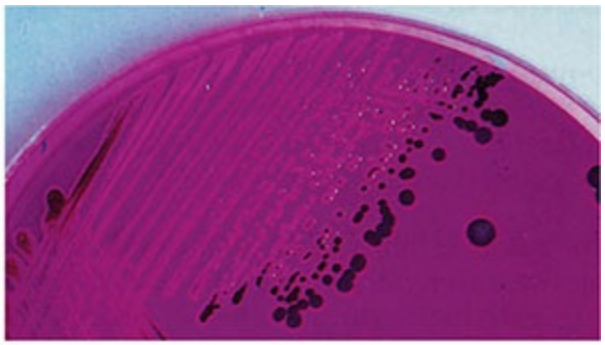

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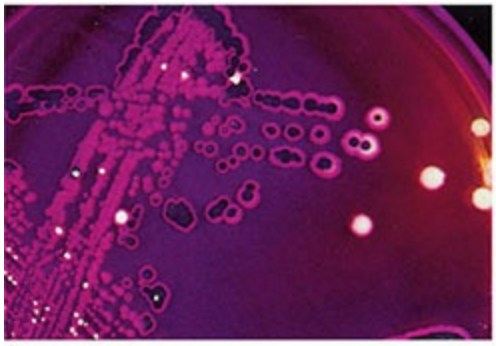

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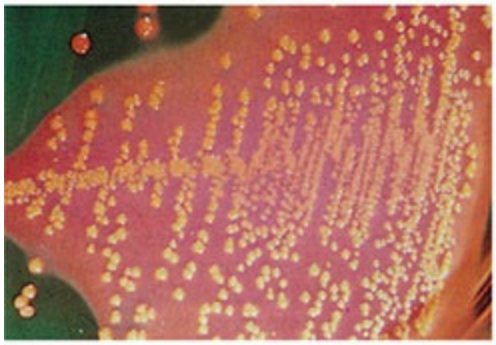

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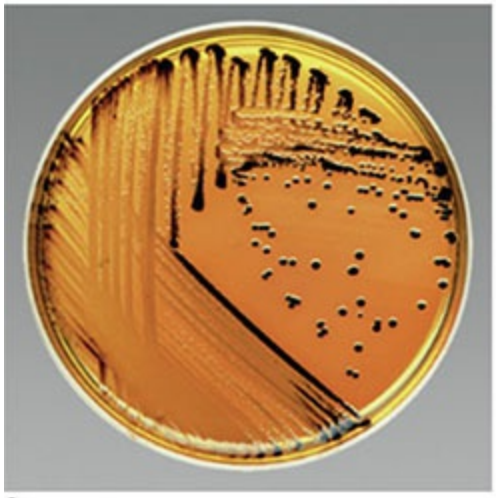

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IP-18

IMAGE PLATE 6-4

DIFFERENTIAL CHARACTERISTICS OF THE *ENTEROBACTERIACEAE*

A. Ortho-nitrophenyl-D-galactopyranoside (ONPG) test showing a positive (yellow) reaction (left) compared with the negative control (right). A positive reaction indicates that the organism is capable of producing β-galactosidase, an enzyme required for the initial degradation of lactose, releasing the yellow-colored *ortho*-nitrophenol.

B. Sulfide indole motility (SIM) semisolid agar tube (right) and Kligler's iron agar (KIA) slant (left), illustrating the differences in sensitivity in detecting hydrogen sulfide (H_2S) by different media. The diffuse delicate blackening in the SIM tube is produced by a motile, weak H_2S-producing organism (in this case *S. enterica* serogroup Typhi). Note that the H_2S produced in the less sensitive KIA tube appears only in the middle of the tube at the interface of the slant and butt and is the typical appearance of *S.* Typhi in this medium.

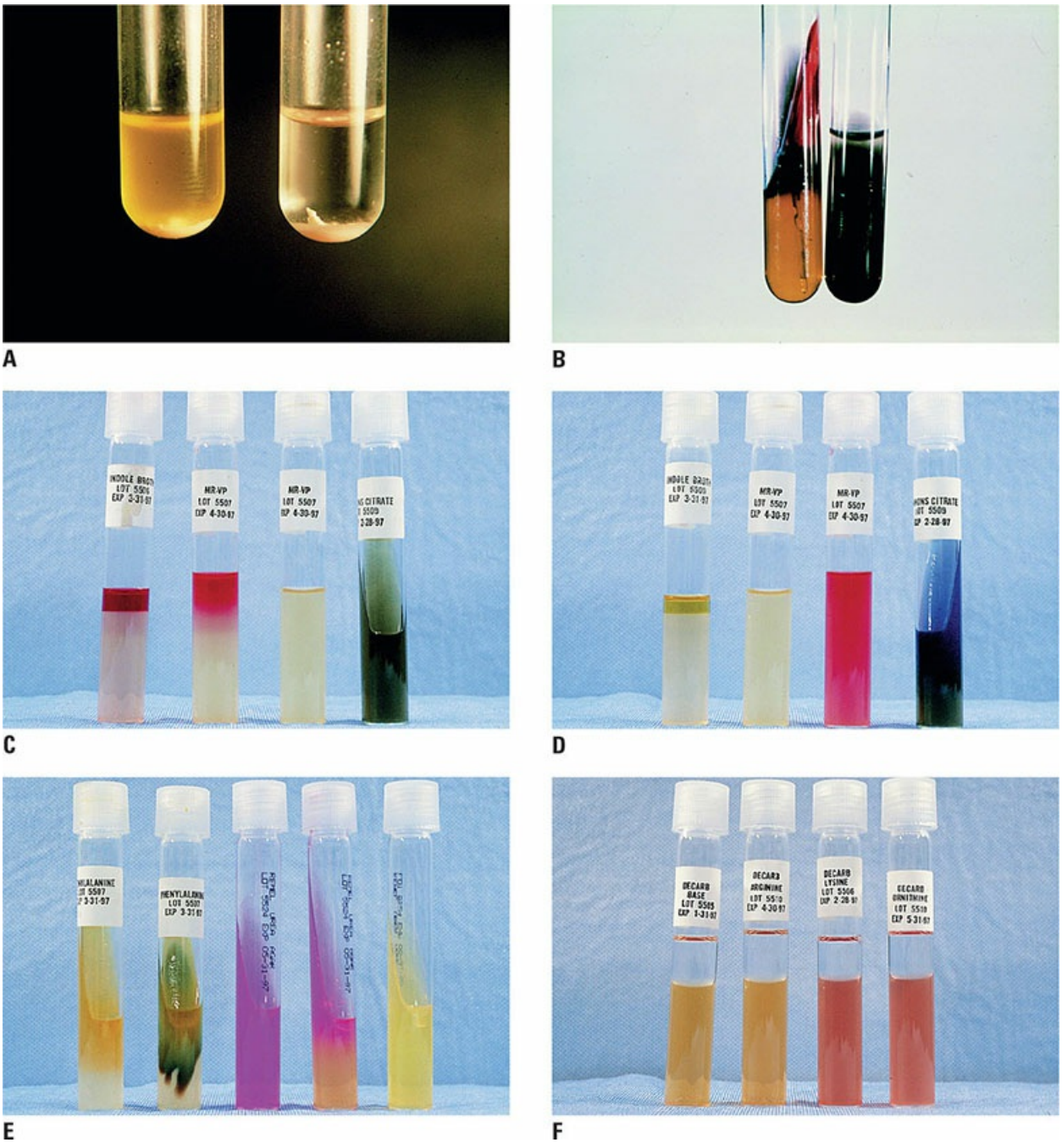
C and **D.** Four tubes demonstrating the indole (I), methyl red (MR), Voges-Proskauer (VP), and citrate (C) tests. These four tests when performed together constitute the classic IMViC reactions. The formation of indole from tryptophan is indicated by a red color on the addition of Kovac's reagent (far left tube, panel **C**). The development of a red color in the MR tube indicates a drop in pH to 4.4 or below, indicative of strong mixed acid fermenters (second tube from left, panel **C**). A red color in the VP tube indicates the presence of acetoin (acetylmethylcarbinol) formed from pyruvate in the butylenes glycol metabolic pathway (third tube from left, panel **D**). Growth on the slant of Simmon's citrate agar and conversion of the bromthymol blue indicator to an alkaline blue color indicates that the organism can utilize sodium citrate as the sole source of carbon (right most tube, panel **D**). **C** shows the reactions for *E. coli* (+, +, −, −). Panel **D** shows the reactions for *Klebsiella/Enterobacter* (−, −, +, +).

E. The two tubes on the left show negative (light yellow) and positive (dark green) phenylalinine deaminase reactions. The green color is produced by the reaction between the $FeCl_3$ reagent and phenylpyruvic acid in the medium, resulting from the deamination of phenylalinine (Chart 6-8). The three tubes on the right are Christensen's urea agar slants. The third from the right shows a strong positive reaction (red color throughout the medium, indicating an alkaline reaction from the degradation of urea), compared with the negative control on the far right (yellow

color throughout). The reaction in the second tube from the right (red color in slant only) is produced by organisms such as *Klebsiella* species and certain *Enterobacter* species that are weak urease producers.

F. Four tubes containing Møller decarboxylase medium covered with a layer of mineral oil to effect an anaerobic environment (Chart 6-7). From left to right: A growth control tube devoid of the amino acids (growth is indicated by conversion to a yellow color due to the fermentation of glucose in the medium), arginine, lysine, and ornithine. The bromcresol purple indicator is yellow at an acid pH and purple at an alkaline pH. Thus, any tube that appears purple indicates an alkaline pH, the reaction produced by organisms that can decarboxylate the amino acid contained in the medium. The organism pictured here is arginine-negative and lysine- and ornithine-positive and is the typical reaction seen with *E. aerogenes*.

IP-19



IP-20

G. Lysine iron agar (LIA) is used to differentiate enteric organisms based on their ability to decarboxylate or deaminate lysine and to form H_2S. Organisms that decarboxylate lysine produce an alkaline (purple) reaction in the butt of the tube (middle tube). Organisms that deaminate lysine produce a red slant over an acid (yellow) butt (leftmost tube). Bacteria that produce H_2S cause blackening of the

medium mainly in the middle and the butt of the tube (also seen in tube on far left). The tube on the far left is lysine deaminase–positive and lysine decarboxylase–negative. The middle tube is lysine deaminase–negative and lysine decarboxylase–positive. The tube on the far right is negative for both lysine deaminase and lysine decarboxylase. (Butt reddish but not yellow.)

H. Tubes of motility medium. Motile organisms will show diffuse growth away from the line of inoculation (left tube); nonmotile organisms show growth only along the stab line (right tube). Also available is motility medium to which 2,3,5-triphenyltetrazolium chloride (TTC) is added. Growth of organisms capable of reducing TTC will appear red along the stab line as well as in the area into which the cells have migrated, making it easier to differentiate between motile and nonmotile bacteria (Image Plate 7-1E). (Photo courtesy of Health and Education Resources, Bethesda, MD.)

IP-21

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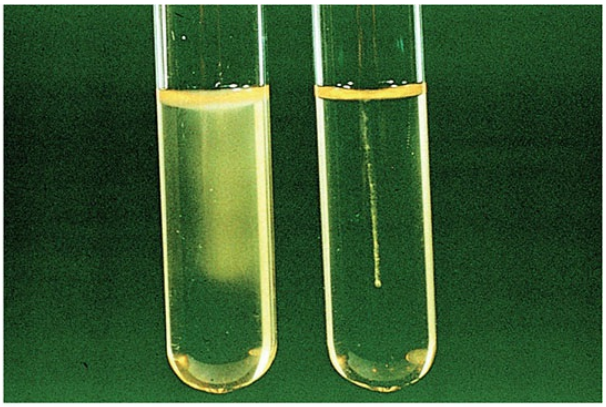

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IMAGE PLATE 6-5

IP-22

Human Plague

A. Hematogenous dissemination of *Y. pestis* from the portal of entry to other organs and tissues may cause intravascular coagulation and endotoxic shock, producing small hemorrhages on the skin. (CDC, Public Health Image Library.)

B. Gram stain of *Y. pestis* showing short, plump, gram-negative rods with bipolar staining. Organisms appear pink at the polar ends with a white area or nonstaining

in the center.

C. Peripheral blood smear stained with Wright's stain showing "safety pin" appearance of *Y. pestis*. Bacteria stain dark blue, leukocytes stain light blue with purple-staining nuclei, erythrocytes stain light tan. (CDC, Public Health Image Library.)

D. Colonies of *Y. pestis* growing on a blood agar plate at 72 hours. *Yersinia pestis* grows well on most standard laboratory media, after 48-72 hours, showing gray-white to slightly yellow opaque raised, irregular "fried egg" morphology; alternatively colonies may have a "hammered copper" shiny surface. (CDC, Public Health Image Library)

Chromogenic Agar

E. BBL CHROMagar O157 is designed for use as a primary plate for stool cultures when screening for *E. coli* O157. Distinguishes *E. coli* O157 (mauve colonies) from *E. coli* non-O157 (blue colonies). Also inhibits most *Proteus*, *Pseudomonas*, and *Aeromonas* strains using selective agents. (Courtesy of BD Diagnostic Systems.)

F and **G.** BBL CHROMagar Orientation medium is a non-selective, differential medium for presumptively identifying bacterial isolates from primary clinical specimens. Allows isolation and presumptive identification of both gram-positive and gram-negative pathogens with a single plate. Useful for identification and enumeration of urinary tract pathogens. Identifies *E. coli* (pink colonies) and *Enterococcus* (blue or turquoise colonies, small) from primary plate. Frame **F** shows Orientation agar with a pure culture of *E. coli*. Frame **G** shows a mixed culture of *E. coli* and *Enterococcus* together with some colonies of *Proteus* spp. (beige with brown halo) (Courtesy of BD Diagnostic Systems.)

H. BBL CHROMagar *Salmonella* is designed for use in *Salmonella* screening of either clinical or industrial samples. Detects *Salmonella* with a highly specific chromogenic reaction (purple colonies with purple halo), while minimizing interference from other hydrogen sulfide-producing colonies such as *Proteus* and *Citrobacter* spp. (Courtesy of BD Diagnostic Systems.)

IP-23

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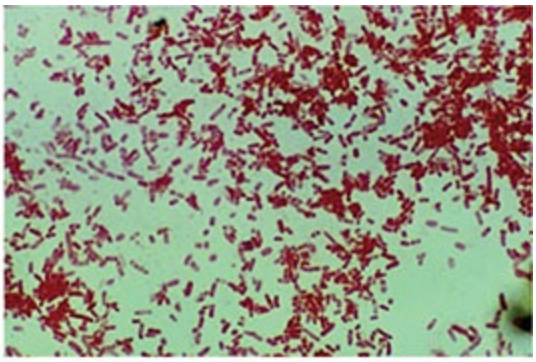

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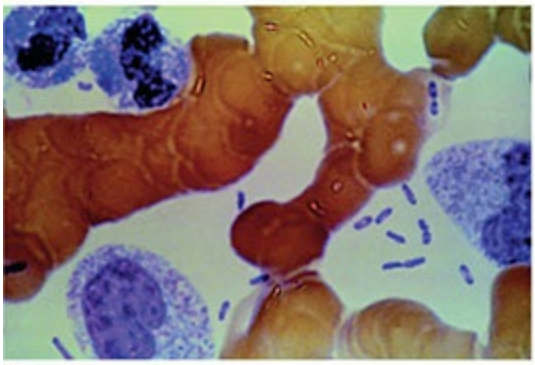

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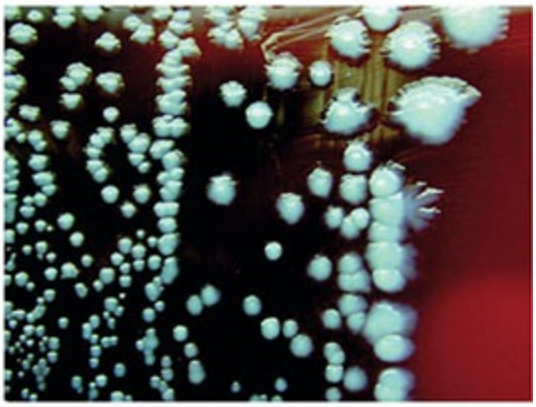

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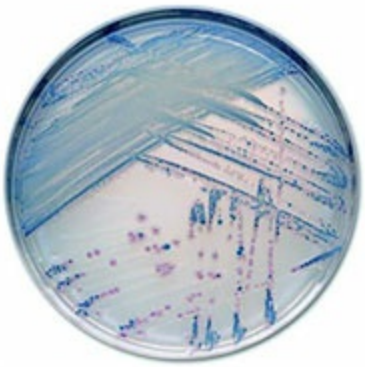

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IP-24

IMAGE PLATE 6-6

COMMERCIAL IDENTIFICATION SYSTEMS

A number of commercially manufactured identification systems are currently available that contain stable reagents and media designed for determining biochemical characteristics. These products range from those that are inoculated and read manually to those that are fully automated.

A. API 20E strips illustrating method of inoculation and the appearance of a strip following inoculation and incubation. A suspension of the organism to be tested is transferred with a pipette into each of the 20 media compartments. Color reactions are read after 18 to 24 hours of incubation at 35°C. The manufacturer supplies worksheets for recording the visual interpretation of the color reactions, which are then converted into a seven-digit biotype number.

B. The BBL Crystal Enteric/Nonfermenter identification system contains a lid with 30 dehydrated substrates on the tips of the plastic prongs. A test suspension is prepared and added to all 30 wells in the base unit. The lid is then aligned with the base and snapped in place, whereby the test inoculum rehydrates the dried substrates and initiates test reactions. After incubation, panels are read upside-down using the BBL Crystal light box. The wells are examined for color changes and a 10-digit profile number is generated and entered on a personal computer in which the BBL Crystal Electronic codebook has been installed, to obtain the identification.

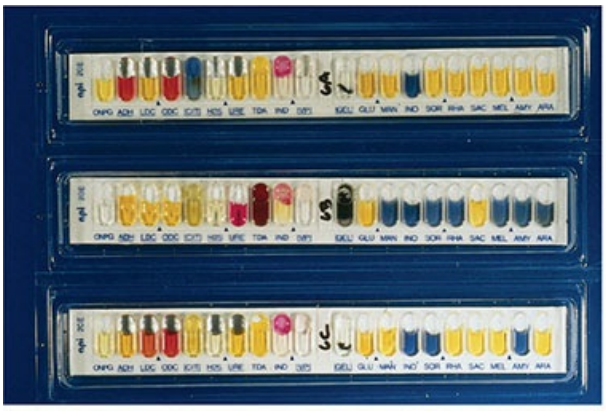
C. The REMEL RapID ONE (Oxidase-Negative *Enterobacteriaceae*) panel has 19 substrates for identification of over 70 organisms belonging to the family *Enterobacteriaceae*. The system consists of a plastic tray with 18 reaction cavities. Reaction cavities contain dehydrated reactants, and the tray allows the simultaneous inoculation of each cavity with a predetermined amount of inoculum. Test 18 is bifunctional, containing two separate tests in the same cavity. Each test is interpreted visually for color changes after 4 hours of incubation at 35°C. The 19 test results plus oxidase are scored in the appropriate boxes of the report form and a seven-digit profile code is generated similar to that shown in A. The biocode number is entered on a PC in which the ERIC (Electronic RapID Compendium) has been installed, to obtain the identification.

D. Biolog GN Microplate system consisting of a 96-well microtiter plate containing 95

carbon substrates and a redox indicator-tetrazolium dye. If a carbon substrate is utilized by the inoculated bacteria, the colorless dye is irreversibly reduced and forms a purple color. With the use of a computer screen, purple wells are coded as positive and colorless wells are coded as negative. The computer then matches the metabolic profile of the inoculated organism with those in the database and generates the most likely identification.

E and **F.** In the MicroScan gram-negative ID panel, the microtiter wells are inoculated with a heavy suspension of the organism to be identified and incubated at 35°C for 15 to 18 hours. The panels can be interpreted visually (Frame **E**), after which the biochemical results are converted into a seven- or eight-digit biotype number that is looked up in a codebook that is supplied by the manufacturer. An automated tray reader, autoSCAN-4 System, can also be used along with the LabPro information manager. Some trays also include antibiotics for performing both microdilution susceptibility testing and bacterial identification in the same panel. The same panels can also be used with the fully automated WalkAway System. (Frame **F**) The instrument processes both rapid and conventional overnight panels on one system and is available in both 40 and 96 panel capacity models. (Courtesy of Dade Behring.)

IP-25



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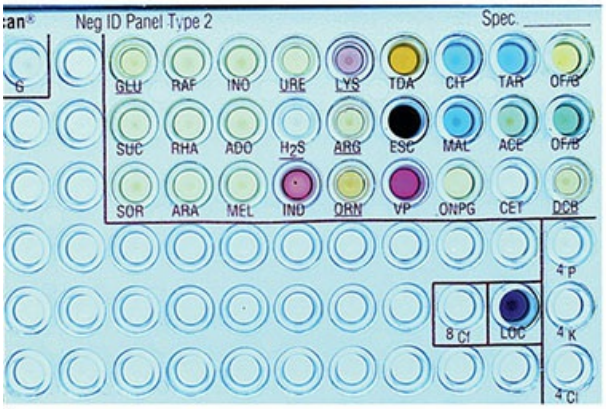

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IP-26

G. The VITEK2 is a system from bioMérieux that performs bacterial identification and susceptibility testing analyses using a standardized inoculum. The VITEK 2 features the AES (Advanced Expert System) that integrates artificial intelligence technologies in the VITEK 2 instrument. AES interprets the results of the antibiotic susceptibility test using a highly developed knowledge database. This base contains

most of the known resistance mechanisms (approximately 2,000 phenotypes, 20,000 MIC distributions). (Courtesy of bioMérieux.)

H. The BD PHOENIX Automated Microbiology System for Identification and Susceptibility Testing offers fully automated ID and susceptibility testing with no off-line tests, handwritten labels, or reagent additions required. The sealed test panel never moves once placed inside, so it cannot dislodge, jam, crack, or leak while in the instrument. The PHOENIX comes with integrated software combined with the BD EpiCenter (Courtesy of BD Diagnostic Systems.)

IP-27

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IP-28

IMAGE PLATE 7-1

IMPORTANT CHARACTERISTICS FOR DISTINGUISHING NONFERMENTATIVE GRAM-NEGATIVE BACILLI

A. Detection of fermentation. The Kligler's iron agar (KIA) tube on the right shows an alkaline slant/alkaline butt reaction characteristic of a nonfermenting organism; the tube on the left shows an alkaline slant/acid deep reaction, indicating fermentation of glucose but nonfermentation of lactose (characteristics of lactose-negative species of the *Enterobacteriaceae*). No acid production in KIA or in triple sugar iron (TSI) agar indicates the inability of nonfermenting bacteria to utilize the lactose or glucose in KIA (or the lactose, glucose, or sucrose in TSI).

B. Cytochrome oxidase test. The formation of a blue color within 10 seconds after smearing a test colony on filter paper saturated with the oxidase reagent (tetramethyl- *p*-phenylenediamine dihydrochloride) indicates cytochrome oxidase activity, a characteristic helpful in identifying many species of nonfermenters. All members of the *Enterobacteriaceae* are cytochrome oxidase-negative.

C. Failure to grow on MacConkey agar or inhibited growth on MacConkey agar is a clue that a gram-negative rod may be a nonfermenter. Although many species of nonfermenters are capable of growing on MacConkey agar, the lack of growth on this medium, as illustrated on the right side of this split frame, excludes the *Enterobacteriaceae*, all members of which grow well on MacConkey (left side).

D. Oxidative utilization of glucose. Illustrated here are two tubes of Hugh–Leifson oxidative fermentative (OF) medium. The tube on the right is open to the atmosphere, whereas the tube on the left is covered with mineral oil to exclude exposure to atmospheric oxygen. Acid (yellow) is seen only in the top portion of the open tube, indicating that the organism is capable of oxidizing glucose but incapable of fermenting glucose.

E. Tubes of Motility B medium containing 2,3,5-triphenyltetrazolium chloride (TTC). Motility is often difficult to observe with nonfermentative bacteria since the organisms tend to grow only in the upper (most aerobic) portion of the tube. The addition of tetrazolium aids in detecting motility because organisms capable of reducing TTC will appear red along the stab line as well as in the area into which the cells have migrated, making it easier to differentiate between motile (left) and nonmotile (right) bacteria.

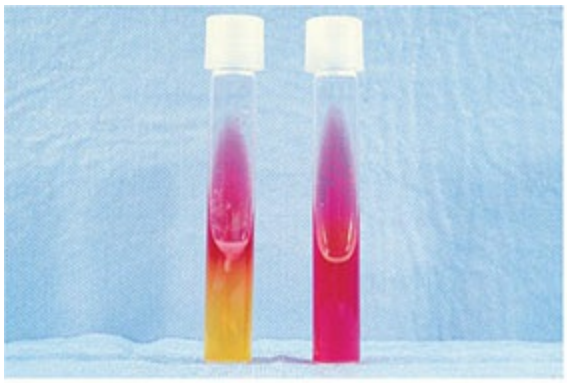
F. Blood agar plate inoculated with a yellow pigment-producing bacterium. Pigment production is an important differential characteristic in identifying nonfermentative gram-negative bacilli. The organism depicted here is *Sphingomonas paucimobilis*.

G. Tubes of Flo and Tech agar inoculated with *Pseudomonas aeruginosa* viewed under visible light. These media are used to enhance the production of two pigments: pyoverdin (fluorescein), which appears as a yellow diffusible pigment on Flo agar (left) in visible light, and pyocyanin, which appears as a turquoise-blue pigment on Tech agar (right) in visible light. Although three species of nonfermenting bacilli produce pyoverdin (*P. aeruginosa, P. fluorescens*, and *P. putida*), only one species (*P. aeruginosa*) produces pyocyanin.

H. Tubes of Flo and Tech agar inoculated with *Pseudomonas aeruginosa* viewed under ultraviolet (UV) light using a Wood's lamp. Note that the Flo agar tube (left) fluoresces, while the Tech agar tube (right) does not fluoresce under UV light. Only pyoverdin pigment, which is enhanced by growing the organism on Flo agar,

fluoresces under UV light.

IP-29



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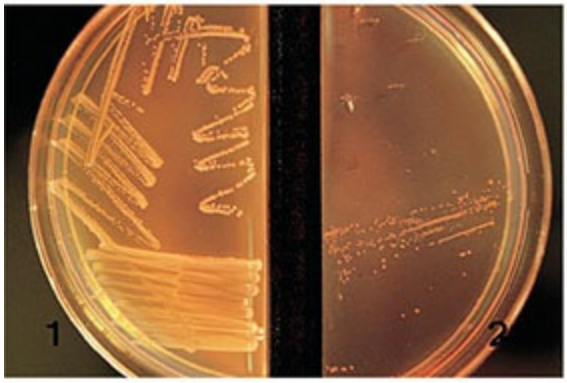

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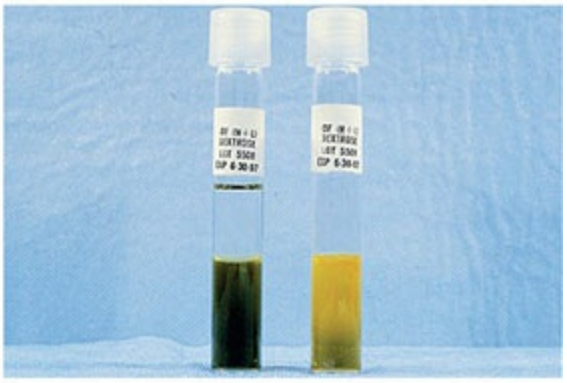

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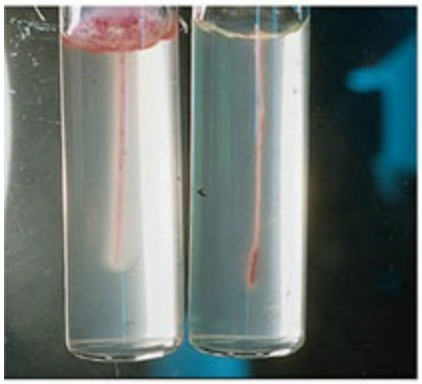

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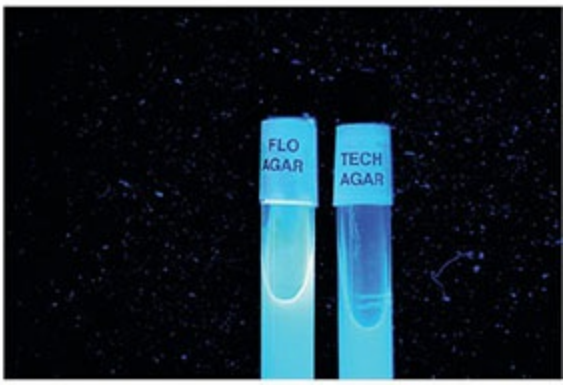

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IP-30

IMAGE PLATE 7-2

Tests Used in the Identification of Nonfermentative Gram-Negative Bacilli

A. Two tubes of Christensen's urea illustrating the fuchsia red color of a positive test (right) compared with a negative control (left). A rapid positive urease reaction (4 hour) is seen with the following species of nonfermentative bacilli: *Bergeyella zoohelcum, Bordetella bronchiseptica, Cupriavidus pauculus,* and *Oligella ureolytica*.

B. Two tubes demonstrating nitrate reduction (left) and nitrite reduction (right). Both tubes contain a Durham tube to demonstrate nitrogen gas formation. The tube on the left depicts the reduction of nitrate to nitrogen gas, and the tube on the right demonstrates the reduction of nitrite to nitrogen gas. Note that there is more gas formed in the tube on the right, indicating that quantitatively more nitrogen gas is formed from nitrite than nitrate during the same incubation period. Production of gas from both nitrate and nitrite is typical of the *Pseudomonas stutzeri* group and *Achromobacter denitrificans*.

C. Three tubes of heart infusion broth showing a positive indole reaction (left), an orange indole reaction (center), and a negative indole reaction (right). The reactions shown here were obtained using the xylene extraction method followed by the addition of Ehrlich's reagent (Chart 1-4). A positive indole reaction is noted by the appearance of a red band at the interface of the tryptophan broth and the xylene layer. The indole-positive nonfermentative bacilli include *Balneatrix, Bergeyella, Chryseobacterium, Elizabethkingia, Empedobacter, Weeksella,* and some unnamed CDC groups. A peculiar orange indole reaction (center tube) is observed with *Delftia acidovorans* and is due to the formation of anthranilic acid from tryptophan by this bacterium. An orange pigment is produced upon the addition of either Ehrlich's of Kovac's reagent. The reaction may take over 1 hour to develop following the addition of reagents.

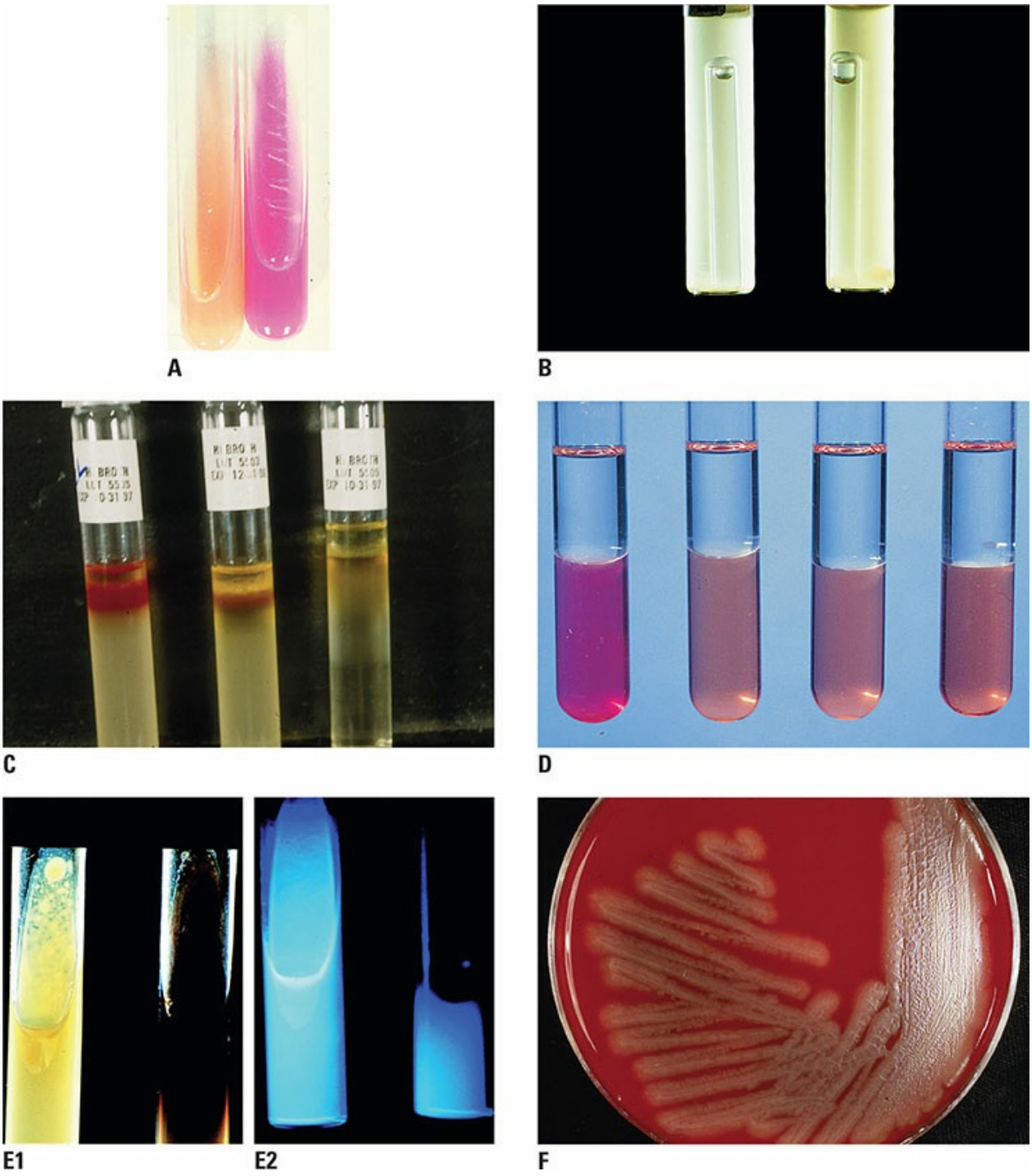
D. Four tubes of Møller decarboxylase medium covered with a layer of mineral oil. Reading from left to right: lysine, arginine, ornithine, and the control tube which is devoid of amino acid. With the nonfermentative bacilli, negative tests remain the same color as the original uninoculated tubes, while positive decarboxylation reactions appear darker purple owing to the formation of alkaline amines causing a more alkaline pH in the tube. The positive lysine decarboxylase reaction illustrated

here occurs with only two species of nonfermenting bacilli: *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex.

E. Split frame showing two tubes of esculin agar viewed under visible light (left), and the same two tubes viewed under ultraviolet light from a Wood's lamp (right). When esculin is hydrolyzed by a microorganism, the glycoside esculin is converted to esculetin and glucose. The esculetin reacts with an iron salt (ferric citrate) in the medium to form a dark brown of black complex (second tube from left). The production of a dark brown of black pigment may be difficult to discern if the organism itself produces a brown or deep-colored pigment. Since esculin is a fluorescent compound, true esculin hydrolysis can be determined by observing for the absence of fluorescence. The brightly fluorescing tube in the right frame (second tube from the far right) depicts the reaction observed with a negative esculin reaction. The tube on the far right shows marked squelching of fluorescence, particularly in the slant, indicating that the test organism is capable of hydrolyzing esculin.

F. Blood agar plate demonstrating the typical appearance of *Pseudomonas aeruginosa* after 24-hour incubation at 35°C. Colonies are large with a spreading periphery and are frequently beta hemolytic. Colonies in the heaviest area of growth appear to have a metallic sheen and a scaling appearance sometimes described as alligator skin morphology. Colonies appearing like this typically produce a sweet grape-like odor.

IP-31



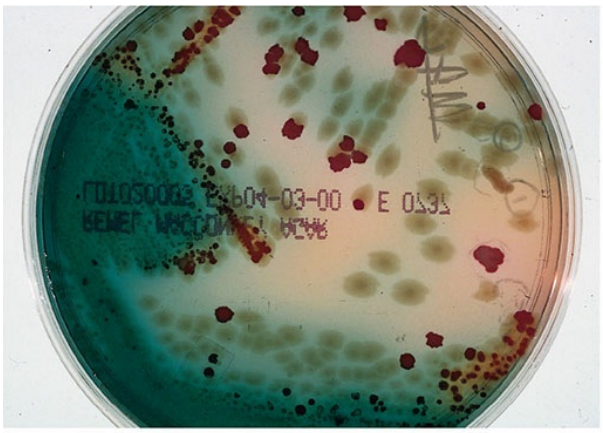
IP-32

G. MacConkey agar plate demonstrating growth of two colony types. The dark purple colonies are lactose-positive and have the typical appearance of *E. coli*. The lactose-negative colonies are producing a turquoise-blue diffusible pigment

noted best in the area of heaviest growth. This is the typical appearance of colonies of *Pseudomonas aeruginosa* that are producing pyocyanin.

H. MacConkey agar plate demonstrating growth of a mucoid variety of *Pseudomonas aeruginosa* typical of the strains isolated from the sputum of patients with cystic fibrosis. Colonies can be extremely mucoid and runny. Note that several colony variants can be seen, which is the typical presentation for these mucoid variants of *P. aeruginosa*. Note that in the heaviest growth area, there appears to be some pyocyanin pigment developing.

IP-33



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IP-34

IMAGE PLATE 7-3

COLONIAL AND MICROSCOPIC MORPHOLOGY OF CERTAIN NONFERMENTATIVE BACILLI

Some species of nonfermentative gram-negative bacilli have characteristic features that can be observed on growth media or in Gram-stained preparations. An awareness of these functions can be an aid in making a correct species identification.

A. *Pseudomonas stutzeri* on blood agar medium. Note the characteristic dry, wrinkled colonies that are typical of this species. One should immediately think of *P. stutzeri* when an oxidase-positive, nonfermenting bacillus produces this type of colony on blood agar.

B. Blood agar plate showing the yellow, dry, and wrinkled appearance of *Pseudomonas luteola*. Colonies are often adherent to the agar and difficult to remove. Colonial morphology resembles that of *Pseudomonas oryzihabitans* and *Pseudomonas stutzeri*. Both *P. luteola* and *P. oryzihabitans* are oxidase-negative, while, *P. stutzeri* is oxidase-positive.

C. MacConkey agar (left) and BAP (right) inoculated with a pure culture of *Brevundimonas vesicularis*. Note that this organism fails to grow on MacConkey agar and appears as deeply yellow colored, slightly wrinkled colonies on BAP. This organism is also susceptible to vancomycin and resistant to polymyxin B, additional features that can help in identification.

D. Blood agar plate illustrating growth of *Stenotrophomonas maltophilia*. The dull yellow pigment is distinctive for this species but at times may be difficult to visualize. Colonies may also appear dark tan to lavender upon continued incubation, particularly if left at room temperature.

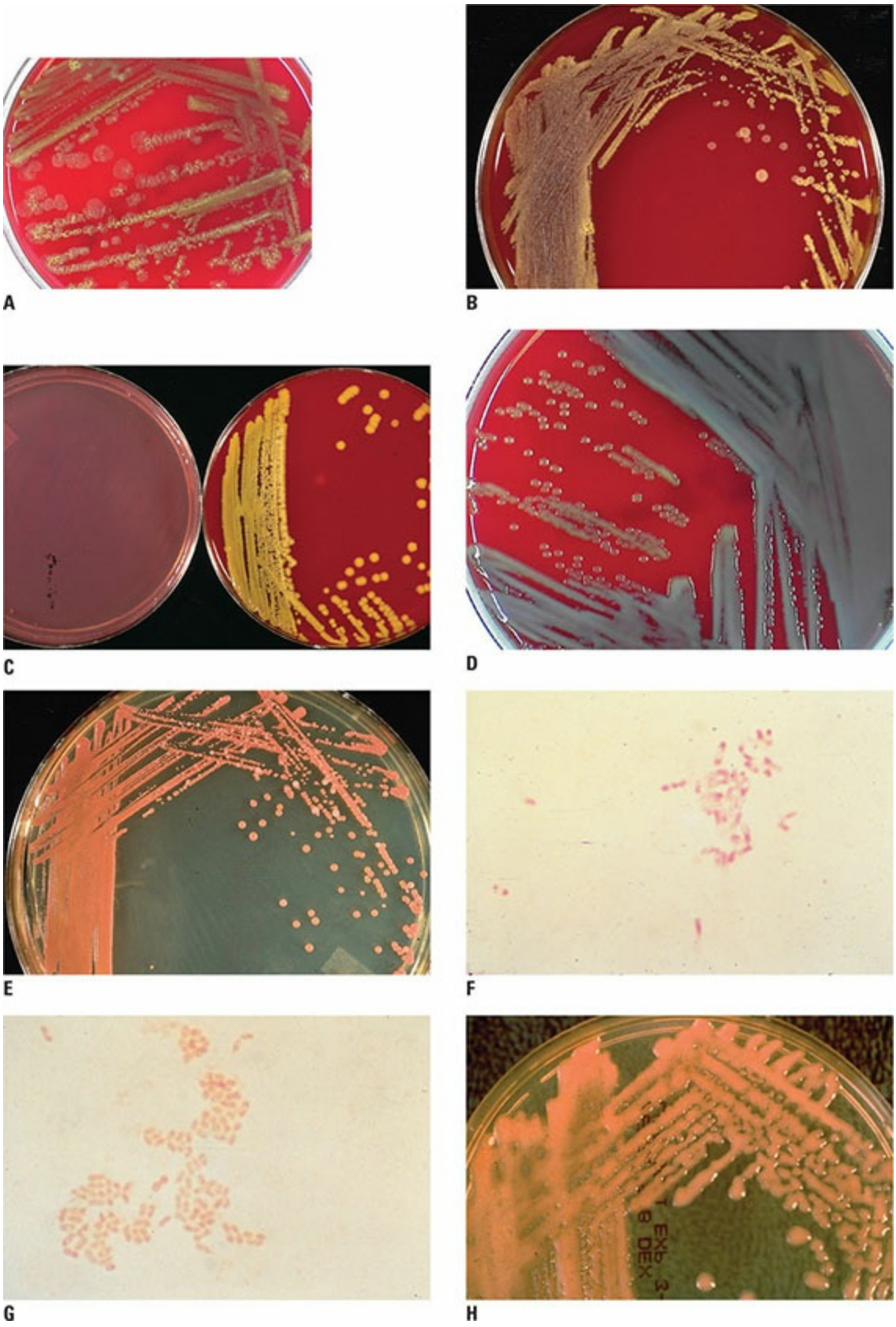
E. *Methylobacterium* species growing on Sabouraud dextrose agar. Colonies appear dry and exhibit a deep pink or coral pigmentation. These colonies absorb ultraviolet light and appear dark when held under a Wood's lamp.

F. Gram's stain of *Methylobacterium* species showing characteristic gram-negative bacilli containing vacuoles.

G. Gram's stain of *Roseomonas* species showing characteristic faint staining, gram-negative coccoid forms, some containing vacuoles.

H. *Roseomonas* species growing on Sabouraud dextrose agar. They appear as light pink, mucoid, almost runny colonies. Like *Methylobacterium* species, *Roseomonas* species grow best on Sabouraud's agar; however, they do not absorb UV light and do not appear dark when held under a Wood's lamp.

IP-35



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IP-36

IMAGE PLATE 7-4

Colonial and Microscopic Morphology of Certain Nonfermentative Bacilli

A. Blood agar plate demonstrating growth of *Alcaligenes faecalis* after 48 hours of incubation at 35°C. Colonies are white and glistening; in older cultures, colonies tend to spread at the outer border of the colonies and turn the agar green. This species typically gives off a fruity odor sometimes described as smelling like green apples.

B. MacConkey agar plate showing growth of *Rhizobium radiobacter*. Even though these bacteria are nonfermenters, they rapidly oxidize lactose and consequently appear as pink-colored, extremely mucoid colonies on MacConkey agar. These organisms can be easily confused with a lactose-positive fermenter; however, they are oxidase-positive, which readily excludes any members of the *Enterobacteriaceae*.

C. Blood agar plate showing growth of *Chryseobacterium indologenes*. Colonies have a deep yellow to orange appearance after 48 hours of incubation particularly if incubated at 30°C. Phenotypically these organisms are difficult to distinguish from *Chryseobacterium gleum*, and some laboratories choose to report such isolates as *C. indologenes/gleum.*

D. Blood agar plate showing characteristic growth of *Myroides odoratus*. Colonies have a deep yellow pigment and may be spready. Most strains exhibit a characteristic fruity odor like that of *Alcaligenes faecalis*.

E and **F.** A useful test for distinguishing between *Neisseria* species and *Moraxella* species is to grow the organisms on blood agar around a penicillin disk. Both organisms are susceptible to penicillin and will demonstrate a zone of inhibition around the penicillin disk (**E**). After overnight incubation, a Gram's stain can be performed on colonies taken from the edge of the zones of inhibition. *Neisseria* species are true cocci and in the presence of subinhibitory concentrations of penicillin will continue to stain as gram-negative diplococci (left side, F). *Moraxella* species are coccobacilli and in the presence of subinhibitory concentrations of penicillin will form bizarre rod-shaped cells (right side, F).

G. Blood (left) and MacConkey agar plates showing growth of *Paracoccus yeei* (CDC group EO-2) after 72 hours incubation at 30°C. Colonies appear white and

mucoid in appearance. On MacConkey agar, they appear slightly pink in color.

H. Gram's stain of *Paracoccus yeei* (CDC group EO-2) prepared from broth culture. These organisms stain peripherally giving the appearance of "O-shaped" cells.

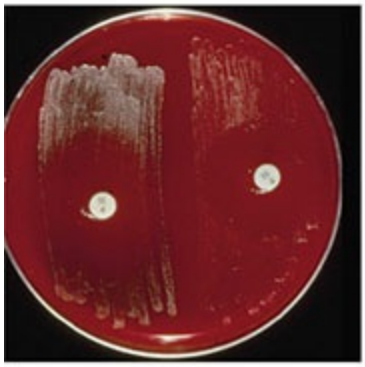
IP-37

A

B

C

D



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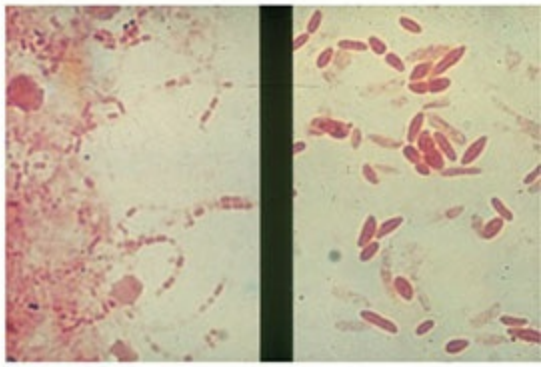

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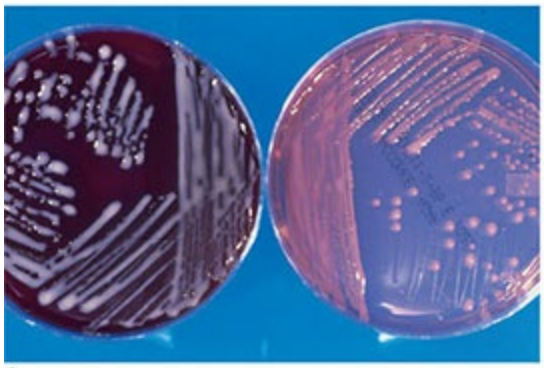

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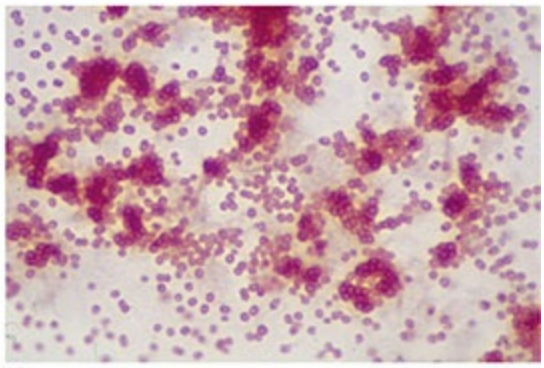


H

IP-38

IMAGE PLATE 7-5

Colonial and Microscopic Morphology of Certain Nonfermentative Bacilli

A. Gram's stain illustrating gram-negative coccobacilli. *Acinetobacter* and *Moraxella* species are the nonfermentative bacilli that characteristically have this staining morphology. The smear depicted here was made from a broth culture of *A. baumannii*. *Acinetobacter* species have a tendency to retain crystal violet and may appear gram-positive in some Gram's stain preparations particularly those made directly from positive blood culture bottles.

B. *Acinetobacter baumannii* on MacConkey agar showing a pink to light lavender appearance that is typical for this species. Often a bluish pigmentation may be observed, particularly striking in colonies grown on EMB agar, where it is described as a "cornflower blue" in appearance.

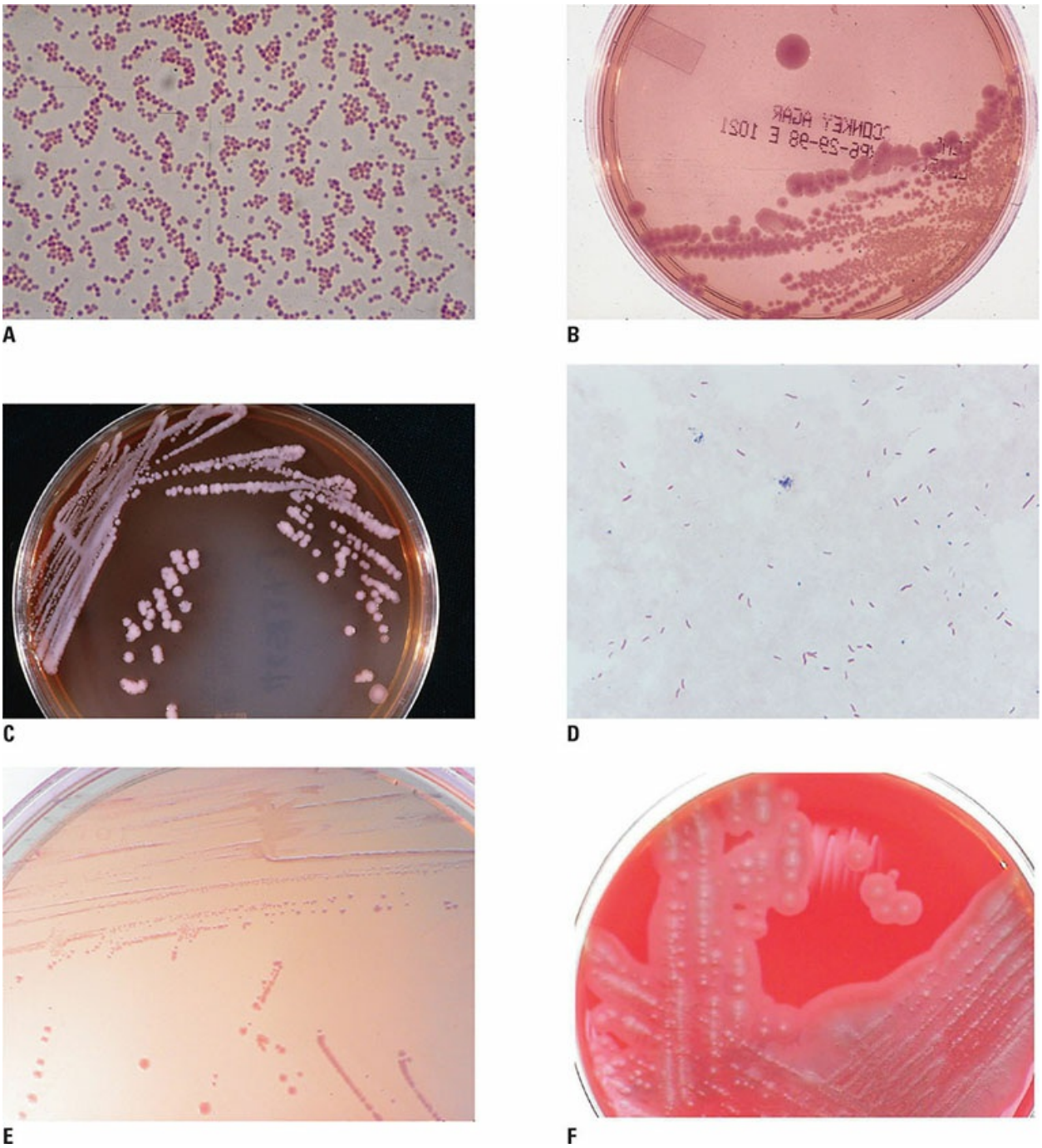
C. Certain glucose-oxidizing acinetobacters may produce a unique brown discoloration of heart infusion agar with tyrosine or blood agar into which glucose is incorporated. This phenomenon may also be observed on MacConkey and Mueller–Hinton agars. Shown here is a clinical isolate of *A. baumannii* producing a brown diffusible pigment on MacConkey agar.

D. Gram's stain of *Laribacter hongkongensis* prepared from 48-hour BAP. Note that the organisms appear as gram-negative, seagull-shaped or spiral rods.

E. *Laribacter hongkongensis* on MacConkey agar after 48-hour incubation in room atmosphere. Colonies are small and lactose-negative, but may have a lavender appearance similar to *Acinetobacter* species due to the uptake of crystal violet contained in the medium.

F. *Kerstersia gyiorum* on BAP at 24-hour incubation in room atmosphere. Colonies are gray, dull, and spread, giving a swarming appearance.

IP-39



IP-40

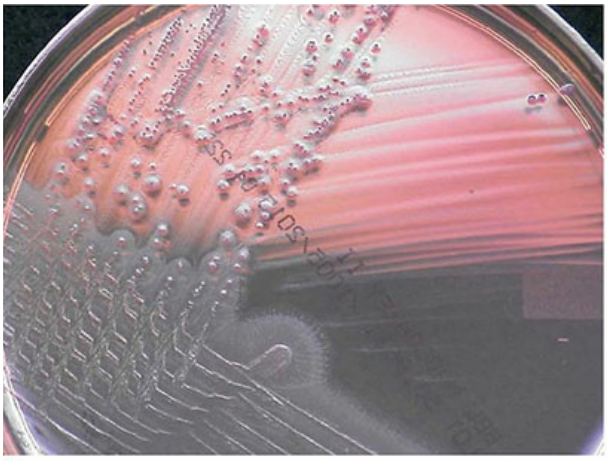
G. *Kerstersia gyiorum* on MacConkey agar at 24-hour incubation in room atmosphere. Colonies are small and lactose-negative with a characteristic spread appearance.

H-1. (Upper Frame) API 20 NE strips illustrating the 24-hour (top) and 48-hour

(bottom) reactions. Note that the first 8 tests (reading left to right) are conventional colorimetric reactions, while the remaining 12 tests are carbon assimilation reactions that are read as positive if turbid and negative in the absence of turbidity. Users of this system should note that the strip is incubated at 30°C rather than the usual 35°C used for most other commercial identification kits.

H-2. (Lower Frame) The Remel RapID NF Plus system consists of a plastic tray containing 10 reaction cavities. Seven of the reaction cavities (4 through 10) are bifunctional, containing two separate tests in the same cavity. The system is inoculated with a heavy suspension of the test organism and incubated for 4 hours at 35°C. Following incubation, bifunctional tests are first scored before the addition of reagent, providing the first test result (top row). The same cavities are scored again after the addition of reagent to provide the second test result (bottom row). The reactions obtained with these 17 tests plus oxidase provide 18 test scores.

IP-41



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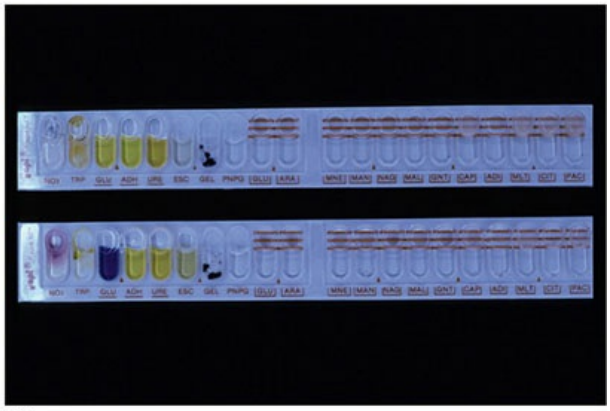


H1

H2

IMAGE PLATE 8-1

LABORATORY IDENTIFICATION OF *CAMPYLOBACTER* SPECIES

A. Gram's stain of *Campylobacter jejuni* illustrating pleomorphic gram-negative bacilli, with short, curved, and spiral forms. Note that some cells connect to form gull-winged and "S" shapes.

B. *C. jejuni* growing on nonselective Brucella agar plate following isolation from stool using the membrane filter technique (described in text). Note that growth has occurred only in the area of the plate underneath where the filter had been placed.

C. Close-up view of *C. jejuni* on blood agar, illustrating raised, gray-white, and somewhat mucoid colonies.

D. Growth of *C. jejuni* on Campy BAP agar, illustrating the tendency of the organism to grow along the streak lines.

E. Tubes showing the rapid hippurate reaction. Purple color develops with the addition of Ninhydrin when hippurate has been hydrolyzed to form glycine and benzoic acid (positive tube on left compared with negative control on right). Of the *Campylobacter* species, only *C. jejuni* gives a positive hippurate reaction.

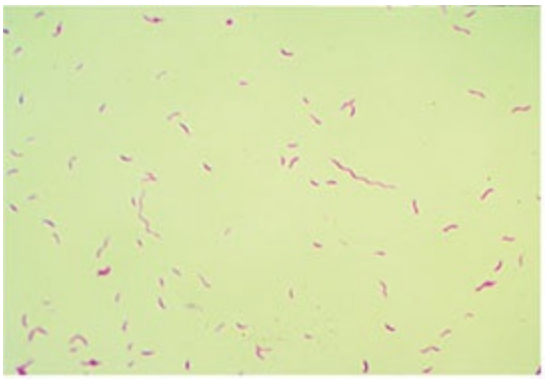
F. Brucella blood agar plate showing growth of *C. jejuni* around cephalothin and nalidixic acid disks. Note that with *C. jejuni* a zone of inhibition forms around the nalidixic acid disk (right), indicating that this species is susceptible to nalidixic acid but resistant to cephalothin. This test is easy to perform and allows presumptive identification of *C. jejuni*.

G. Triple sugar iron (TSI) agar slant reactions illustrating the hydrogen sulfide (H_2S) reactions of several species. The tube to the extreme left illustrates the lack of H_2S, characteristic of *C. jejuni, C. fetus* subsp. *fetus*, and *C. fetus* subsp. *venerealis*. Tubes 2, 4, and 5 (reading from left) illustrate a strong butt reaction, characteristic of *C. sputorum* biovar *bubulus*, *C. sputorum* biovar *fecalis,* or *C. sputorum* biovar *sputorum*. Tube 3 illustrates a strong slant reaction characteristic of *C. mucosalis*.

H. Silver-stained tissue section of superficial gastric mucosa demonstrating clusters of blue-black staining bacilli along the epithelial lining, consistent with the bacillary forms of *Helicobacter pylori*. When observed in a Gram-stained preparation, the

individual cells are long, thick, and curved.

IP-43



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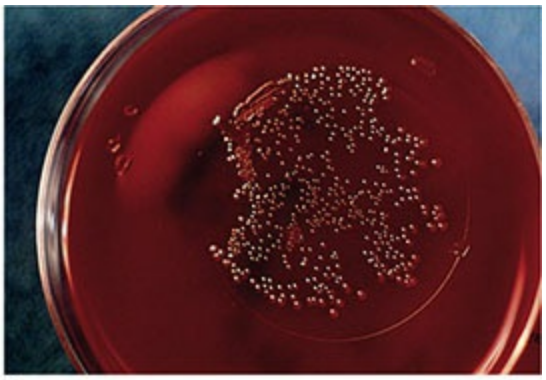

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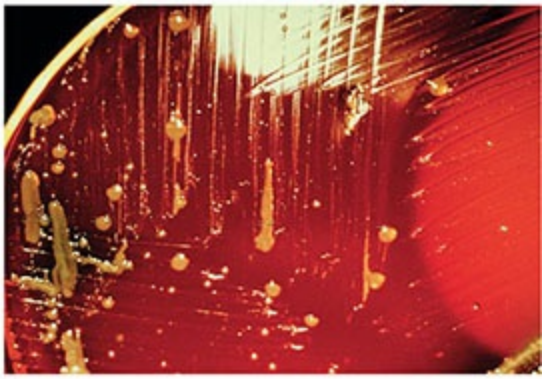

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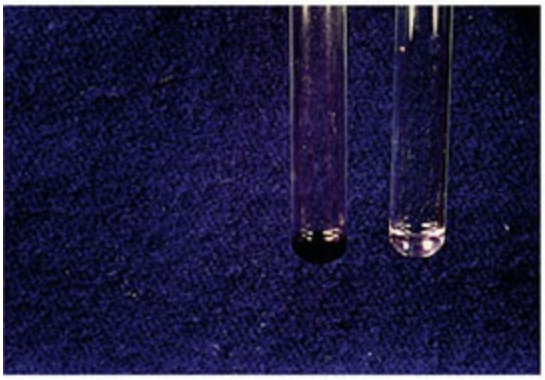

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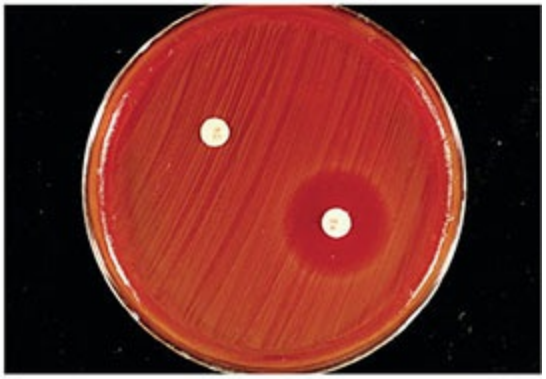
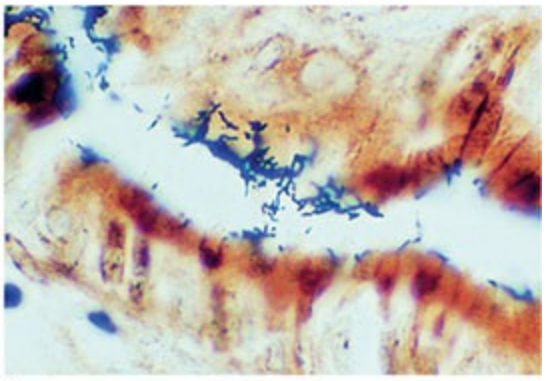


F

Different Reaction in TSI AGAR SLANTS
Growth in CAMPY Atmosphere
Campylobacter species

G



H

IP-44

IMAGE PLATE 8-2

LABORATORY IDENTIFICATION OF *VIBRIO CHOLERAE* AND OTHER *VIBRIO* SPECIES

A. Appearance of *Vibrio cholerae* on thiosulfate citrate bile sucrose (TCBS) agar. The yellow colonies result from citrate utilization and the formation of acid from utilization of the sucrose in the medium. The appearance of yellow colonies on this medium is virtually diagnostic of *V. cholerae*.

B. Colonies of *V. parahaemolyticus* growing on TCBS agar illustrating the characteristic semi-translucent, green-gray appearance.

C. Gelatin agar with white, opaque colonies of *V. cholerae*. Note the opalescence of the agar adjacent to the colonies, indicating hydrolysis and denaturation of the gelatin.

D. Gram's stain of *V. vulnificus* illustrating gram-negative bacterial cells with the curved, rod-shaped morphology typical of *Vibrio* species.

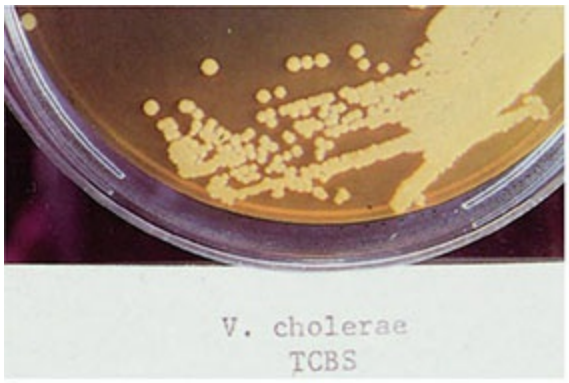
E. Positive string test with *V. cholerae*. When colonies of *V. cholerae* are mixed in a drop of 0.5% sodium deoxycholate, they produce a viscous suspension that can be drawn into a string when the inoculating loop is slowly raised from the side.

F. Positive slide agglutination test for *V. cholerae* using polyvalent O antiserum.

G. Blood agar plate illustrating the relatively large, intensely-hemolytic colonies of the El Tor biotype of *V. cholerae*.

H. Chicken erythrocyte agglutination test. Classical strains of *V. cholerae* do not agglutinate chicken erythrocytes (top), in contrast to the El Tor biotype (bottom), which is capable of agglutinating the erythrocytes.

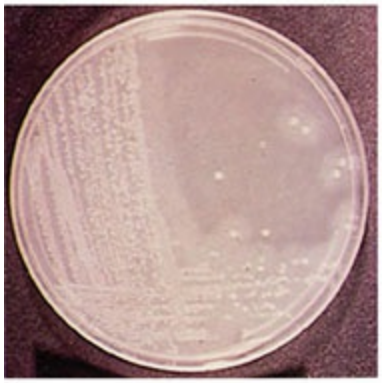
IP-45


V. cholerae
TCBS

A

V. parahaemolyticus
TCBS AGAR

B



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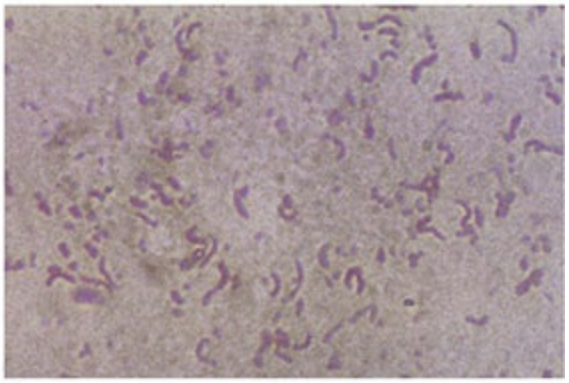

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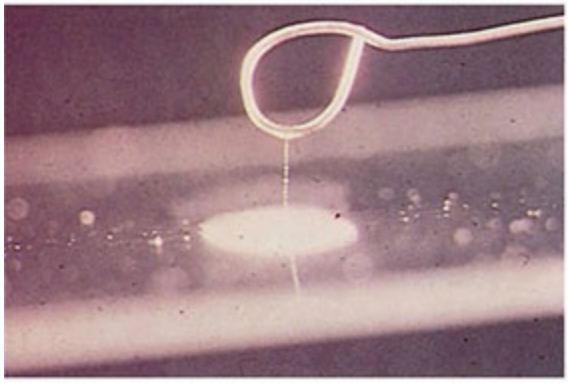
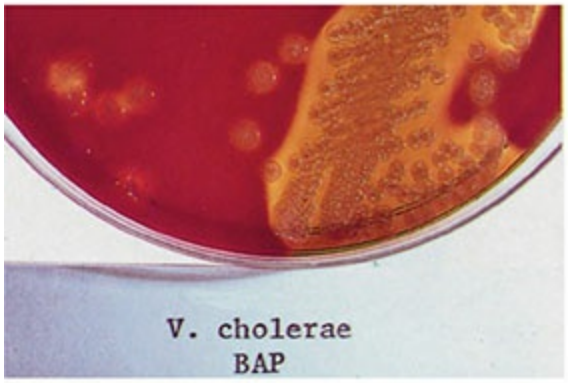
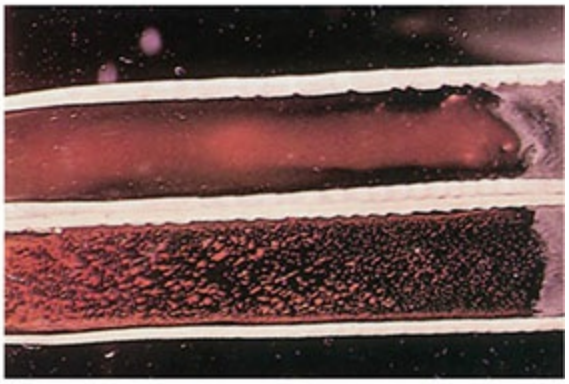


E

F


V. cholerae
BAP

G



H

IP-46

IMAGE PLATE 9-1

IDENTIFICATION OF *HAEMOPHILUS* SPECIES AND *AGGREGATIBACTER*

A. A Gram-stained smear of centrifuged cerebrospinal fluid showing polymorphonuclear cells and scattered gram-negative bacilli of *Haemophilus influenzae*. These organisms characteristically appear as small, poorly staining coccobacilli on Gram's stains of clinical specimens. Occasionally, longer, filamentous bacilli may be seen.

B. Growth of moist, smooth, gray colonies of *H. influenzae* type b on chocolate agar after 24 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. This medium contains hematin (X factor) and is enriched with other cofactors, such as NAD (V factor), that allow the growth of *Haemophilus* and other fastidious microorganisms.

C. Satellite growth of *Haemophilus* around streaks of *Staphylococcus aureus* on sheep blood agar. X factor, or hemin, is provided by the lysed sheep erythrocytes surrounding the *Staphylococcus* streak, while the staphylococci themselves provide the V factor, or NAD. These factors enable the tiny, dewdrop colonies of *Haemophilus* to grow adjacent to the *Staphylococcus* streak.

D. Growth factor test for identification of *H. influenzae*. The organism is inoculated onto a plate of trypticase-soy or brain–heart infusion agar, and disks containing X factor and V factor are placed in proximity to one another on the inoculated plate surface. After incubation in a CO_2-enriched environment at 35°C to 37°C, factor requirements are determined by observing the pattern of organism growth relative to the two disks. In this picture, growth is observed between the X factor and V factor disks, indicating that the organism requires both exogenous X and exogenous V factors for growth.

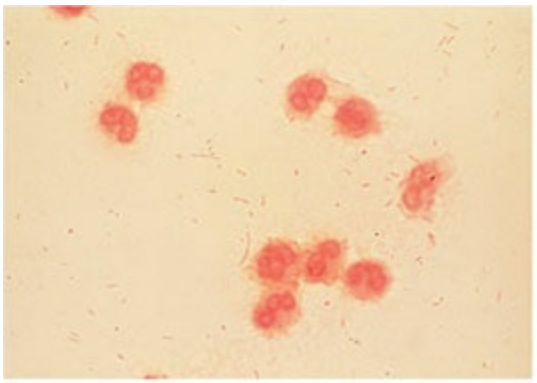
E. Growth factor test for *Haemophilus parainfluenzae*. The organism is inoculated as described for **D** above, but in this photograph, filter paper strips that are impregnated with X factor, V factor, and both X and V factors are placed on the lawn of inoculum. After incubation in a CO_2-enriched environment at 35°C to 37°C, growth is observed around the V factor strip and around the XV factor strip, but not around the X factor strip. This growth pattern indicates that the organism requires only V factor for growth.

F. The ALA–porphyrin agar test. This is an alternative method for determining the X factor requirements of *Haemophilus* isolates. The agar medium contains δ-aminolevulinic acid (ALA). Organisms are inoculated onto the medium, and after overnight incubation at 35°C to 37°C in a CO_2-enriched environment, the growth is observed under a Wood's (ultraviolet) light. If the colony growth fluoresces "brick red," the organism is able to synthesize X factor (hemin) from ALA and does not require exogenous X factor. This picture shows growth of both *H. influenzae* (left), with its negative ALA-porphyrin test (i.e., requires exogenous hemin for growth), and *H. parainfluenzae* (right), with its positive ALA-porphyrin test (i.e., synthesizes hemin from ALA).

G. ALA-porphyrin disk method. This picture also shows the ALA-porphyrin test, but in this approach, a δ-aminolevulinic acid-impregnated disk is used. The disk is moistened with water, and some of the colony growth is rubbed onto the surface of the disk. After incubation for 4 hours at 35°C to 37°C, the disks are observed in the dark under a Wood's light. If the disk fluoresces "brick red," the organism is able to synthesize its own X factor. If no fluorescence is observed, the organism requires exogenous X factor. In this picture, *H. influenzae* (left) shows no fluorescence (i.e., requires exogenous X factor), while *H. parainfluenzae* (right) shows fluorescence (i.e., synthesizes X factor from ALA).

H. Nitrate reduction and biotyping reactions for *H. influenzae* biotype I. Isolates of *H. influenzae* and *H. parainfluenzae* reduce nitrate to nitrite and can be grouped into distinct biotypes by their reactions in three biochemical tests: production of indole, ornithine decarboxylase, and urease. This picture shows the nitrate and biotyping reactions of *H. influenzae* biotype I. Tests shown (left to right) are a positive nitrate reduction test, indole production in tryptone broth following Xylene extraction (positive), Møller's decarboxylase broth base (negative), Møller's ornithine decarboxylase broth (positive), and a urea slant (positive).

IP-47



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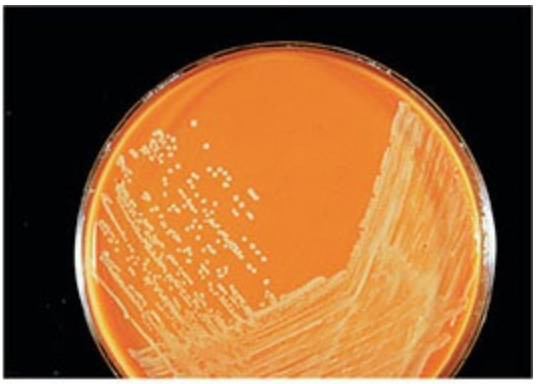

B

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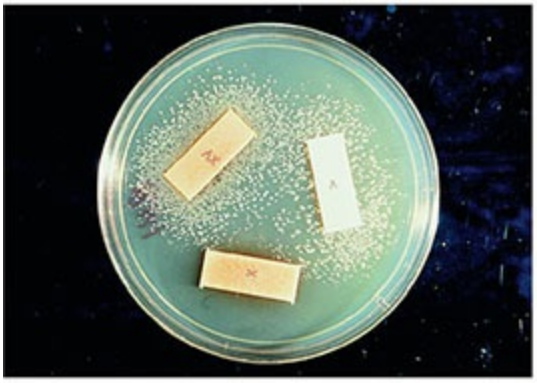

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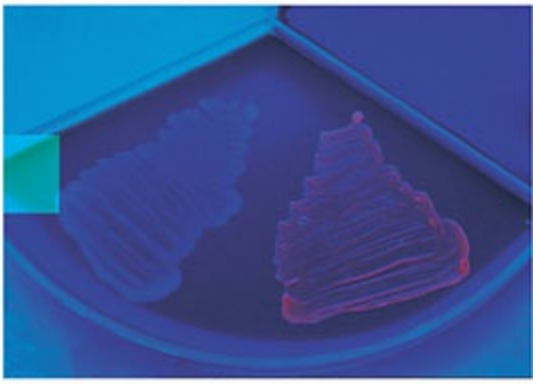

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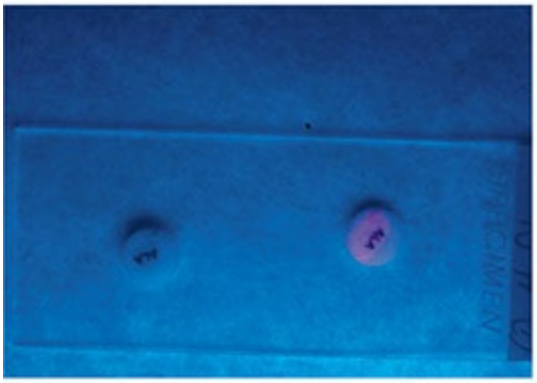


G

H

IMAGE PLATE 9-2

IDENTIFICATION OF *HAEMOPHILUS* SPECIES (CONTINUED)

A. The MicroScan *Haemophilus-Neisseria* Identification (HNID) panel (Dade-MicroScan, West Sacramento, CA). The manual microtiter format system identifies both *Haemophilus* and *Neisseria* species and provides biotype designations for *H. influenzae* and *H. parainfluenzae*. This picture shows a panel inoculated with *H. influenzae* biotype I (urease [URE]-positive, ornithine decarboxylase [ODC]-positive, and indole [IND]-positive. (Courtesy of Dade-MicroScan.)

B. A lesion of chancroid on the external female genitalia. Lesions of chancroid may resemble chancres of syphilis; however, chancroid lesions are usually painful and soft, while lesions of primary syphilis are usually painless and indurated. The etiologic agent of chancroid, *Haemophilus ducreyi*, may be cultured from these lesions.

C. RapID NH panel (Remel) inoculated with *H. ducreyi*. The top panel is before addition of reagents to the three bifunctional wells of the cuvette (PO_4/NO_2, ORN/NO_3, and URE/IND), while the bottom panel is after reagent addition for development of the NO_2, NO_3, and IND reactions. The only positive reactions observed with *H. ducreyi* on the RapID NH panel are the phosphatase reaction (top, eighth reaction from the left [yellow]) and the nitrate reductase reaction (bottom, ninth reaction from the left [red]).

D. Gram-stained smear of *Aggregatibacter aphrophilus*, showing small, pale-staining coccobacilli.

E. Growth of *A. aphrophilus* on sheep blood agar (left) and chocolate agar (right) after 48 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. *A. aphrophilus* does not require either exogenous X or V factors and, consequently, is able to grow on sheep blood agar. Colonies are characteristically small and have a slight yellowish pigmentation.

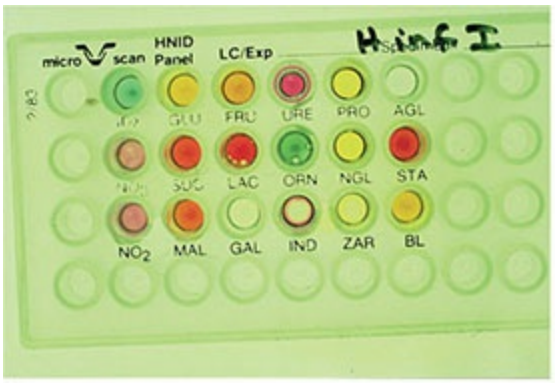
F. *Haemophilus paraphrophilus* on sheep blood agar (left) and chocolate agar (right) after 48 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. *H. paraphrophilus* is biochemically identical to *A. aphrophilus*, but it requires V factor for growth. Consequently, *H. paraphrophilus* grows on chocolate agar

(right) but not on sheep blood agar (left).

G. Biochemical tests for identification of *A. aphrophilus*. The tubes shown in this photograph (left to right) include nitrate broth, indole–tryptone broth, Møller's decarboxylase base broth, Møller's lysine decarboxylase broth, Møller's ornithine decarboxylase broth, and a urea agar slant. As shown here, *A. aphrophilus* is nitrate-positive, but negative for indole, lysine decarboxylase, ornithine decarboxylase, and urease.

H. Rapid carbohydrate utilization tests for *A. aphrophilus*. In this procedure, a balanced salts-phenol red solution (BSS) is dispensed in a series of tubes in 0.10-ml aliquots. A single drop of a filter-sterilized carbohydrate solution (20% w/v) is added to each tube. A heavy suspension of the organism is prepared in the BSS without added carbohydrate, and a single drop of this suspension is added to each of the BSS-carbohydrate tubes. After 4 hours of incubation at 35°C to 37°C, the reactions are interpreted. With the production of acid, the phenol red indicator turns from red to yellow. In this frame, the tubes (left to right) are the BSS organism suspension (without added carbohydrate), BSS with glucose (G), maltose (M), sucrose (S), lactose (L), mannitol (MN), xylose (X), and mannose (MA). As can be seen from these reactions, *A. aphrophilus* produces acid from glucose, maltose, sucrose, lactose, and mannose, but not from mannitol or xylose. Details of this procedure, which can also be used for identifying *Neisseria* species, are presented in Chart 11-1.

IP-49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HNID Panel
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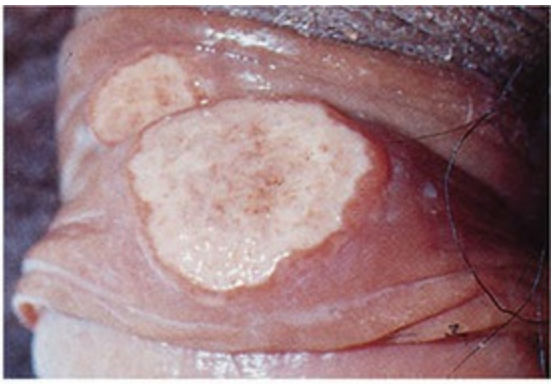

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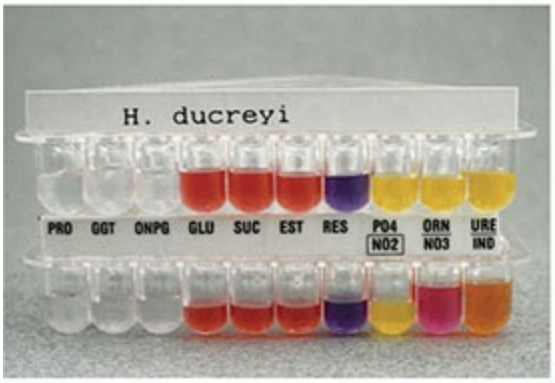
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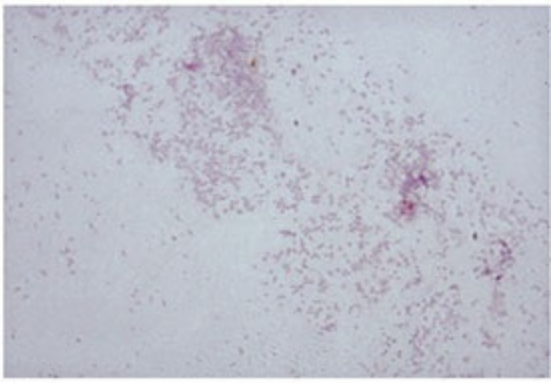

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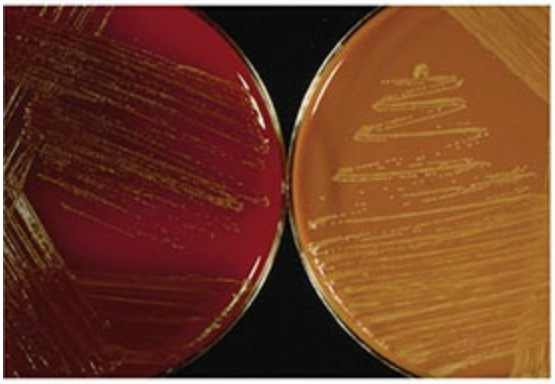

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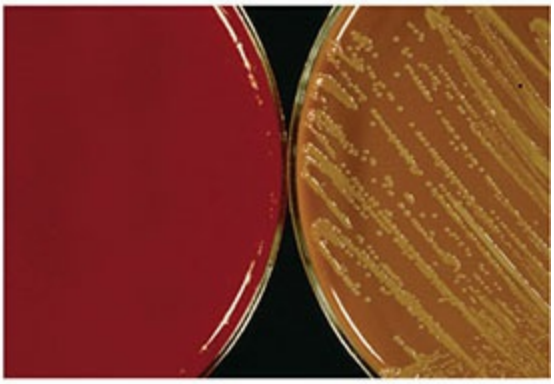

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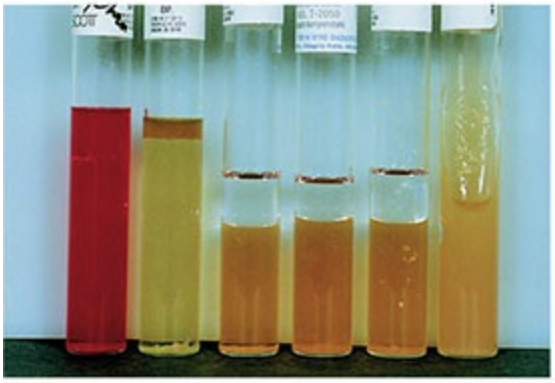

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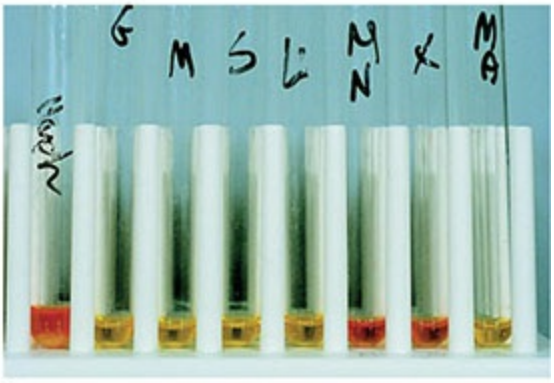
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IMAGE PLATE 9-3

Aggregatibacter, *Cardiobacterium*, and *Eikenella* Species

A. Gram-stained smear of *Aggregatibacter actinomycetemcomitans*, showing the pale-staining, gram-negative bacilli and coccobacilli of this species.

B. *A. actinomycetemcomitans* growing on chocolate agar after 72 hours of incubation at 35°C to 37°C in 5% to 7% CO_2.

C. Rapid carbohydrate utilization tests for *A. actinomycetemcomitans*. As shown here, *A. actinomycetemcomitans* produces acid from glucose (G), mannitol (MN) and mannose (MA), but not from maltose (M), sucrose (S), lactose (L), or xylose (X). Failure to produce acid from lactose, along with a positive catalase test, helps to differentiate *A. actinomycetemcomitans* from *A. aphrophilus* (see Image Plate 9-2H above) Nitrate reduction, lysine and ornithine decarboxylases, and urease reactions are the same for both *A. actinomycetemcomitans* and *A. aphrophilus* (see Image Plate 9-2G above).

D. Gram-stained smear of *Cardiobacterium hominis*. These organisms are often gram-variable, but are staining uniformly gram-negative in this photograph. The cells of *C. hominis* are generally longer than the other HACEK bacteria and show considerable pleomorphism (e.g., cells with pointed or swollen ends, teardrop-shaped and dumbbell-shaped cells, etc.). The characteristic palisading of the cells in "picket fence" arrangements and the clustering of cells to form compact rosettes are also evident in this picture.

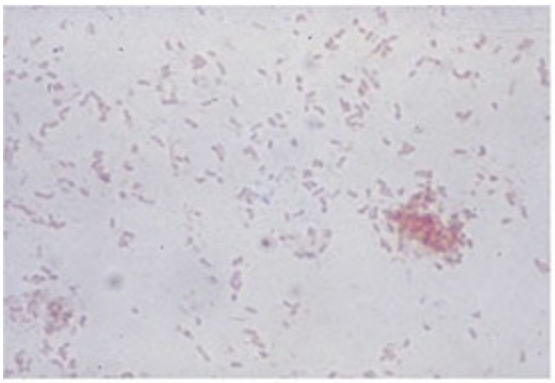
E. Growth of *C. hominis* on sheep blood agar after 72 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. Colonies of this organism are small, opaque, and glistening. Some strains may also pit the agar like *E. corrodens*.

F. Gram-stained smear of *Eikenella corrodens*. The cells of *E. corrodens* appear as "regular" gram-negative bacilli, and are not coccobacillary or pleomorphic like other members of the HACEK bacteria.

G. Growth of *E. corrodens* on sheep blood agar after 72 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. The incident light on the plate in this photograph demonstrates the pitting of the agar surface that is characteristic of most strains. Nonpitting strains may also be identified, and both pitting and nonpitting variants may be observed in the same culture.

H. Biochemical tests for identification of *E. corrodens*. The tubes shown here are (left to right) nitrate broth, indole–tryptone broth, Møller's decarboxylase base, Møller's lysine decarboxylase broth, Møller's ornithine decarboxylase broth, and a urea slant. *E. corrodens* is nitrate-positive, urease-negative, and is the only HACEK member that is both lysine and ornithine decarboxylase–positive. *E. corrodens* also differs from the other HACEK bacteria in not producing acid from carbohydrates either fermentatively or oxidatively.

IP-51



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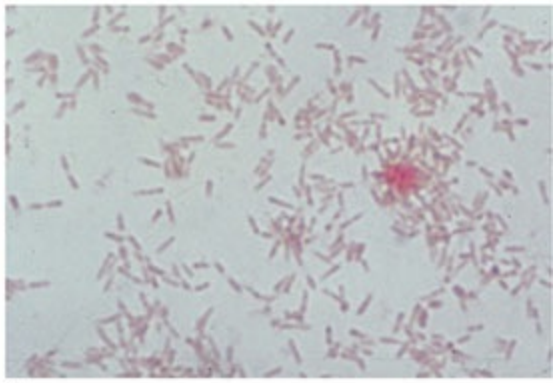

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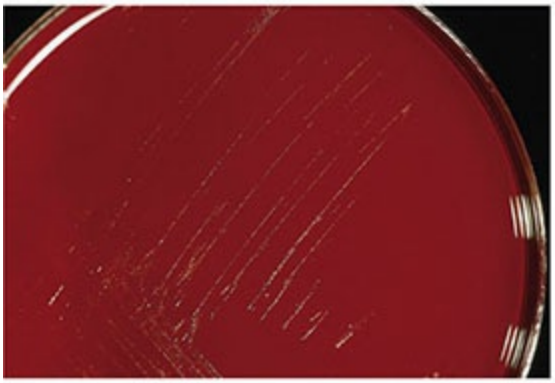

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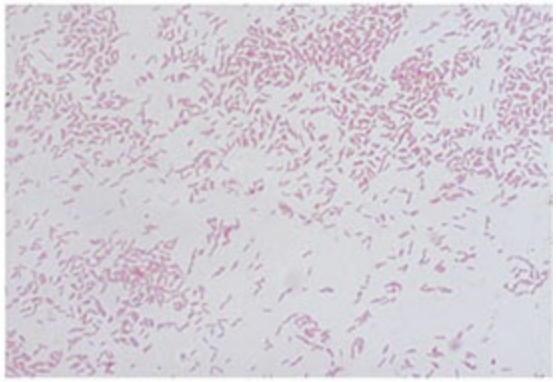

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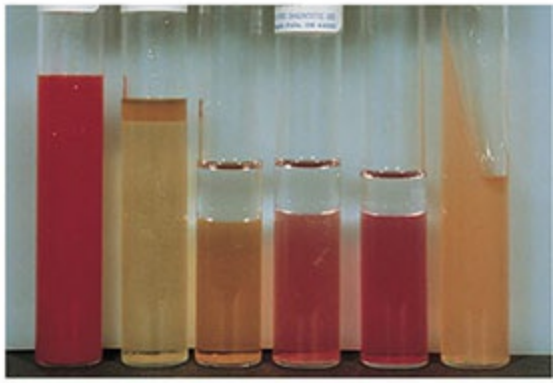


H

IP-52

IMAGE PLATE 9-4

KINGELLA, *CAPNOCYTOPHAGA*, AND *DYSGONOMONAS* SPECIES

A. *Kingella kingae* on sheep blood agar. The plate on the left is after 24 hours of incubation at 35°C to 37°C in 5% to 7% CO_2, while the plate on the right is the same isolate after 48 hours of incubation. The β-hemolysis produced by this organism may be apparent only underneath and immediately adjacent to the colony after 24 hours of incubation, but after 48 hours of incubation, the β-hemolysis is more apparent.

B. Colonies of *Kingella denitrificans* on chocolate agar after 24 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. Colonies of this organism resemble the small colony types of *Neisseria gonorrhoeae*.

C. RapID NH panel (Remel) inoculated with *K. denitrificans*. Two identical panels are depicted in this photograph. The upper panel is before the addition of nitrate and indole reagents to the appropriate bifunctional cuvette wells. Positive reactions on the panel for this oxidase-positive, catalase-negative organism include prolyl aminopeptidase (PRO), glucose fermentation (GLU), and positive nitrite reduction (NO_2), and nitrate reduction (NO_3) tests. All other test results are negative.

D. Human isolate of *Capnocytophaga* species on sheep blood agar after 48 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. This photograph shows the characteristic colony morphology of these organisms and illustrates the fringe of "gliding motility" at the periphery of the colonies. Toward the centers of the areas of growth, the colonies have a mottled appearance.

E. Gram-stained smear of human *Capnocytophaga* species. These organisms characteristically appear as gram-negative, slightly curved, fusiform bacilli with pointed ends. In this respect, these bacteria resemble the obligately anaerobic species *Fusobacterium nucleatum*.

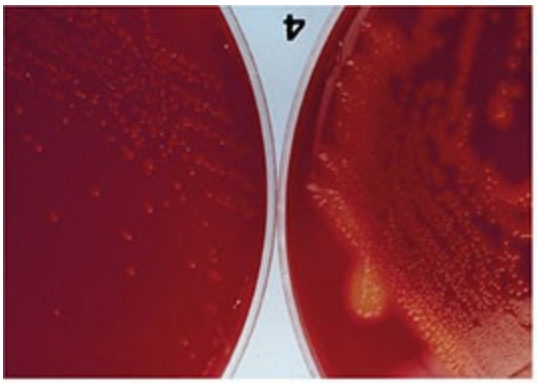
F. Colonies of *Capnocytophaga canimorsus* on sheep blood agar after 5 days of incubation at 35°C in 5% to 7% CO_2. Colonies of this species are entire, circular, convex, and shiny, as shown here.

G. Gram-stained smear of *C. canimorsus*. This photograph shows the fusiform cells typical of the genus. Like the *Capnocytophaga* species that are found in the

human oropharynx, some of the cells of this species are also slightly curved.

H. Colonies of *Dysgonomonas capnocytophagoides* (formerly CDC group DF-3). This organism grows as pinpoint colonies after 24 hours of incubation that subsequently form larger, gray to white colonies. This photograph shows a 48-hour culture of *D. capnocytophagoides* growing on sheep blood agar after incubation at 35°C to 37°C in 5% to 7% CO_2. Colonial growth of this organism has been reported to have a characteristically "sweet" odor.

IP-53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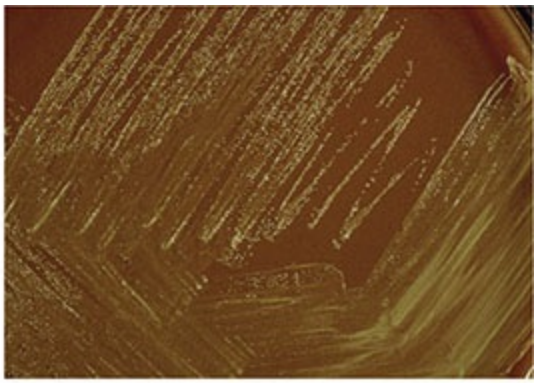

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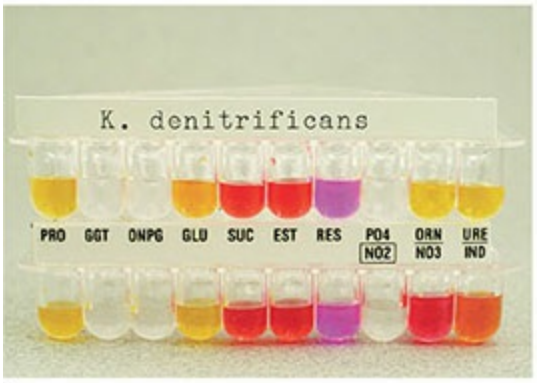
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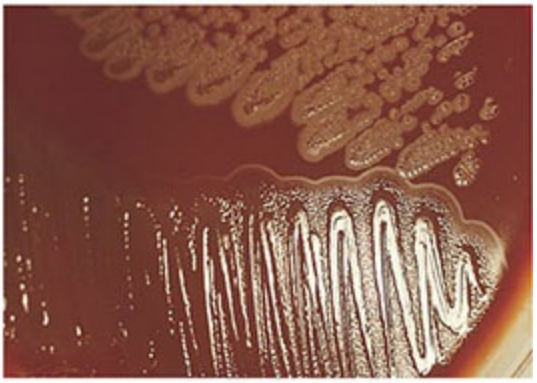

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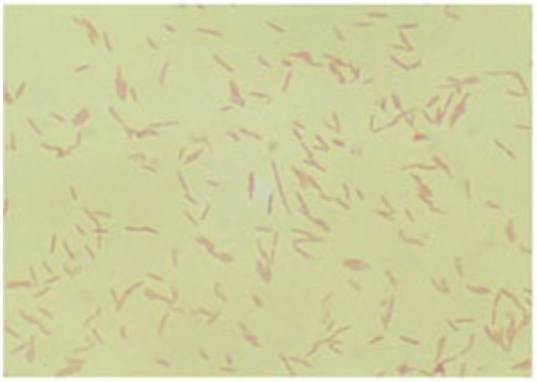

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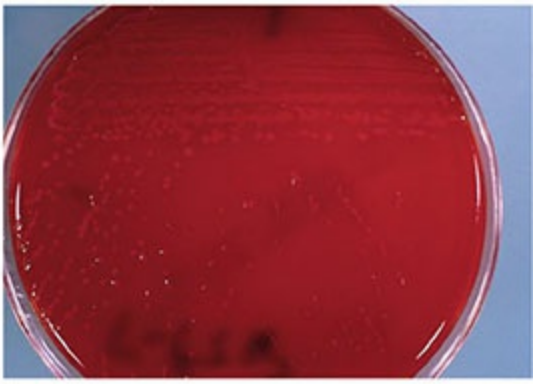

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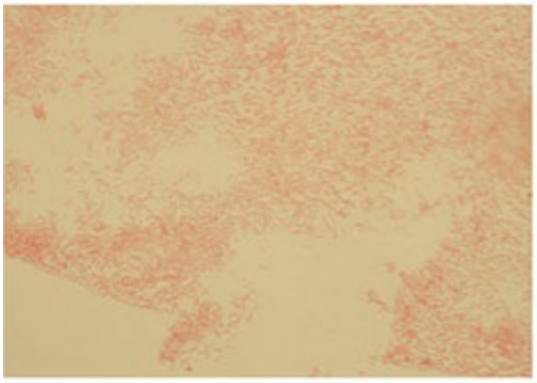

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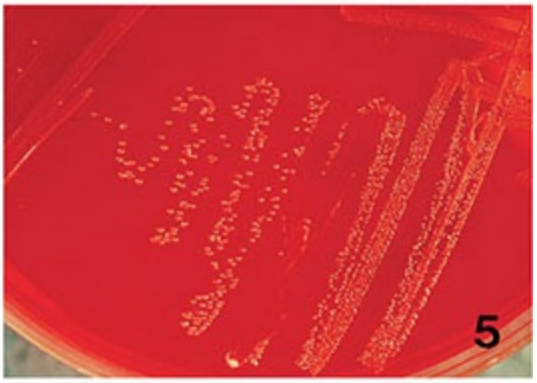

5

H

IMAGE PLATE 9-5

Pasteurella, *Brucella*, and *Bordetella* Species

A. Colonies of *Pasteurella multocida* growing on sheep blood agar after 48 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. The organism is nonhemolytic and does not grow on MacConkey, EMB, or other selective/differential enteric media. *P. multocida* is most frequently isolated from bite wounds from domesticated animals, especially cats and dogs.

B. Gram-stained smear of *P. multocida*, showing the small, gram-negative coccobacilli that are characteristic of this species and other species in the genus *Pasteurella*.

C. Positive spot-indole reaction of *P. multocida*. A piece of filter paper is saturated with a few drops of *p*-dimethylaminocinnamaldehyde reagent, and colony growth of the organism is removed from the agar surface and rubbed onto the reagent along with a negative control organism. The appearance of a teal-blue color on the filter paper is a positive test, whereas the negative control organism results in a pale pink color, as shown here. *P. multocida* is also oxidase-positive.

D. *Brucella melitensis* growing on sheep blood agar after 48 hours of growth in 5% to 7% CO_2-enriched atmosphere at 35°C to 37°C. The colonies are small, nonpigmented, nonhemolytic, entire, and convex.

E. Direct fluorescence antibody (DFA) test for *Bordetella pertussis*. The DFA test is an important adjunct to culture for the detection of *B. pertussis* in nasopharyngeal specimens. This photograph shows a positive DFA preparation, with the organisms appearing as apple-green fluorescent coccobacilli. As demonstrated here, the bacteria may appear singly or in clusters resulting from the trapping of bacteria in mucous strands. (Photo courtesy of Marty Roe, Children's Hospital, Denver, CO.)

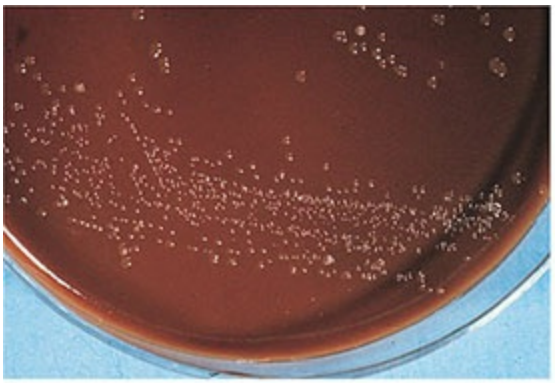
F. Gram-stained smear of *B. pertussis*, showing the characteristic, small, coccobacillary morphology.

G. Growth of *B. pertussis* on Regan-Lowe agar without (left) and with (right) cephalexin incorporated into the medium. The small white colonies present on both plates are colonies of *B. pertussis*, while the large colonies on the medium lacking cephalexin represent contaminant organisms present in the specimen. Culture for pertussis should include media with and without antibiotics, since some strains of

both *B. pertussis* and *B. parapertussis* may be inhibited by cephalexin.

H. Colonies of *B. pertussis* on Regan-Lowe agar after 72 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. This medium contains horse blood (10% w/v) in a charcoal agar base. The high concentration of blood and the incorporation of activated charcoal in the medium help to neutralize any toxic materials that may be present in the specimen and to facilitate recovery of the bacteria.

IP-55



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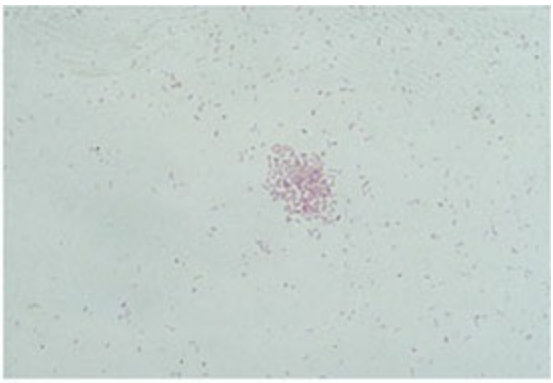

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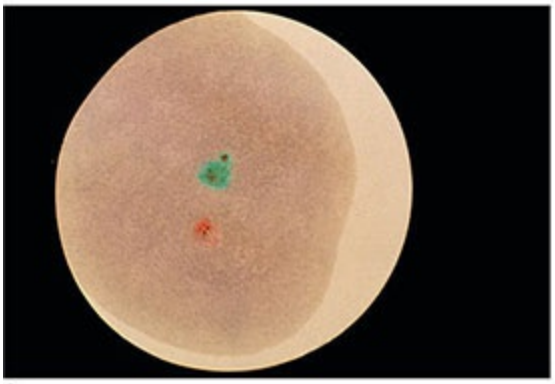

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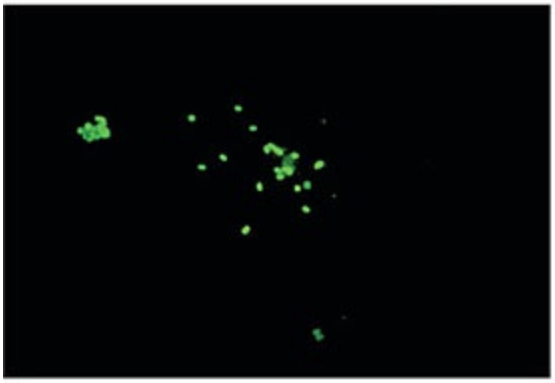

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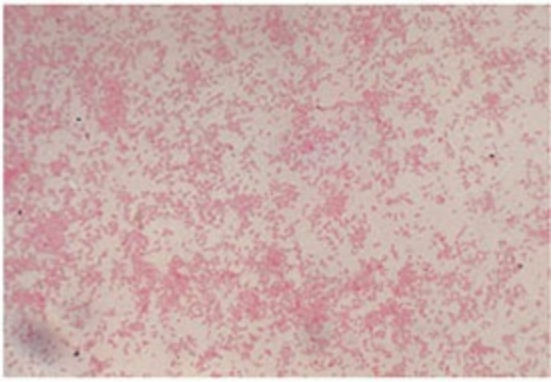

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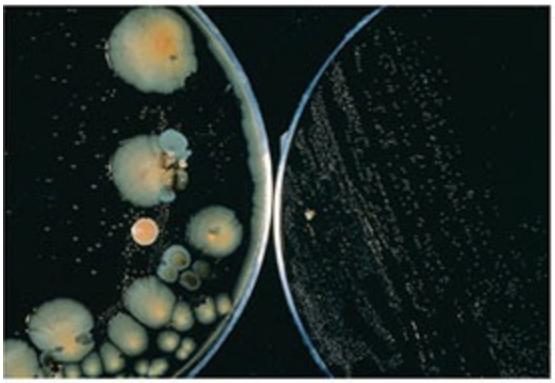

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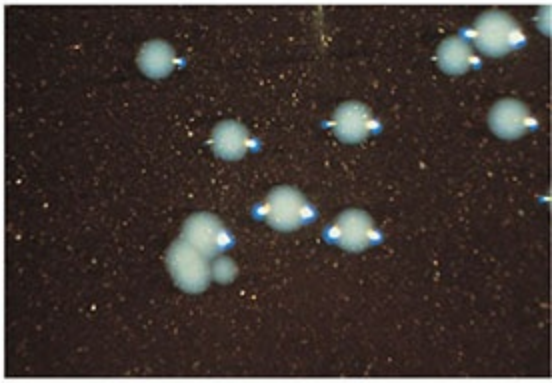


H

IP-56

IMAGE PLATE 10-1

LABORATORY DIAGNOSIS OF LEGIONELLOSIS

A. Gram's stain of *Legionella pneumophila* culture using basic fuchsin instead of safranin for the counterstain. The organisms are thin rods of various lengths.

B. Gram-Weigert-stained touch preparation of open-lung biopsy specimen revealing small, thin intracellular and extracellular bacilli (original magnification, 100× objective lens). Note the short, blunt rods in the macrophage nearest the center of the slide. *L. pneumophila* serogroup 1 was the only organism isolated in culture; a tissue imprint of the lung was DFA-positive when stained with the conjugate specific for this organism.

C. Heavy growth on buffered charcoal yeast extract agar (BCYE) after 3 or more days of incubation and no growth on blood agar are characteristic of *Legionella* species.

D. Dissecting microscopic view of *L. pneumophila* colonies on BCYE. Note the crystalline-like internal structures within 3- to 5-mm colonies that have entire margins (original magnification, approximately 40×).

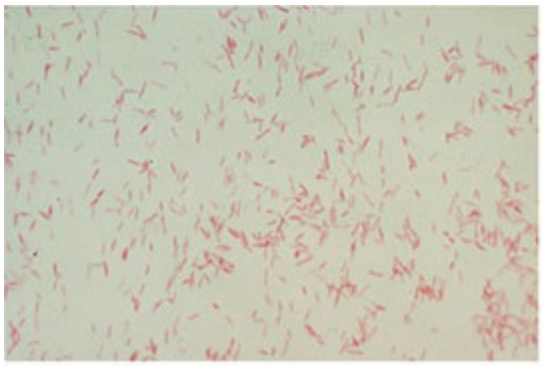
E. Blue-white autofluorescence of *L. bozemanii* on BCYE; photographed under long-wave ultraviolet light. Other species that autofluoresce blue-white include *L. dumoffii*, *L. gormanii*, *L. anisa*, *L. tucsonensis*, *L. cherrii*, *L. parisiensis*, and *L. steigerwaltii*. This characteristic is absent in *L. pneumophila*, *L. micdadei*, *L. feeleii*, *L. longbeachae*, *L. oakridgensis*, and many other *Legionella* species.

F. Modified Kinyoun's acid-fast stain of *L. micdadei* grown on BCYE. Some of the rod-shaped bacteria in the preparation are acid-fast (red), whereas some are not acid-fast (blue). These are partially acid-fast. Using the traditional Ziehl–Neelsen stain, or an auramine–rhodamine stain, they are not likely to be acid-fast (see text for details).

G. Paraffin section of lung tissue from a patient with acute Legionnaires' disease. The section shows an area of consolidation. Inflammatory exudates consisting of fibrin, many neutrophils, few macrophages, and some erythrocytes fill alveolar spaces and alveolar ducts (hematoxylin and eosin stain; original magnification, approximately 200×).

H. This traditional Gram's stain from a legionellosis demonstrates polymorphonuclear leukocytes and macrophages, but there are "no organisms seen." This is typical in the traditional Gram's stain, since legionellae stain poorly with the safranin counterstain (Gram's stain, 1000× magnification).

IP-57



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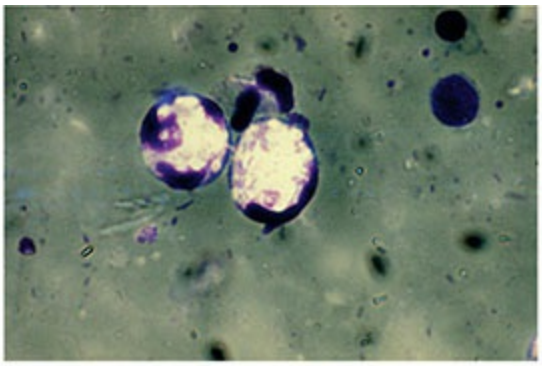

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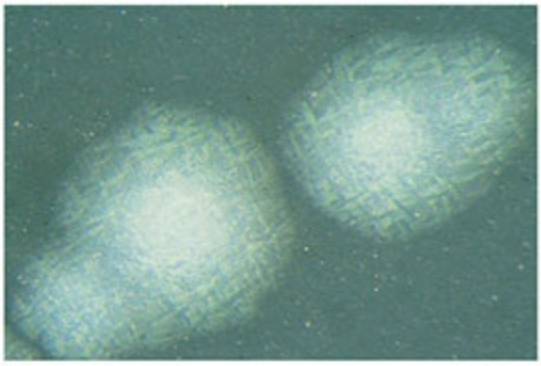

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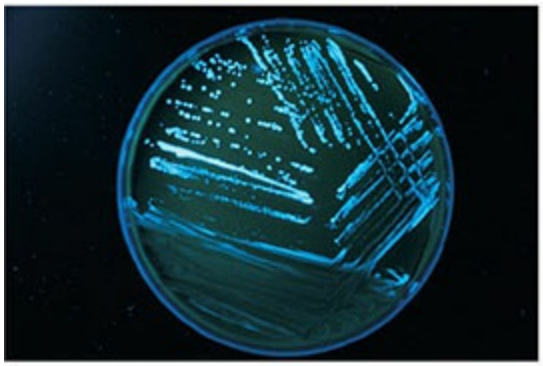

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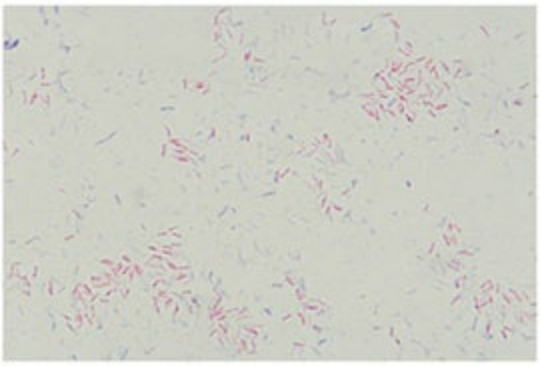

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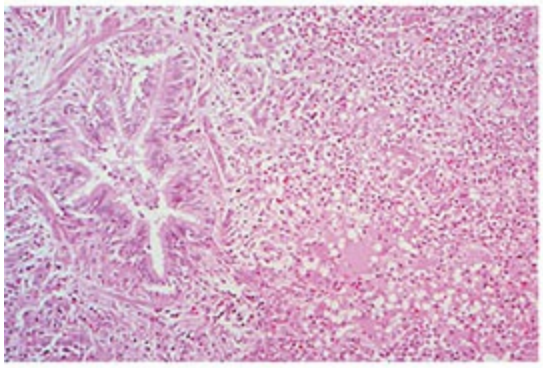

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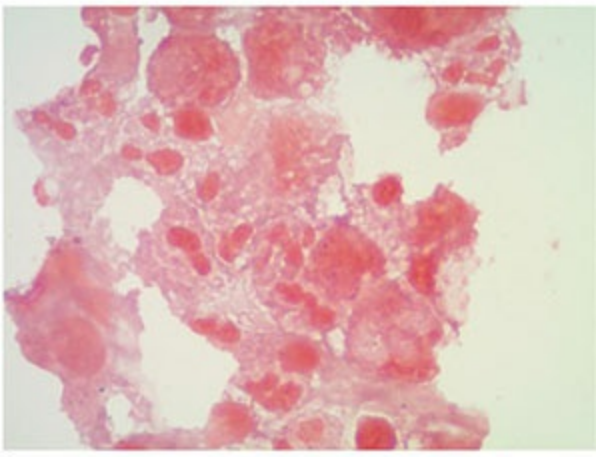


H

IP-58

IMAGE PLATE 11-1

IDENTIFICATION OF *NEISSERIA* SPECIES

A. Gram-stained smear of a urethral discharge from a male with gonococcal urethritis. Note the presence of intracellular gram-negative diplococci within pale-staining segmented neutrophils.

B. Skin lesion of disseminated gonococcal infection (DGI) on the index finger of the left hand. Skin lesions associated with DGI are usually located on the extremities.

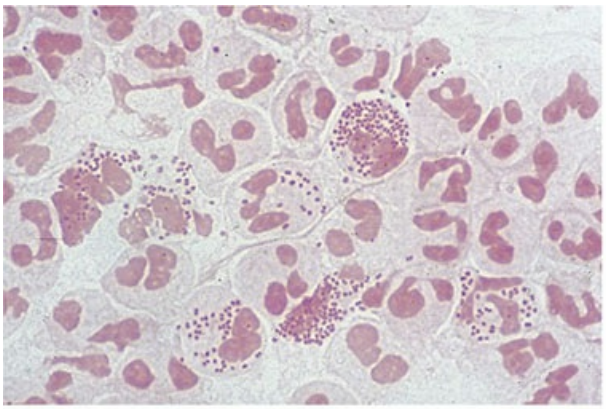
C. Typical colonies of *Neisseria gonorrhoeae* on modified Thayer–Martin (MTM) medium after 24 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. MTM is a chocolate agar–based formulation that contains vancomycin, colistin, and nystatin to inhibit gram-positive, gram-negative, and fungal organisms, respectively. Pathogenic *Neisseria* species, notably *N. gonorrhoeae* and *N. meningitidis*, grow well on MTM medium.

D. Close-up view of colonies of *N. gonorrhoeae* growing on MTM agar after 24 hours of incubation at 35°C to 37°C in 5% to 7% CO_2.

E. Conventional cystine-tryptic digest semisolid agar (CTA) for identification of *Neisseria* species. This photograph shows the typical battery of carbohydrates used for identification. The battery includes (left to right) CTA-glucose, CTA-maltose, CTA-sucrose, and CTA-lactose (the control CTA tube without carbohydrate is not shown). The CTA basal medium contains a phenol red indicator, and a change in the color of the medium from red to yellow indicates acid production from the respective carbohydrate. In this photograph, acid has been produced from glucose only, identifying the organism as *N. gonorrhoeae*.

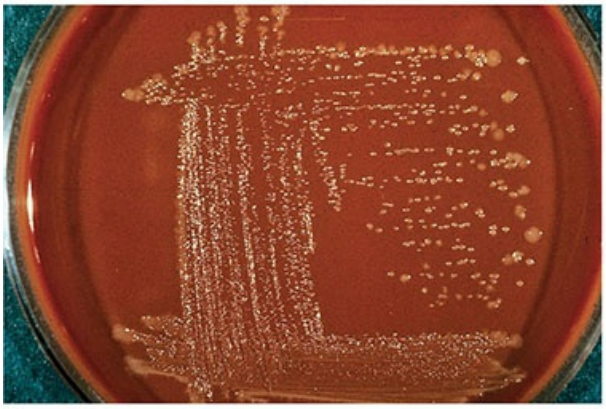
F. Close-up view of colonies of *Neisseria meningitidis* growing on sheep blood agar after incubation for 24 hours at 35°C to 37°C in 5% to 7% CO_2.

IP-59

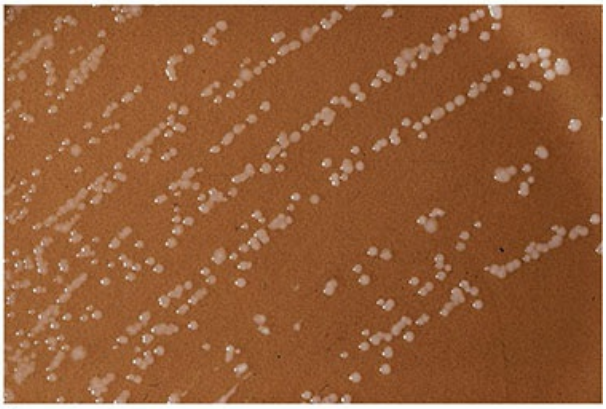


A

B

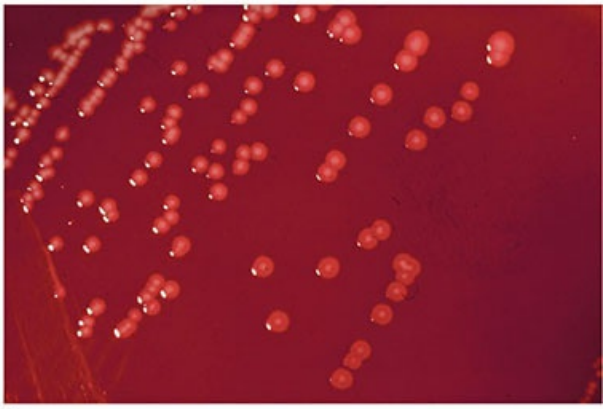


C



D

E



F

IP-60

G. Rapid carbohydrate utilization test for identification of *Neisseria* species (see Chart 11-1). The rapid carbohydrate utilization test is performed using a balanced salts–phenol red solution (BSS, 0.10 mL per tube), with a single drop of carbohydrate (20% w/v aqueous solution, filter-sterilized) added for each carbohydrate tested. A dense suspension of the organism is prepared in BSS

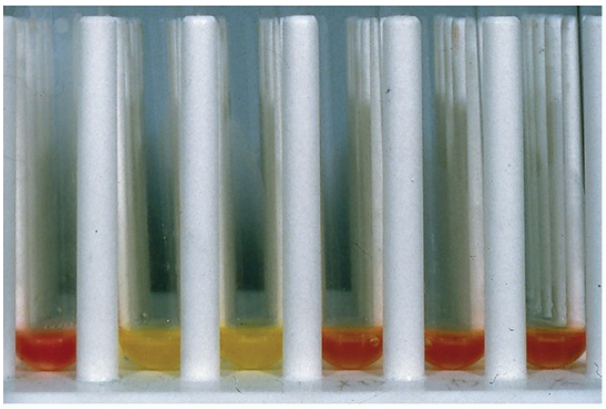
without carbohydrate, and a single drop of this heavy suspension is added to each of the BSS carbohydrate tubes. The inoculated tubes are incubated at 35°C for 4 hours. This photograph shows a set of BSS-carbohydrates composed of (left to right) BSS only (for the organism suspension), BSS-glucose, BSS-maltose, BSS-fructose, BSS-sucrose, and BSS-lactose. Because acid has been produced in the BSS-glucose and BSS-maltose tubes (color change from red to yellow), the organism is identified as *N. meningitidis*.

H. The CarboFerm Neisseria kit consists of an 8-well reagent cuvette that comes packaged as 12 strips snapped into a plastic frame. The frame is labeled with row designations A through H. The wells of the cuvette are sealed with a plastic cap strip, which is removed prior to inoculation. The wells in row A contain dehydrated peptone medium base with phenol red indicator and no added carbohydrates (control wells). The wells in rows C, D, E, and F contain the same medium base with added carbohydrates; these wells contain glucose (wells in row C), maltose (wells in row D), lactose (wells in row E), and sucrose (wells in row F) along with the dehydrated peptone medium and phenol red indicator. The wells in row H contain bromo-chloro-indolyl butyrate (butyrate esterase substrate) impregnated on a filter paper disk at the bottom of the well. The wells in rows B and G are empty. The kit also includes screw-capped test tubes containing inoculation fluid. The kit is designed to identify the gram-negative, oxidase-positive diplococci belonging to the genus *Neisseria* and *Moraxella catarrhalis*.

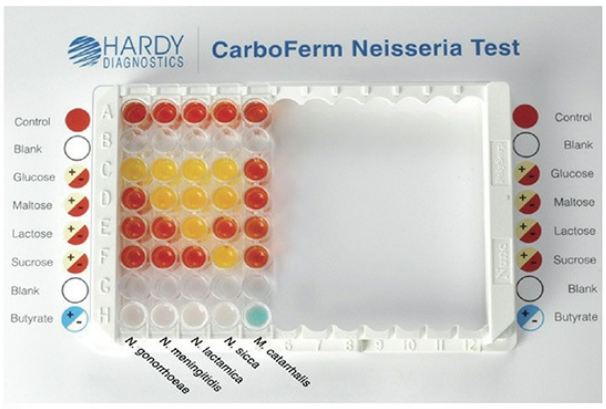
In order to inoculate the CarboFerm Neisseria, an organism suspension equal to or exceeding the turbidity of a No. 4 McFarland standard is prepared in the inoculation buffer. This inoculum is prepared from an 18- to 24-hour culture of the organism on chocolate, blood, or selective media (e.g., modified Thayer–Martin medium). Four to five drops of the inoculum are placed in wells A (control), C (glucose), D (maltose), E (lactose), F (sucrose), and H (butyrate esterase substrate). The cuvette is placed in an ambient air incubator at 35°C.

After 15 minutes, well H (the butyrate esterase substrate) is examined for the presence of a blue color. If the filter paper disk is teal-blue in color, the organism is identified as *M. catarrhalis*, and testing is completed. If the disk in well H is colorless (white), the butyrate esterase reaction is recorded as negative, and the cuvette is re-incubated for a total of 4 hours. At this time, the carbohydrate control and each of the carbohydrate wells are inspected for color changes. A red or red-orange color is recorded as negative, while orange, yellow-orange, and yellow are recorded as positive.

IP-61



G



H

IP-62

IMAGE PLATE 11-2

IDENTIFICATION OF *NEISSERIA* SPECIES AND *MORAXELLA CATARRHALIS*

A. GONOCHEK II (EY Laboratories, San Mateo, CA). Three chromogenic substrates are included in a single plastic tube to detect glycosidase and aminopeptidase enzymes specifically found in *N. meningitidis*, *N. lactamica*, and *N. gonorrhoeae*. Hydrolysis of substrates in the tube results in the small inoculum volume turning various colors. Identification patterns observed are: blue (upper left)—*N. lactamica* (β-galactoside hydrolysis); yellow (upper right)—*N. meningitidis* (γ-glutamyl- *p*-nitroanilide hydrolysis); and red (lower left)—*N. gonorrhoeae* (prolyl-naphthylamide hydrolysis). Lack of a color reaction (**lower** right) following the 30-minute incubation presumptively identifies the organism as *Moraxella catarrhalis*.

B. BactiCard *Neisseria* (Remel Laboratories, Lenexa KS). This identification strip contains four chromogenic enzyme substrate tests for identification of the pathogenic *Neisseria* species and *Moraxella catarrhalis*. After hydration of each of the four test circles with a drop of buffer, growth from selective media or a suitable subculture is applied onto each of the four test areas. If a blue-green color develops in the IB (butyrate esterase) test area within 2 minutes (strip at left), the organism is identified as *M. catarrhalis*. If no color develops in this area, the strip is incubated for an additional 13 minutes. If a blue-green color develops in the BGAL (β-galactosidase) test area (strip at far right) during this time, the organism is identified as *N. lactamica*. If no color reaction develops in this area during the incubation period, a single drop of color-developing reagent is added to the PRO and GLUT test areas. The appearance of a red color in the PRO (prolyl

aminopeptidase) test area identifies the isolate as *N. gonorrhoeae* (second strip from the left), whereas the development of a red color in the GLUT (γ-glutamyl aminopeptidase) test area identifies the isolate as *N. meningitidis* (third strip from the left)

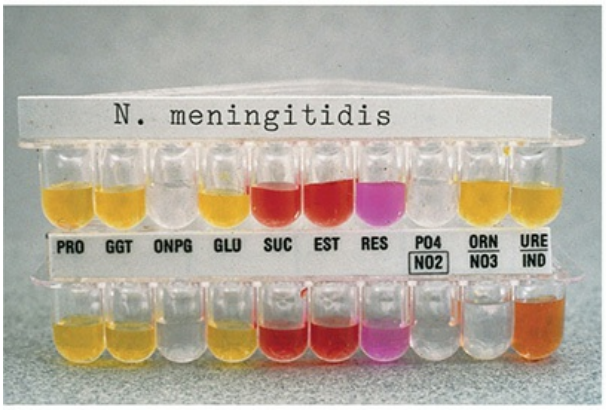
C. RapID NH system (Remel). The RapID NH system is a 4-hour commercial system for identification of *Neisseria* species, *Haemophilus* species, and several other species of fastidious gram-negative bacteria. The photograph actually shows two duplicate panels inoculated with *N. meningitidis*. The top panel in the photo is the system prior to the addition of reagents, while the bottom panel is the system after the addition of reagents to the last three bifunctional test wells. The reactions that identify the isolate as *N. meningitidis* are the positive prolyl aminopeptidase (PRO) and γ-glutamyl aminopeptidase (GLUT) reactions, the positive glucose (GLU) reaction, and the positive NO_2 (reduction of nitrite) test.

D. MicroScan HNID panel (Dade-MicroScan, West Sacramento, CA). The MicroScan HNID panel is a 4-hour test panel for the identification of *Haemophilus* and *Neisseria* species, Positive tests on the panel shown in the photograph include the nitrate reduction (NO_3) and nitrite reduction (NO_2) tests, production of acid from glucose (GLU), sucrose (SUC), maltose (MAL), fructose (FRU), and prolyl aminopeptidase (PRO). These characteristics identify this isolate as *N. mucosa*.

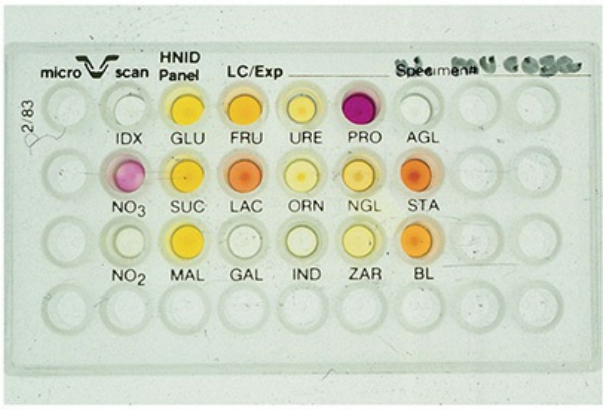
IP-63

A

B



C



D

IP-64

E. API NH panel (bioMérieux, Durham, NC). The API NH is a strip format identification system for the 2-hour identification of *Neisseria* and *Haemophilus* species. The strip includes seven single-substrate cupules and three bifunctional cupules. The single-substrate tests include (left to right), β-lactamase-test (PEN), acid production from glucose (GLU), fructose (FRU), maltose (MAL), sucrose (SAC), ornithine decarboxylase (ODC), and urease (URE). The bifunctional cupules include butyrate esterase plus prolyl aminopeptidase (LIP/PRO A), alkaline phosphatase (PAL) plus γ-glutamyl aminopeptidase (PAL/GGT), and β-galactosidase plus indole (GAL/IND). The entire strip is read first, including the LIP, PAL, and GAL tests, and then a color development reagent is added to the PRO A and GGT wells, and indole reagent is added to the IND well. These last three cupules are then reread to provide the fourth number of a four-digit biocode. The carbohydrate cupules, LIP, PRO A, GGT, and GAL address the identification of *Neisseria* species and *Moraxella catarrhalis*, while the carbohydrate cupules, ODC, URE, PAL, GAL, and IND are used for identifying and biotyping *Haemophilus* species. In this photograph, the GLU and PRO A tests are positive,

providing a biocode of 1001. Consultation with the API NH database provides an identification of *N. gonorrhoeae*. The PEN well is an acidometric β-lactamase detection cupule that is not used for identification purposes. The PEN test is positive here, indicating that the isolate is β-lactamase positive.

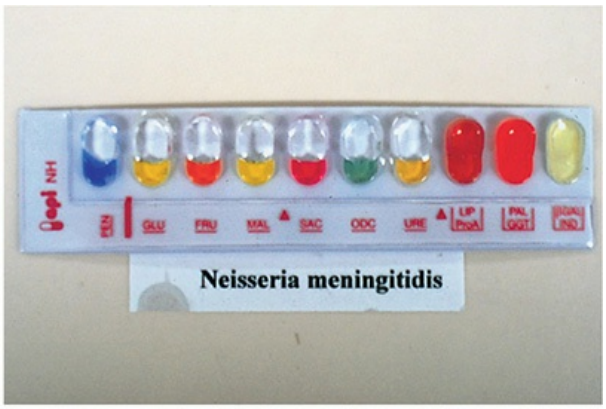
F. API NH inoculated with *N. meningitidi* s. Positive tests on the strip include glucose (GLU), maltose (MAL), prolyl aminopeptidase (PRO A), and GGT (γ-glutamyl aminopeptidase). These reactions result in an API biocode of 5003, which provides an identification of *N. meningitidis*. This isolate is β-lactamase-negative, as indicated by the blue color in the PEN cupule.

G. The M.cat butyrate disk test (Carr-Scarborough Microbiologicals, Decatur, GA). *M. catarrhalis* strains produce a butyrate esterase enzyme that is able to hydrolyze indoxyl butyrate. An indoxyl butyrate-impregnated filter paper disk is moistened with water, and growth from a colony is rubbed onto the disk. The butyrate esterase enzyme hydrolyzes the compound and a blue-green color appears on the disk within 2 minutes. This figure shows three positive M. Cat. butyrate disks on a glass slide.

H. API NH inoculated with *M. catarrhalis*. In this photograph, the only positive test is the LIP cupule (blue is a positive reaction). The API biocode for these reactions is 0010, which identifies it as *M. catarrhalis*. The PEN cupule is not used to determine the API biocode, but only to detect β-lactamase production. The blue color in the PEN cupule indicates that this strain is β-lactamase.

IP-65

E



F

G

H

IP-66

IMAGE PLATE 12-1

IDENTIFICATION OF STAPHYLOCOCCI AND RELATED SPECIES

A. Adult patient with staphylococcal cellulitis of the upper lip.

B. Adult patient with the bullous, vesicular lesions of facial staphylococcal impetigo. Culture of the serosanguinous fluid within these vesicles reveals large numbers of *Staphylococcus aureus*.

C. Neonate with staphylococcal scalded skin syndrome. This syndrome is seen in newborns infected with *S. aureus* strains that produce exfoliatins (i.e., epidermolytic toxins). These toxins dissolve the mucopolysaccharide matrix of the epidermis, resulting in intraepithelial splitting of the cellular linkages in the stratum granulosum. Bullous formation occurs over large areas of the body, with subsequent sloughing of the superficial skin layers, as shown here.

D. Gram-stained smear from a lesion of staphylococcal cellulitis. The organisms appear as small extracellular gram-positive cocci arranged in clusters along with the pink-staining polymorphonuclear leukocytes.

E. Colonies of *S. aureus* on sheep blood agar. This photograph shows typical colonies of *S. aureus* after 24 hours of incubation at 35°C to 37°C. A zone of β-hemolysis is seen immediately surrounding the colonies.

F. Gram-stained smear from a broth culture of *S. aureus*, showing the typical gram-positive cocci in grape-like clusters.

G. Positive catalase test. The catalase test differentiates staphylococci and micrococci from the streptococci, enterococci, and the *Streptococcus*-like bacteria (Chapter 13). The test is performed by placing growth from a colony onto a glass slide and adding a drop of 3% hydrogen peroxide to the inoculum. Immediate and vigorous bubbling owing to the production of oxygen gas, as shown here, indicates a positive test. Micrococci and staphylococci are catalase-positive, whereas streptococci and enterococci are catalase-negative.

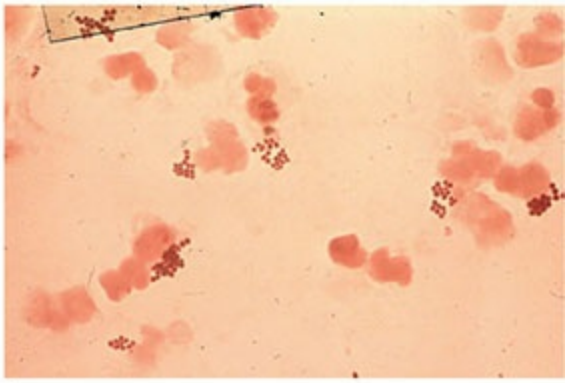
H. Colonies of *Micrococcus luteus* on sheep blood agar. This species produces yellow-pigmented colonies. Other micrococci and organisms in related genera may be nonpigmented or may produce orange- or red-pigmented colonies on agar media. Micrococci and related organisms may be differentiated from staphylococci by resistance to furazolidone, susceptibility to bacitracin, or other methods as shown in Table 12-1.

IP-67

A

B

C



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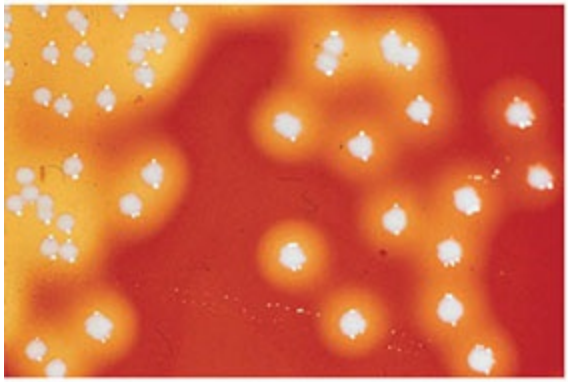

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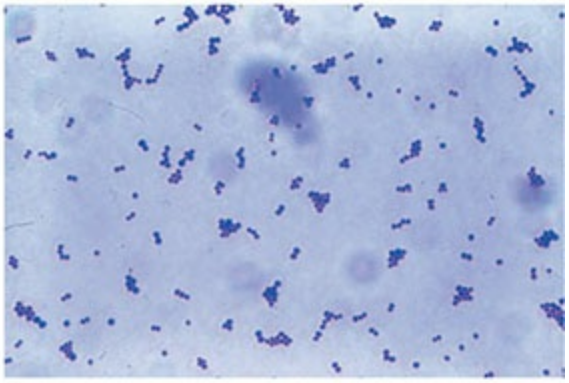

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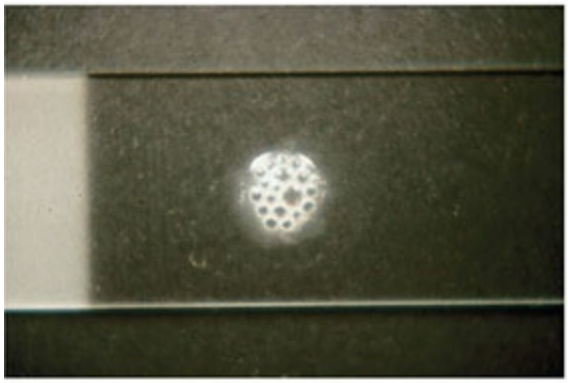

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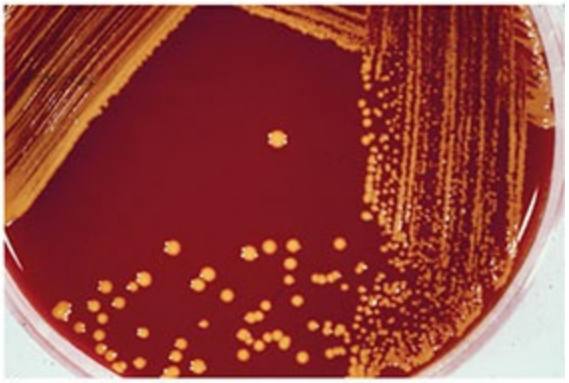


H

IP-68

IMAGE PLATE 12-2

IDENTIFICATION OF STAPHYLOCOCCI

A. Furazolidone disk test with a *Micrococcus* species. Several tests are available for differentiating micrococci and related genera from staphylococci. The furazolidone susceptibility test is a reliable overnight method for making this differentiation. Micrococci are resistant to furazolidone and will usually grow right up to the edge of the 6-mm furazolidone disk.

B. Furazolidone disk test with a *Staphylococcus* species. The growth of staphylococci is inhibited by furazolidone and will show an area of growth inhibition around the disk, as depicted in this figure.

C. Modified oxidase test. This test is a rapid, 30-second method for differentiating staphylococci from micrococci and related species. The oxidase reagent (tetramethyl- *p*-phenylenediamine dihydrochloride) is made up of dimethyl sulfoxide (DMSO), which allows the reagent to penetrate the staphylococcal cell wall. The test is performed by rubbing a portion of colony growth from a plate onto a filter paper disk that is impregnated with the reagent. Most micrococci and related species are modified oxidase-positive, producing a blue-purple color on the disk within 30 seconds, whereas most *Staphylococcus* species are modified oxidase-negative.

D. Slide coagulase test. This test is a rapid method for identifying *S. aureus*. Most strains of this species produce a cell-bound coagulase or "clumping factor" that is detected by mixing a saline suspension of the organism with EDTA-rabbit plasma. A saline control (left) must be included with each test to assess autoagglutination. Not all *S. aureus* strains possess clumping factor, so negative slide coagulase tests must be confirmed with the tube coagulase test for colonies with a morphology suggestive of *S. aureus* (Image Plate 12-2E).

E. Tube coagulase test. In this test, extracellular coagulase produced by *S. aureus* forms a complex with a component in plasma, called coagulase-reacting factor. This complex, in turn, reacts with fibrinogen in the plasma to form fibrin and, consequently, the development of a visible clot in the tube. A positive test is shown in the tube at the bottom of the figure, whereas a negative test is shown in the tube at the top of the figure.

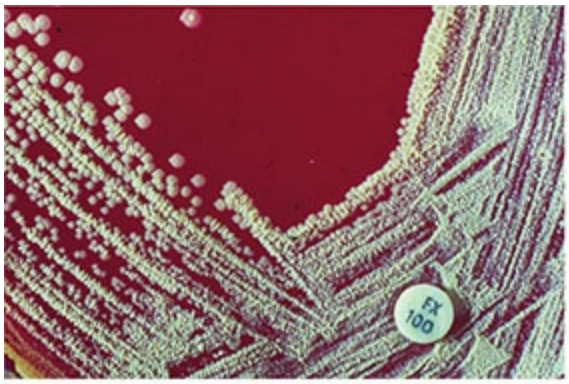
F. Latex agglutination test for identification of *S. aureus*. This alternative coagulase

test procedure uses latex beads that are coated with plasma. Fibrinogen bound to the latex detects clumping factor, and the immunoglobulins, also present on the latex, detect protein A on the surface of the *S. aureus* cell. Newer formulations of these tests also contain latex beads coated with antibodies against *S. aureus* capsular types 5 and 8, which enable more reliable detection of oxacillin-resistant *S. aureus* strains. Mixing of colonial growth of *S. aureus* with the latex reagent results in rapid agglutination (left). A coagulase-negative *Staphylococcus*, which produces a negative latex agglutination reaction, is also shown (right).

G. DNase medium with toluidine-blue. This medium detects the production of deoxyribonuclease by *S. aureus*. The organisms are spot-inoculated heavily onto the agar surface and the plate is incubated at 35°C to 37°C for 24 hours. A positive test is indicated by the appearance of a pink color under and around the inoculum. As shown here, *S. aureus* ("SA" on plate) is DNase-positive, while the control (*S. saprophyticus*, "SS" on plate) is DNase-negative. The DNase test serves as a helpful adjunct test for *S. aureus* strains that have weak or equivocal coagulase test results.

H. API ID32 Staph system (bioMérieux) for identification of staphylococci and micrococci. This strip-format identification system contains 26 biochemical substrates. Positive and negative reactions are recorded to generate a biotype number that is used along with a computerized database (apiweb) to identify the organism.

IP-69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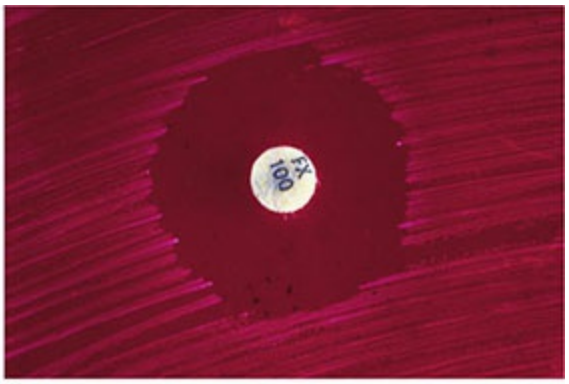
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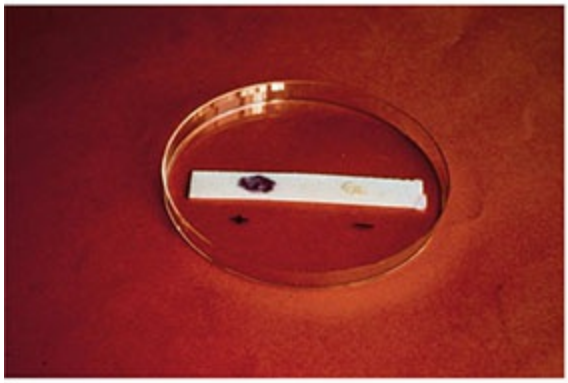

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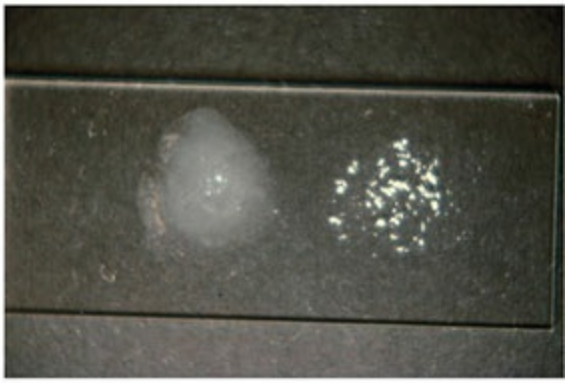

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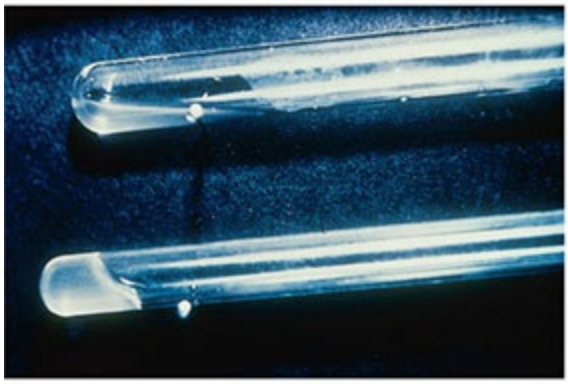

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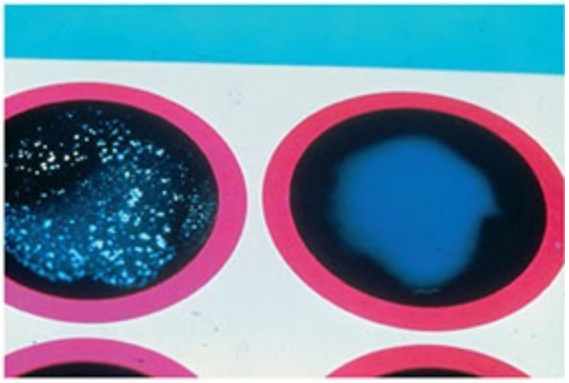

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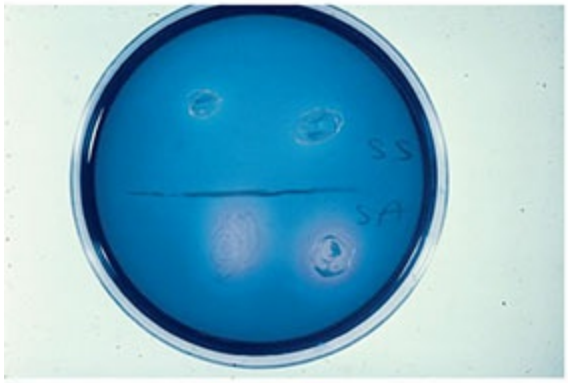

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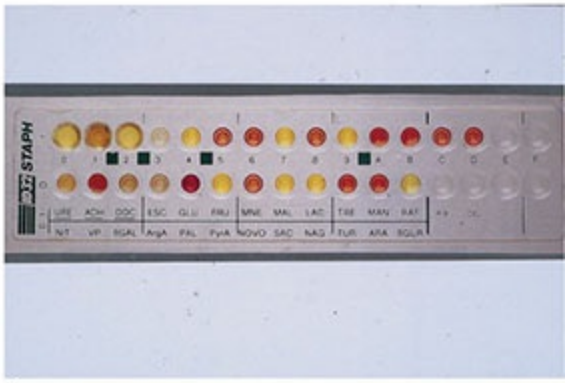

STAPH
URE ACH DOC ESC GLU FRU MNE MAL LAC TRE MAN RAF
NIT VP BGAL ArgA PAL PyrA NOVO SAC NAG TUR ARA SQLR

H

IMAGE PLATE 12-3

IDENTIFICATION OF STAPHYLOCOCCI (CONTINUED)

A. Colonies of *Staphylococcus epidermidis* on sheep blood agar (SBA) after 24 hours of incubation at 35°C to 37°C.

B. *S. saprophyticus* on SBA with furazolidone and novobiocin disks. *S. saprophyticus* is a cause of urinary tract infections in young women. Like other staphylococci, this species is susceptible to furazolidone (FX), as shown by the zone of inhibition around the FX disk. The other disk (designate c-) on this slide is a novobiocin disk. *S. saprophyticus* is one of several *Staphylococcus* species that is resistant to novobiocin.

C. API Staph identification panel inoculated with *S. saprophyticus*. This picture shows the reactions on the first half of the panel. The interpretations of the test reactions shown are as follows:

CHO Control	Negative	GLU	Positive	FRU	Positive
MNE	Negative	MAL	Positive	LAC	Positive
TRE	Positive	MAN	Positive	XLT	Negative
MEL	Negative				

D. API Staph identification panel inoculated with *S. saprophyticus*. This picture shows the second half of the panel. The interpretations of the test reactions shown are as follows:

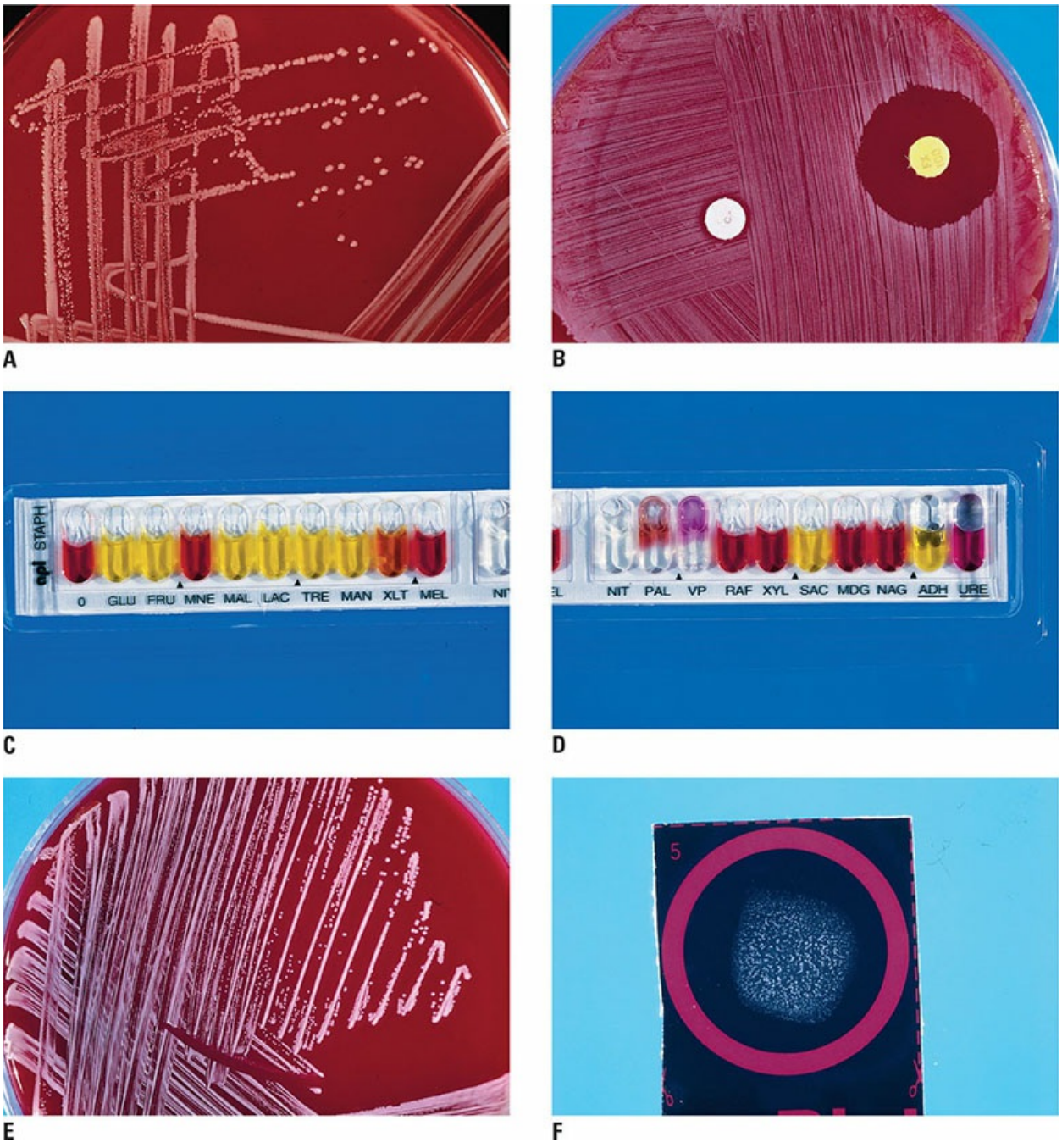
		NIT	Negative	PAL	Positive
VP	Positive	RAF	Negative	XYL	Negative
SAC	Positive	MDG	Negative	NAG	Negative
ADH	Negative	URE	Positive		

The API biocode numbers for these reactions, shown in Image Plate 12-3C and D, is 6634112. Consultation with the computer-assisted API Staph database provides a list of possible identifications, including *S. saprophyticus* (71.9% likelihood), *S. warneri* (19.3% likelihood), or *S. hominis* (6.6% likelihood). Since *S. saprophyticus* is the only species that is novobiocin-resistant, the organism can be identified as *S. saprophyticus*.

E. This slide shows an isolate growing on sheep blood agar after incubation at 35°C to 37°C for 24 hours. This isolate was recovered from several blood cultures of a 38-year-old patient with presumed staphylococcal endocarditis. This isolate is nonpigmented and nonhemolytic, and was also furazolidone- and novobiocin-susceptible (not shown).

F. This photograph shows the latex agglutination reaction of the isolate from Image Plate 12-3E. The isolate is coagulase-positive by the latex test. Since the isolate did not "look" like *S. aureus*, the technologist inoculated an API Staph identification strip. These reactions are shown in Image Plate 12-3G and H.

IP-71



IP-72

G. API Staph identification strip inoculated with the blood culture isolate shown in Image Plate 12-3E. This photograph shows the reactions on the first half of the strip. The interpretations of these test reactions are as follows:

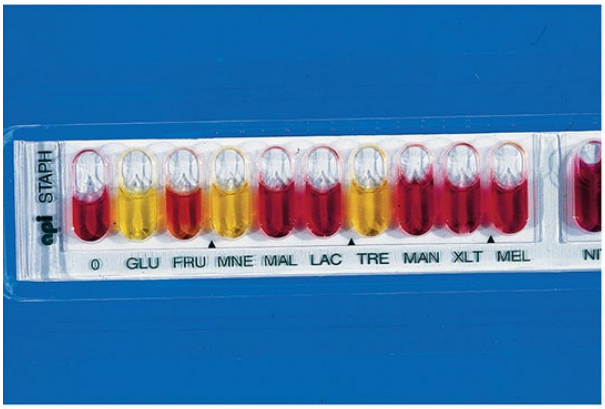
CHO Control	Negative	GLU	Positive	FRU	Negative
MNE	Positive	MAL	Negative	LAC	Negative
TRE	Positive	MAN	Negative	XLT	Negative
MEL	Negative				

H. API Staph identification strip inoculated with the blood culture isolate from Image Plate 12-3E. This photograph shows the reactions on the second half of the strip. The interpretations of these test reactions are as follows:

	NIT	Positive	PAL	Positive
VP Positive	RAF	Negative	XYL	Negative
SAC Negative	MDG	Negative	NAG	Positive
ADH Positive	URE	Negative		

The API biocode for the reactions shown in Image Plate 12-3G and H is 2116141. Consultation with the API Staph computer-assisted database provides an identification of this isolate as *S. schleiferi*. This is consistent with the positive latex reaction shown in Image Plate 12-3F, as this species can be slide, latex, and/or tube coagulase-positive.

IP-73



G

H

IP-74

IMAGE PLATE 13-1

IDENTIFICATION OF STREPTOCOCCI

A. Patient with erysipelas due to group A β-hemolytic streptococci. This infection

involves the soft tissues and cutaneous lymphatics and results in systemic manifestations of infection, including fever. Note the bullous lesions adjacent to the chin.

B. Gram-stained smear of streptococci growing in a broth culture. As their name suggests, streptococci characteristically grow in chains. These chains are most frequently seen when the organisms are grown in broth. On Gram-stained smears prepared from growth on agar media, the organisms usually appear in pairs or in shorter chains.

C. Gram-stained smear of *Streptococcus pneumoniae*. This photograph shows the typical appearance of pneumococci in blood culture broth. These bacteria characteristically grow in pairs in which the cells have a slightly elongated "lanceolate" morphology. With some cells in this photograph, a clear area or "halo" may be observed surrounding the organism pairs, indicating the presence of the polysaccharide capsule of *S. pneumoniae.*

D. β-Hemolytic streptococci on sheep blood agar. β-Hemolytic streptococci produce hemolysins that lyse the sheep erythrocytes, resulting in a clearing of the medium surrounding the colonies. The group A *Streptococcus* shown here demonstrates this type of hemolysis, as do groups B, C, and G streptococci. The zones of β-hemolysis surrounding colonies of group B streptococci, however, are not as large relative to the size of the colony as those seen with groups A, C, and G β-hemolytic streptococci.

E. α-Hemolytic streptococci on sheep blood agar. Streptococci initially may be classified on the basis of their hemolytic properties on sheep blood agar. Partial hemolysis of the erythrocytes results in a "greening" of the agar medium surrounding the colonies (α-hemolysis). Streptococci that are β-hemolytic include *S. pneumoniae*, the viridans group of streptococci, and most *Enterococcus* (formerly *Streptococcus*) species.

F. α-Hemolytic streptococci on sheep blood agar. More intense β-hemolysis is noted in areas where the medium has been "stabbed," pushing some of the bacteria under the medium surface. The hemolysis in these areas is due to the combined activities of streptolysin O and streptolysin S, the principal hemolysins of group A streptococci. Streptolysin O is oxygen-labile and does not show maximal activity on the surface of the agar; the surface β-hemolysis is largely due to streptolysin S, which is oxygen-stable.

G. Direct latex agglutination test for group A streptococci. Both latex agglutination and rapid enzyme immunoassays are currently available for the direct detection of group A streptococci in throat swab specimens. With the latex agglutination method

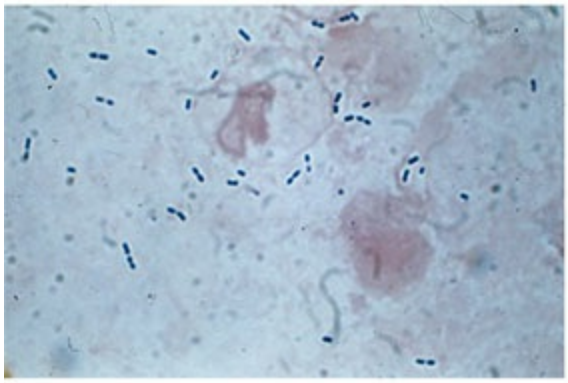
shown here, the throat swab is enzymatically treated to extract the group A antigen and the extract is reacted with a latex bead suspension coated with anti–group A streptococcal antibodies and with a control, uncoated latex suspension. Agglutination of the latex with the test latex reagent (left) but not the control latex (right) is a positive test. Although most of these tests are highly specific for the group A cell wall antigen, the sensitivities of these assays vary widely.

H. Strep OIA (Biostar, Boulder, CO). The Strep OIA is an immunoassay for direct detection of group A streptococci in throat swab specimens. The swab is extracted with acetic acid, neutralized, and then mixed with anti–group A antibodies bound to horseradish peroxidase (HRP). A drop of this mixture is placed on the surface of an OIA slide, which is coated with anti–group A streptococcal antibodies. After a 2-minute incubation, the slide is rinsed and HRP substrate is added and allowed to react on the slide for 4 minutes. After rinsing, the slide is read by examining the hue of light reflected from the reaction area on the OIA slide. If group A streptococcal antigen is present, the slide shows a purple spot (left). If no antigen is present, the slide surface retains its gold color, with or without a tiny blue dot, as shown here (right).

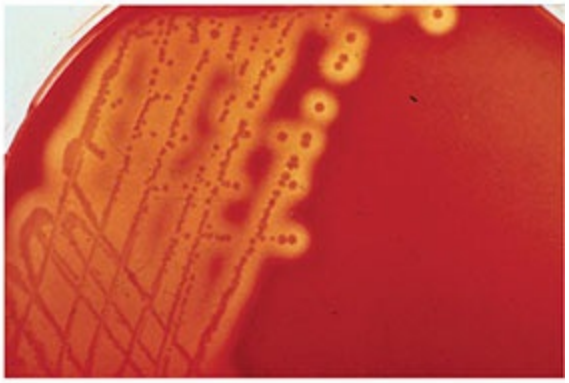
IP-75

A

B



C



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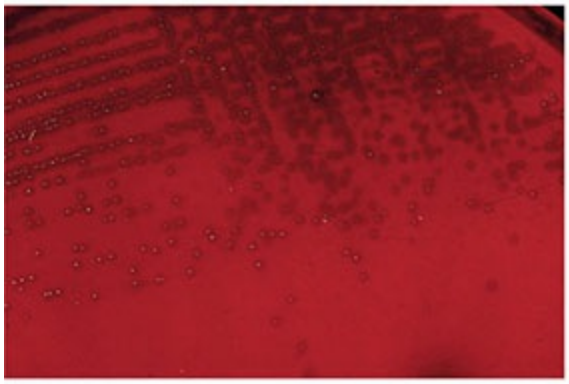
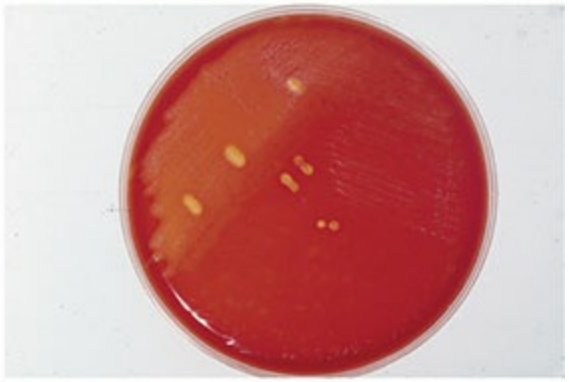
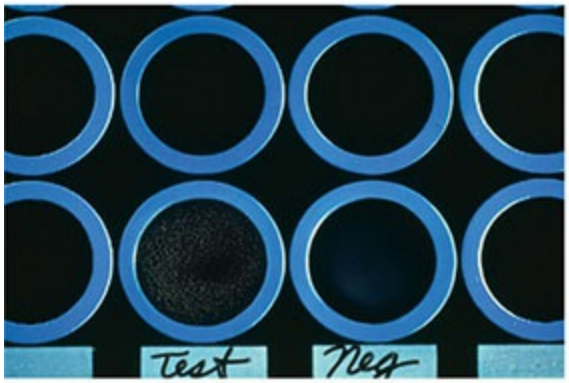


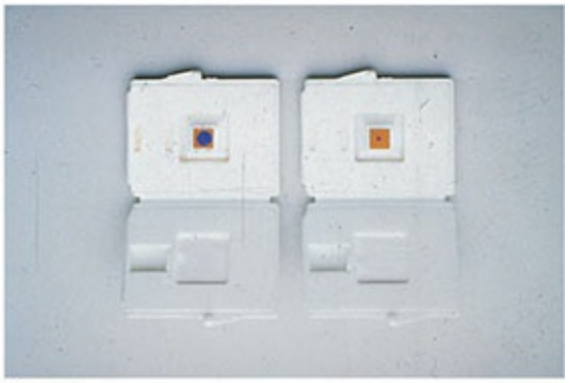
E



F



G



H

IMAGE PLATE 13-2

IDENTIFICATION OF STREPTOCOCCI AND ENTEROCOCCI

A. *S. pneumoniae* colonies on sheep blood agar. Two characteristics of *S. pneumoniae* can be used for presumptive identification. On the left is shown a typical α-hemolytic, mucoid strain of *S. pneumoniae* growing on sheep blood agar. Its appearance is due to the production of large amounts of capsular polysaccharide. On the right is a close-up photograph illustrating the collapse of the central portion of the colonies owing to organism autolysis, resulting in the so-called checker-piece and nail-head colony morphologies shown here.

B. Bacitracin susceptibility test. Group A streptococci are susceptible to low concentrations of bacitracin. The Taxo A bacitracin disk (BD Microbiology Systems) shown here contains 0.04 unit of bacitracin. This photograph shows the bacitracin test reactions for a group A β-hemolytic *Streptococcus* (left) and a group B β-hemolytic *Streptococcus* (right). Any zone of inhibition around the bacitracin disk is considered a positive test.

C. Susceptibility to bacitracin and sulfamethoxazole-trimethoprim. The β-hemolytic *Streptococcus* shown here is susceptible to bacitracin (right), but also shows a large zone of growth inhibition around the sulfamethoxazole-trimethoprim (SXT) disk (left). Group A β-hemolytic streptococci are susceptible to bacitracin, but are resistant to SXT, while group B β-hemolytic streptococci are resistant to both bacitracin and SXT. Some non–group A β-hemolytic streptococci (e.g., groups C, F, and G) are susceptible to bacitracin, but will also be susceptible to SXT, as shown here. Therefore, the performance of these two tests together increases the specificity of the presumptive identification that would be obtained by using the bacitracin test alone. This isolate would be called "presumptive β-hemolytic *Streptococcus*, not groups A or B, by bacitracin/SXT."

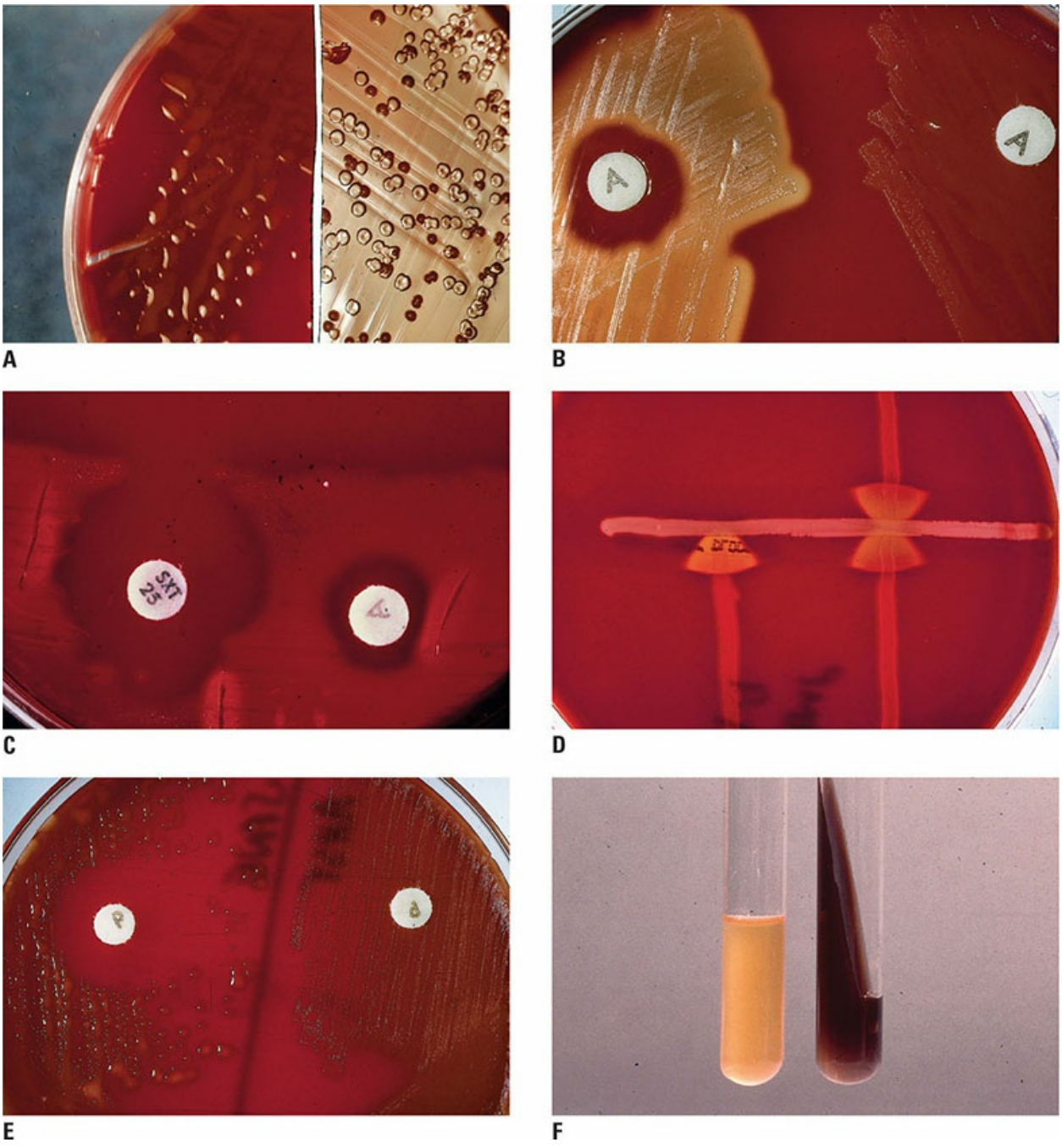
D. CAMP test for presumptive identification of group B β-hemolytic streptococci. The CAMP test reaction depends on the interaction between CAMP factor, a product of the group B *Streptococcus*, with the β-hemolysin of *Staphylococcus aureus*. Possible group B streptococci are streaked at right angles to a streak of *S. aureus* (without the streaks touching) on a sheep blood agar plate. Following overnight incubation at 35°C to 37°C, an arrowhead-shaped area of synergistic hemolysis is found in the intersecting area where the CAMP factor and the β-hemolysin have diffused into the medium. Nonhemolytic variants of group B

streptococci will also be CAMP test-positive. This photograph shows three positive CAMP tests.

E. Susceptibility to optochin for identification of *S. pneumoniae*. Susceptibility to ethyl hydrocuprene hydrochloride (optochin) is used to differentiate *S. pneumoniae* from other viridans streptococci. α-Hemolytic colonies are subcultured onto a sheep blood agar plate and an optochin disk (P disk; BD Microbiology Systems) is placed on the inoculum. The presence of a zone of growth inhibition of 14 mm around a 6-mm optochin disk after overnight incubation at 35°C to 37°C identifies the organism as *S. pneumoniae*.

F. Bile esculin hydrolysis and salt-tolerance tests. These tests are used for the presumptive identification of *Enterococcus* species and group D streptococci. The bile esculin test (right) is performed by inoculating a slant of bile esculin medium and incubating it overnight at 35°C to 37°C. Enterococci and group D streptococci are able to grow in the presence of 40% bile and to hydrolyze esculin to esculetin (which forms the black precipitate). The salt-tolerance test (left) is performed with a broth medium containing 6.5% NaCl. *Enterococcus* species will grow, whereas nonenterococcal group D streptococci will not grow in this medium. The photograph shows a positive reaction for both tests, identifying this organism as a group D *Enterococcus* species.

IP-77



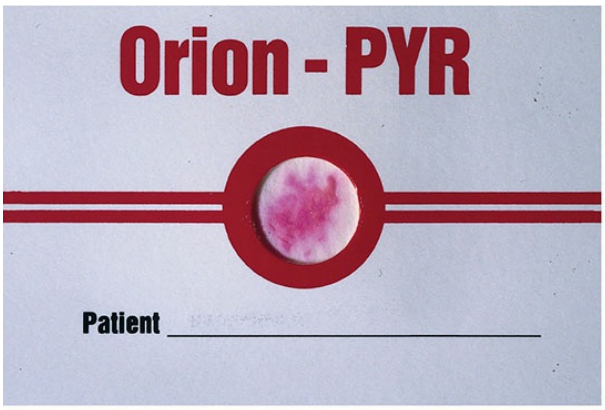
IP-78

G. PYR test. Hydrolysis of L-pyrrolidonyl-naphthylamide (PYR) is a presumptive test for identification of group A β-hemolytic streptococci and *Enterococcus* species that can replace the bacitracin-SXT disk tests and the salt-tolerance tests, respectively. In the disk test shown, colony growth is applied to a moistened disk impregnated with the PYR substrate. After 2 minutes, a drop of

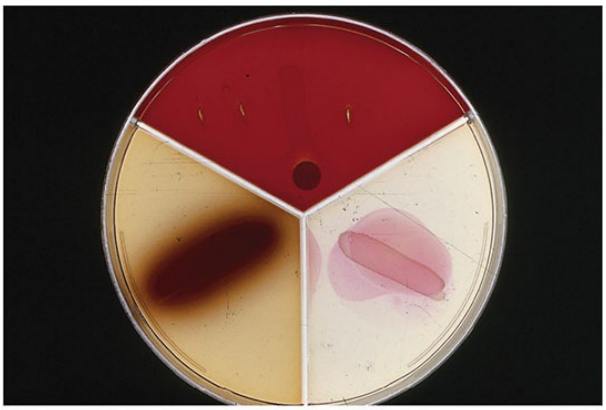
dimethylaminocinnamaldehyde reagent is placed on the disk to detect the free naphthylamide that is released on hydrolysis of PYR (red).

H. Tri-Plate for presumptive identification of streptococci. This single plate is designed to provide the results of several tests for the presumptive identification of streptococci and enterococci. The three sectors of the plate contain sheep blood agar, bile esculin agar, and PYR agar, respectively. Determination of hemolysis and a CAMP test are performed in the blood agar sector, and the bile esculin and PYR reactions are read in the other two sectors. This photograph shows the Tri-Plate inoculated with an *Enterococcus* species: α-hemolytic, CAMP test-negative, bile esculin–positive, and PYR-positive.

IP-79



G


H

IP-80

IMAGE PLATE 13-3

Identification of Streptococci and Enterococci and *Streptococcus*-Like Bacteria

A. Latex agglutination test for serologic grouping of β-hemolytic streptococci. Although the Lance field extraction method and the capillary precipitin tests are the time-honored techniques for definitive identification of the groupable streptococci, other methods, such as the one shown here, have been adopted in most laboratories and are considered standard procedures. In this test, the cell wall grouping antigen is extracted from the bacterial cell wall enzymatically, and the extract is subsequently reacted with latex beads to which group-specific antibodies

are bound. A positive test is indicated by agglutination of the latex beads in the homologous latex reagent. As shown here, agglutination has occurred with the group A reagent, but not with the group B, C, D, F, or G reagents, thereby identify the isolate as a group A β-hemolytic *Streptococcus*.

B. Quellung test for identification of *S. pneumoniae*. This microscopic "precipitin test" can be used to identify pneumococci or to determine the capsular serotype of individual pneumococcal isolates. Reaction of the anticapsular antibodies with the carbohydrate material of the capsule causes a microprecipitin reaction on the surface of the organism and a change in the refractive index of the capsule itself. Microscopically, the capsule appears to "swell." A small amount of methylene blue is added to the preparation to allow visualization of the cells and to provide contrast so that subtle refractile changes in the capsule can be more easily discerned.

C. Pneumoslide latex agglutination test (BD Microbiology Systems). The Pneumoslide test uses latex beads that are sensitized with antipneumococcal antibodies. Mixture of the sensitized latex with colony material from a culture plate results in visible agglutination of the suspension, thereby identifying the isolate as *S. pneumoniae*.

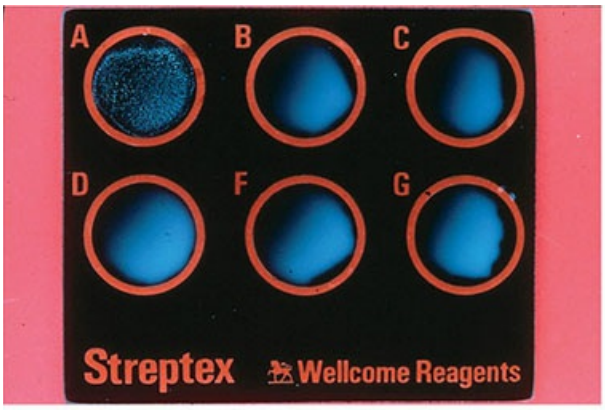
D. *Abiotrophia/Granulicatella* species. These organisms were formerly called satelliting or nutritionally variant streptococci because they require thiol compounds, cysteine, or the active form of vitamin B_6 (pyridoxal or pyridoxamine) for growth on media. This requirement may be met by cross-streaking a *Staphylococcus* across an inoculum of the probable nutritionally variant isolate, as shown in this photograph. The small colonies of the organism are observed growing adjacent to the *Staphylococcus* streak following incubation in a manner similar to the satellite test for presumptive identification of *Haemophilus* species. Members of the satelliting streptococci have now been placed in either the genus *Abiotrophia* or the genus *Granulicatella*.

E. *Enterococcus faecium*. This photograph shows a sheep blood agar plate inoculated with *E. faecium*. Colonies of this organism are smooth, gray, and nonhemolytic or α-hemolytic. The swab, which was used to pick up some of the colony growth from the plate, shows that the organism is nonpigmented. This becomes important for the identification of certain other enterococcal species.

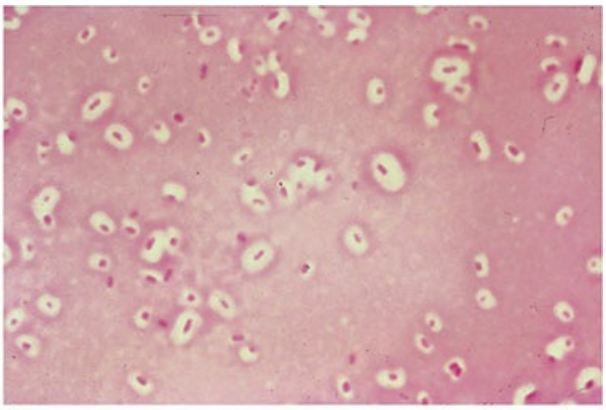
F. API Strep identification panel (bioMérieux) inoculated with *E. faecium*. This photograph shows the first half of the identification panel. Interpretations of the reactions shown are as follows:

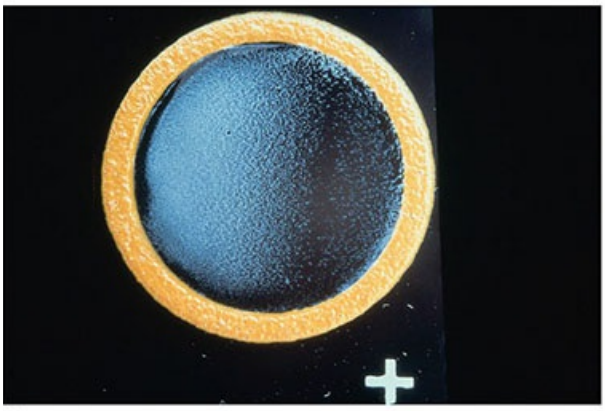
VP	Positive	HIP	Negative	ESC	Positive
PYRA	Positive	α-GAL	Positive	β-GUR	Negative
β-GAL	Positive	PAL	Negative	LAP	Positive

IP-81

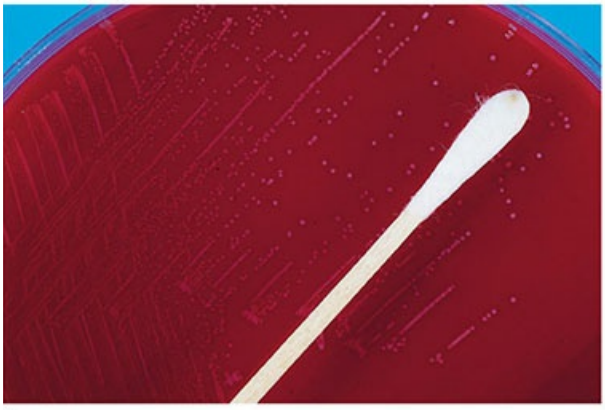


A


B


C

D


E

F

IP-82

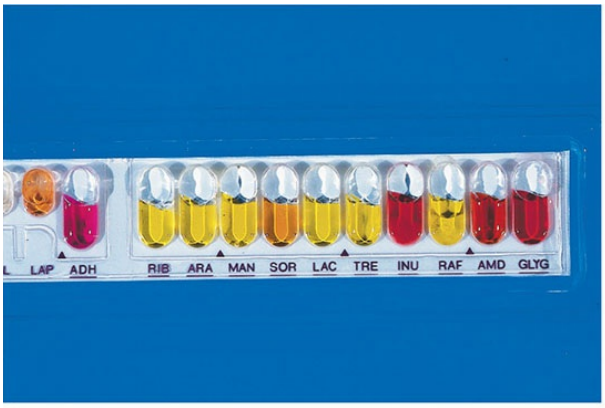
G. API Strep identification panel inoculated with *E. faecium*. This photograph shows the second half of the identification panel. Interpretations of the reactions shown are as follows:

ADH	Positive	RIB	Positive	ARA	Positive
MAN	Positive	SOR	Positive	LAC	Positive
TRE	Positive	INU	Negative	RAF	Positive
AMD	Negative	GLYG	Negative	HEM	Negative

The API biocode for the reactions shown in Image Plate 13-3F and G is 5357750. Consultation with the API computer-assisted database provides an identification of *E. faecium*, with 99% likelihood.

H. Vancomycin screening agar. Vancomycin screening agar consists of brain–heart infusion agar containing vancomycin (6 g/mL). This medium can be used to detect vancomycin-resistant enterococci, the majority of which are *E. faecium*. As shown in this photograph, isolates A, B, C, and D are vancomycin-resistant and are able to grow on this medium. Isolate E is a strain of *E. faecalis*, most of which are vancomycin-susceptible, as indicated by growth inhibition on this medium.

IP-83



G

H

IP-84

IMAGE PLATE 13-4

IDENTIFICATION OF ENTEROCOCCI AND VIRIDANS GROUP STREPTOCOCCI

A. *Enterococcus casseliflavus* on sheep blood agar. *E. casseliflavus* is one of two motile *Enterococcus* species. In addition, *E. casseliflavus* produces a yellow pigment. This pigment is evident on inspection of the swab in the photograph, which has been used to pick up some of the colony growth.

B. *Enterococcus gallinarum* in semisolid motility agar. As mentioned above, *E. gallinarum* and *E. casseliflavus* are the two motile *Enterococcus* species. In this photograph, *E. gallinarum* has been stab-inoculated into a tube of semisolid motility medium containing tetrazolium. On incubation of the inoculated tube, the motile organisms move out from the streak line to create turbidity in the medium. The purple color results from the motile organisms reducing the tetrazolium to formazan, which appears as a purple precipitate anywhere that viable organisms are found.

C. *Streptococcus* bovis on sheep blood agar. *S. bovis* is a nonenterococcal group D *Streptococcus*. This organism is bile esculin–positive, but does not grow in 6.5% NaCl broth and is PYR-negative. Isolation of *S. bovis* from blood cultures is associated with colonic neoplasia.

D. API Strep identification panel inoculated with *S. bovis*. This photograph shows the first half of the identification panel. The interpretations of the reactions shown are:

VP	Positive	HIP	Negative	ESC	Positive
PYRA	Negative	α-GAL	Positive	β-GUR	Negative
β-GAL	Positive	PAL	Negative	LAP	Positive

E. API Strep identification panel inoculated with *S. bovis*. This photograph shows the second half of the identification panel. The interpretations of the reactions shown are:

ADH	Negative	RIB	Negative	ARA	Negative
MAN	Positive	SOR	Negative	LAC	Positive
TRE	Positive	INU	Positive	RAF	Positive
AMD	Positive	GLYG	Positive	β-HEM	Negative

The API biocode for the reactions shown in Image Plate 13-4D and E is 5250573. Consultation with the API computer-assisted database provides an identification of this isolate as *S. bovis* I (99.9% probability). According to the new nomenclature for this organism complex, *S. bovis* I is now called *Streptococcus gallolyticus* subsp. *gallolyticus* (Table 13-7).

F. *Streptococcus mitis* growing on sheep blood agar after 24 hours of incubation. This

particular isolate appears to be γ-hemolytic or nonhemolytic. Other strains of this species may be α-hemolytic.

G. API Strep identification panel inoculated with *S. mitis*. This photograph shows the first half of the identification panel. Interpretations of the reactions are as follows:

VP	Negative	HIP	Negative	ESC	Negative
PYRA	Negative	α-GAL	Negative	β-GUR	Negative
β-GAL	Negative	PAL	Positive	LAP	Positive

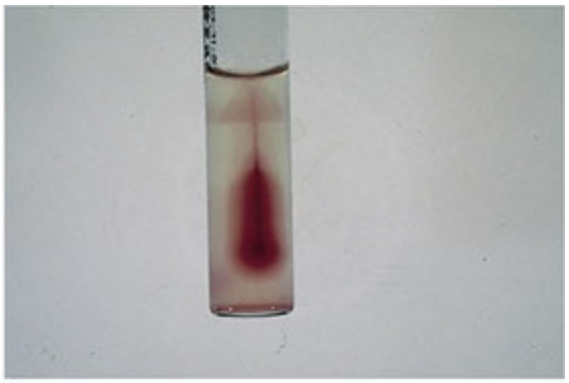
H. API Strep identification panel inoculated with *S. mitis*. This photograph shows the second half of the identification panel. The interpretations of the reactions are as follows:

ADH	Negative	RIB	Negative	ARA	Negative
MAN	Negative	SOR	Negative	LAC	Positive
TRE	Positive	INU	Negative	RAF	Negative
AMD	Positive	GLGY	Negative	β-HEM	Negative

The biocode for the reactions shown in Image Plate 13-4G and H is 0060411. Consultation with the API computer-assisted database provides an identification of S. mitis (80%)/oralis (20%). *S. mitis* and *S. oralis* belong to the "mitis group" of viridans streptococci. Some isolates of *S. mitis* have been shown to be resistant to penicillin and may be resistant to other antimicrobial agents as well.

IP-85

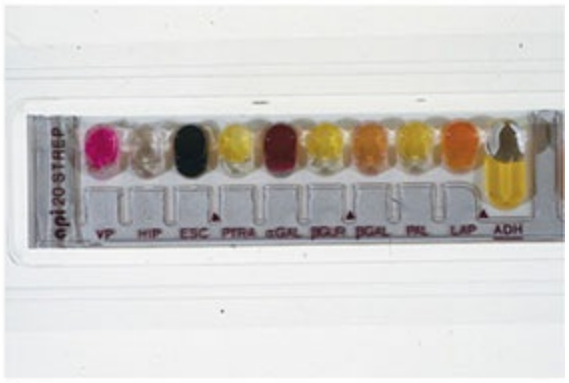
A



B

7

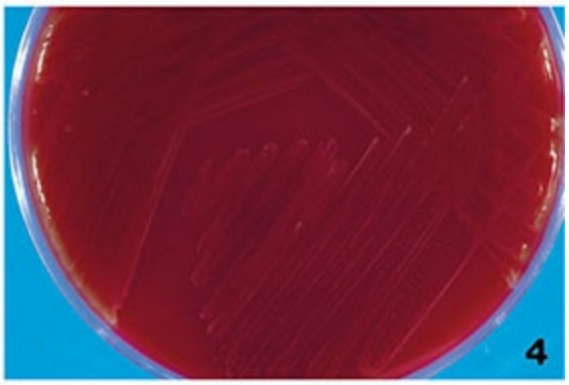
C


api 20 STREP
VP HIP ESC PTRA αGAL βGUR βGAL PAL LAP ADH

D

LAP ADH
RIB ARA MAN SOR LAC TRE INU RAF AMD GLTG

E


4

F

api 20 STREP
VP HIP ESC PTRA αGAL βGUR βGAL PAL LAP ADH
5

G

LAP ADH
RIB ARA MAN SOR LAC TRE INU RAF AMD GLTG
6

H

IP-86

IMAGE PLATE 14-1

LISTERIA AND *ERYSIPELOTHRIX* SPECIES

A. Gram-stained smear of *Listeria monocytogenes*, showing short, gram-positive bacilli.

B. Small, β-hemolytic colonies of *L. monocytogenes* on sheep blood agar (SBA) after 72 hours of incubation at 35°C to 37°C.

C. *L. monocytogenes* growing on SBA after 24 hours (left) and 48 hours (right) of incubation. After 24 hours, the β-hemolytic nature of the organism may not be obvious. After 48 hours, the zone of hemolysis extends beyond the margin of the colony, but is still rather subtle.

D. *L. monocytogenes* on bile esculin agar (right). The tube on the left is an uninoculated bile esculin agar slant. Like the enterococci, *L. monocytogenes* is able to hydrolyze esculin in the presence of 40% bile. Esculin hydrolysis is indicated by blackening of the medium.

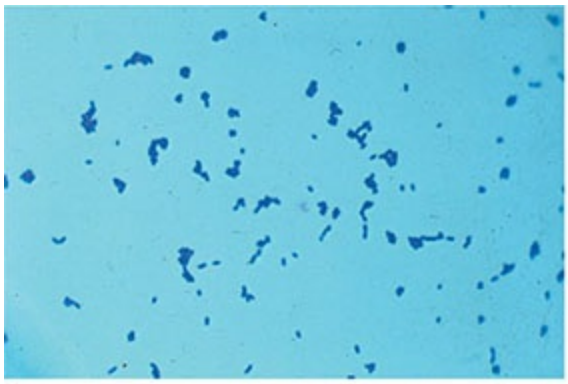
E. Umbrella-shaped zone of motile growth produced by *Listeria monocytogenes* after 24 hours of growth in semisolid motility medium at 25°C.

F. API Coryne inoculated with *Listeria monocytogenes*. The API biocode of the isolate shown is 0170164. Consultation with the API Profile Index provides an identification as "Listeria *monocytogenes*/innocua, 97.1% likelihood." Differentiation of *L. monocytogenes* and *L. innocua* is determined by assessment of hemolysis on SBA and by reactivity of *L. monocytogenes* in the CAMP test with *S. aureus*.

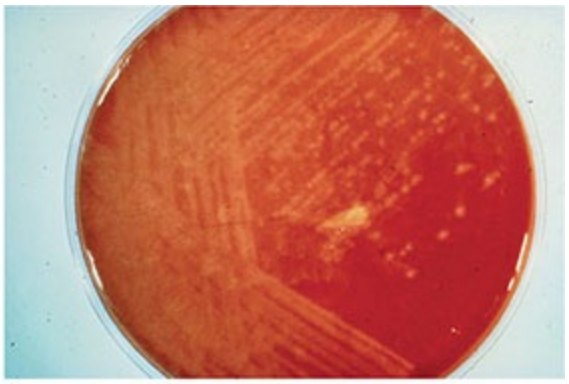
G. Gram-stained smear of *Erysipelothrix rhusiopathiae*, showing the long, thin, gram-positive rods of this species.

H. Colonies of *Erysipelothrix rhusiopathiae* on SBA after 24 hours of incubation at 35°C.

IP-87



A



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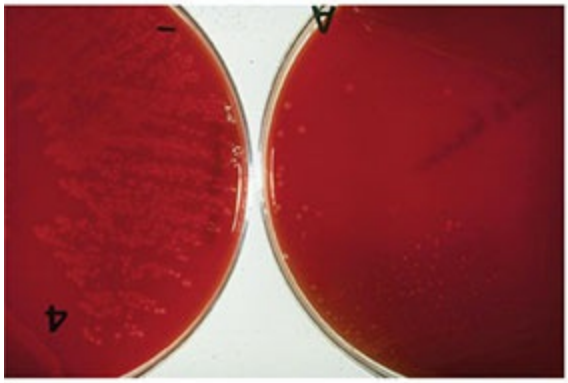

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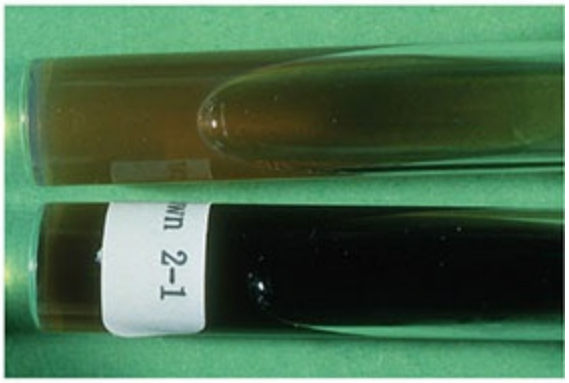

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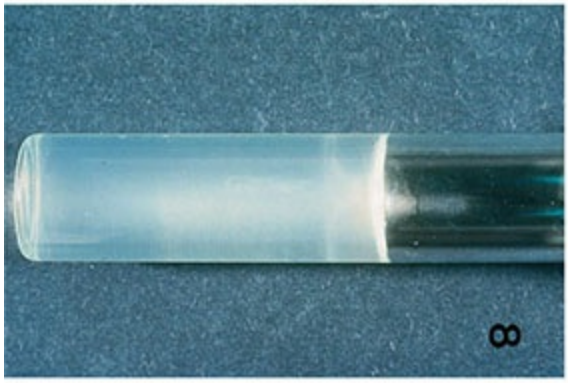
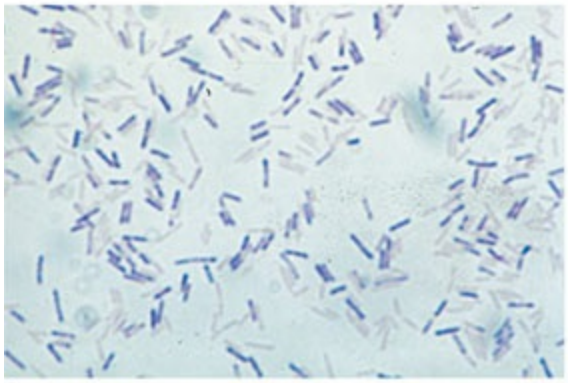


E

F



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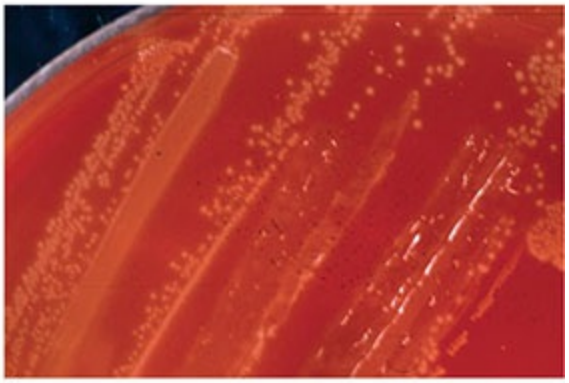


H

IP-88

IMAGE PLATE 14-2

ERYSIPELOTHRIX AND *BACILLUS* SPECIES

A. *Erysipelothrix rhusiopathiae* after overnight growth in a streak-and-stab inoculated tube of Kligler's iron agar. Note the production of H_2S along the line of the stab in the butt of the tube.

B. Gram-stained smear of a *Bacillus* species. Note the presence of central/subterminal, nonstaining endospores that do not swell the cell.

C. Gross specimen of intestinal tract from a patient who died of gastrointestinal anthrax. The appendix is noted in the lower left portion of the photograph. Lesions of gastrointestinal anthrax are usually noted in the cecum and adjacent areas of the bowel. Symptoms include abdominal pain, hematemesis, and bloody diarrhea.

D. Gram's stain (left) and malachite-green spore stain (right) of *B. anthracis*. *B. anthracis* grows as rather large gram-positive bacilli, with the individual cells having squared-off or slightly concave ends. Often, the organisms are arranged in chains that have the appearance of bamboo, as depicted here. The spore stain shows the ovoid spores that do not swell the cell. (Courtesy of Elmer W. Koneman.)

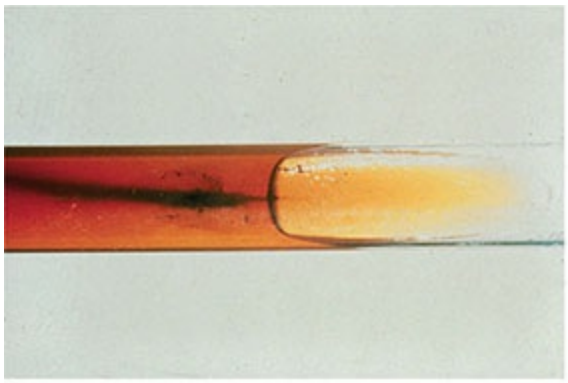
E. Off-white, dull, nonhemolytic colonies of *Bacillus anthracis* on SBA.

F. Close-up of *B. anthracis* colonies on SBA. Colonies of *B. anthracis* have a ground-glass morphology with comma-shaped outgrowths along the colony margins, as shown here. These outgrowths are even more apparent microscopically. Such colonies have been described as "Medusa head" colonies, after the Greek sorceress who had hair composed of serpents. (Courtesy of Elmer W. Koneman.)

G. Close-up of the rough, nonhemolytic colonies of *B. anthracis*. A few of the colonies have been touched with an inoculating loop, resulting in the colonies "standing up" like beaten egg whites.

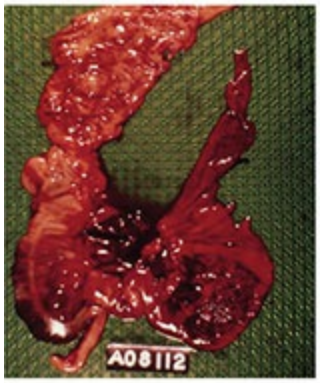
H. β-Hemolytic colonies of *Bacillus cereus* on SBA. *B. cereus* is one of the more frequently isolated *Bacillus* species in the clinical laboratory. Unlike *B. anthracis*, this species is hemolytic, motile, and α-lactamase-positive.

IP-89



A

B



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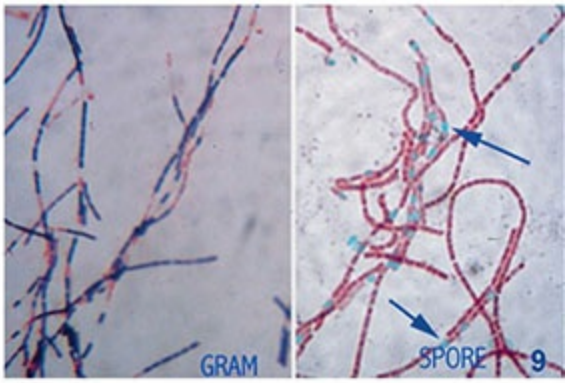

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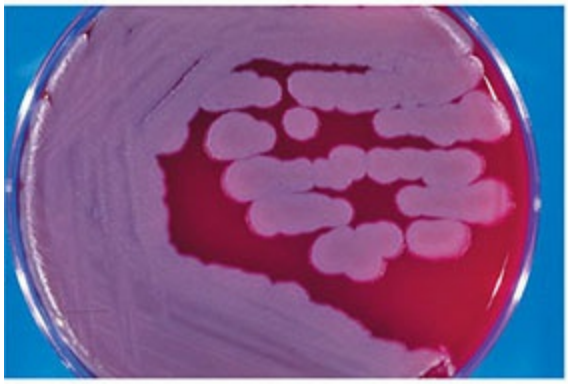
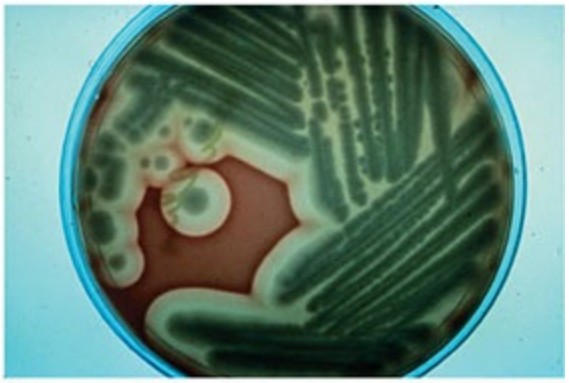


E

F

G



H

IMAGE PLATE 14-3

IP-90

CORYNEBACTERIUM SPECIES

A. Gram-stained smear showing the typical morphology of members of the genus *Corynebacterium*. Regularly shaped rods, club-shaped bacilli, "Chinese-letter" and "picket-fence" cellular arrangements can be observed in this photograph.

B. BHI agar inoculated with a lipophilic and a nonlipophilic *Corynebacterium* species. On the right side of the plate, a lipophilic species has been inoculated as a lawn on the plate, and a drop of 0.1% Tween 80 has been placed in the center of the inoculum. A nonlipophilic *Corynebacterium* species is inoculated as a lawn on the left half of the plate. After incubation for 24 hours at 35°C to 37°C, growth of the lipophilic species is observed only where the drop of Tween 80 was placed, while the nonlipophilic species grows all over the surface of the medium on the right side of the plate.

C. *Corynebacterium amycolatum* growing on SBA after 3 days of incubation at 35°C. Note the dry, matte appearance of the colonies and the "wrinkling" of the growth in confluent areas. *C. amycolatum* is the most frequently isolated *Corynebacterium* species and is found on the skin.

D. API Coryne panel inoculated with *Corynebacterium amycolatum*. The photograph shows first half of the identification panel. The interpretations of the reactions shown are:

NIT	Positive	PYZ	Positive	PYRA	Negative
PAL	Positive	β-GUR	Negative	β-GAL	Negative
α-GLU	Negative	β-NAG	Negative	ESC	Negative
URE	Negative				

E. API Coryne panel inoculated with *Corynebacterium amycolatum*. This photograph shows the second half of the identification panel The interpretations of the reactions shown are:

GEL	Negative	CHO Control	Negative		
GLU	Positive	RIB	Positive	XYL	Negative
MAN	Negative	MAL	Negative	LAC	Negative
SAC	Positive	GLYG	Negative	CAT	Positive

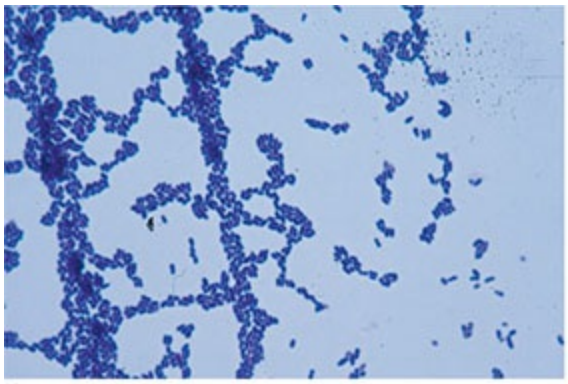
The API Coryne biocode for the reactions shown in Image Plate 14-3D and E is 3100305, which gives a cross-call identification of *Corynebacterium* group G, *C. striatum,* or *C. amycolatum. C. amycolatum* is nonlipophilic, while group G coryneform strains are lipophilic; so this isolate is not a group G coryneform. Additional tests (e.g., gas-liquid chromatography of glucose broth cultures) are needed to differentiate *Corynebacterium amycolatum* and *Corynebacterium striatum* from one another. This isolate produced propionic acid from glucose as determined by gas-liquid chromatographic analysis of culture broth, so this organism can be identified as *C. amycolatum*.

F. Local inflammation and pseudomembrane formation in the oropharynx produced by toxigenic strains of *Corynebacterium diphtheriae*.

G. Growth of *Corynebacterium diphtheriae* on modified Tinsdale agar. On this medium, *C. diphtheriae* grows as black colonies with brown halos. Isolates with this colonial morphology are first identified biochemically as *C. diphtheriae*, and subsequent testing involves demonstrating the production of diphtheria toxin.

H. Growth of *C. diphtheriae* from Loeffler's coagulated serum medium. This medium is inoculated with material from the posterior pharynx (i.e., the diphtheritic pseudomembrane), and a smear is prepared after about 8 hours of incubation and stained with methylene blue. As shown here, *C. diphtheriae* appears as pleomorphic beaded bacilli. The beaded appearance is due to the production of metachromatic granules by the organism on this medium.

IP-91



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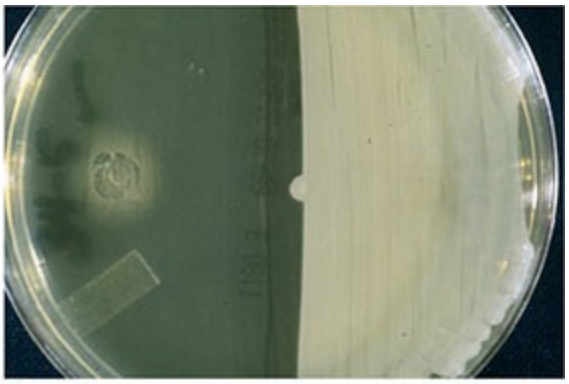
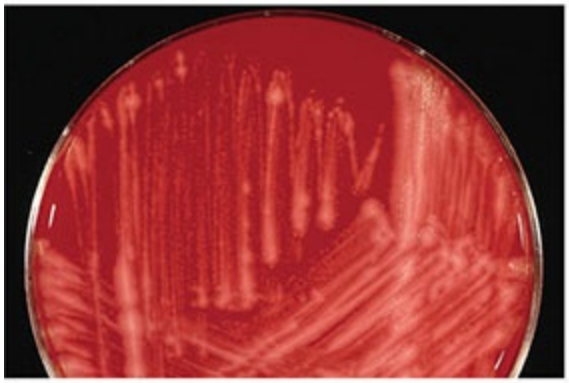


B



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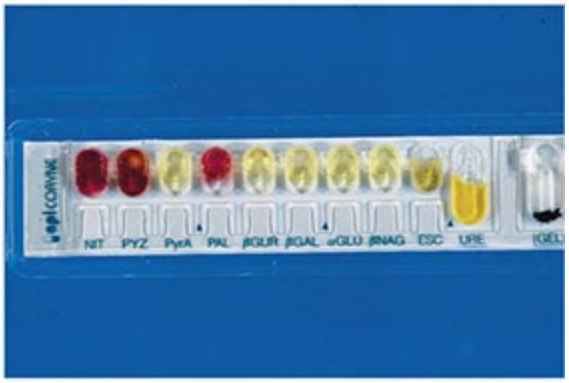
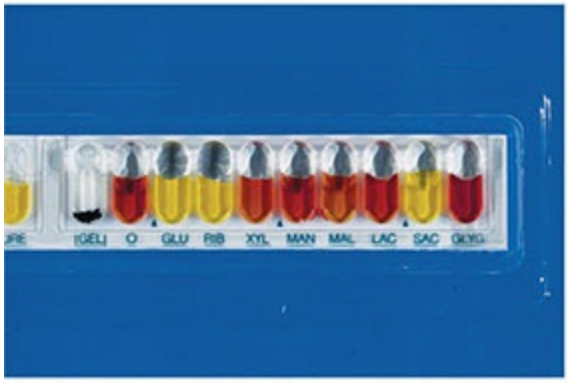
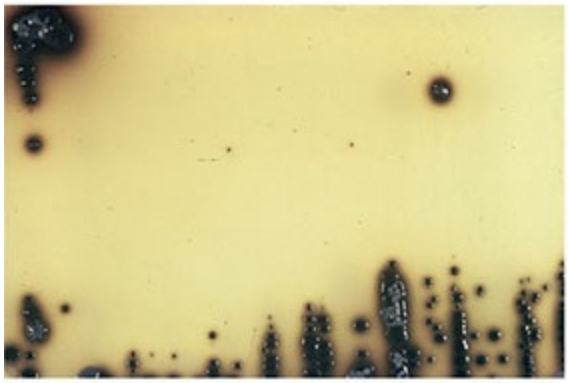

NIT
PYZ
PyrA
PAL
βGLR
βGAL
αGLU
βNAG
ESC
URE

D

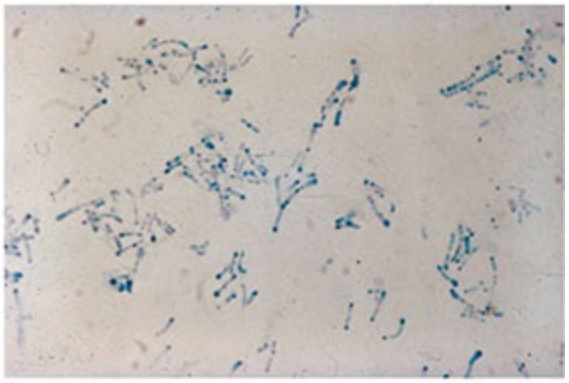

[GEL]
O
GLU
RIB
XYL
MAN
MAL
LAC
SAC

E

F



G



H

IMAGE PLATE 14-4

Corynebacterium Species

A. Growth of *C. diphtheriae* on SBA after 24 hours of incubation at 35°C. Colonies of this organism are nonlipophilic, white, and creamy. This isolate was inoculated onto the API Coryne identification kit, and the biochemical reactions for identification are shown in **B** and **C** below.

B. API Coryne inoculated with *C. diphtheriae*. This photograph shows the first half of the identification panel. The interpretations of the reactions shown are:

NIT	Positive	PYZ	Negative	PYRA	Negative
PAL	Negative	β-GUR	Negative	β-GAL	Negative
α-GLU	Positive	β-NAG	Negative	ESC	Negative
URE	Negative				

C. API Coryne inoculated with *C. diphtheriae*. This photograph shows the other half of the identification panel. The interpretations of the reactions shown are:

GEL	Negative	CHO Control	Negative		
GLU	Positive	RIB	Negative	XYL	Negative
MAN	Negative	MAL	Positive	LAC	Negative
SAC	Negative	GLYG	Negative	CAT	Positive

The API Coryne biocode for these reactions is 1010124, which corresponds to an identification of *Corynebacterium diphtheriae* (97.8% probability). Additional testing must be performed to determine whether the isolate produces diphtheria toxin.

D. *Corynebacterium jeikeium* on SBA after 48 hours of incubation at 35°C to 37°C. This lipophilic species produces small, nonhemolytic colonies on SBA.

E. API Coryne inoculated with *Corynebacterium jeikeium*. This photograph shows the first half of the identification panel. The interpretations of the reactions shown are:

NIT	Negative	PYZ	Positive	PYRA	Negative
PAL	Positive	β-GUR	Negative	β-GAL	Negative
α-GLU	Negative	β-NAG	Negative	ESC	Negative
URE	Negative				

F. API Coryne inoculated with *C. jeikeium*. This photograph shows the second half of the identification panel. The interpretations of the reactions shown are:

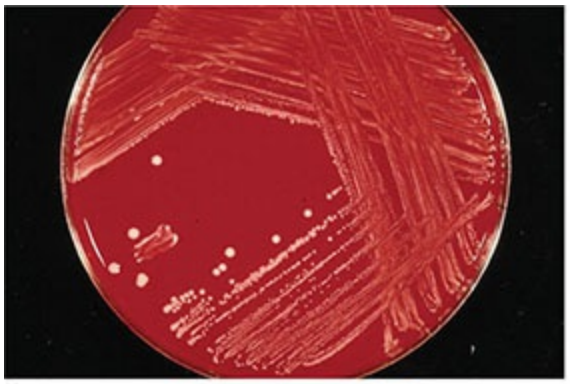
GEL	Negative	CHO Control	Negative		
GLU	Positive	RIB	Positive	XYL	Negative
MAN	Negative	MAL	Negative	LAC	Negative
SAC	Negative	GLYG	Negative	CAT	Negative

The API Coryne biocode for these reactions is 2100304, which corresponds to an identification of *C. jeikeium* (93.6% probability).

G. Gram-stained smear showing the rather plump, gram-positive bacilli of *Corynebacterium glucuronolyticum* ("*C. seminale*").

H. *C. glucuronolyticum* on SBA after 24 hours of incubation at 35°C to 37°C.

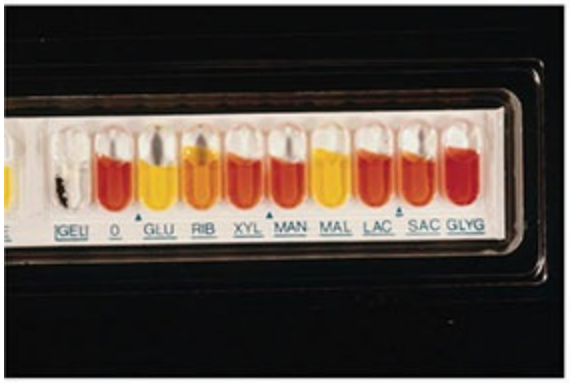
IP-93



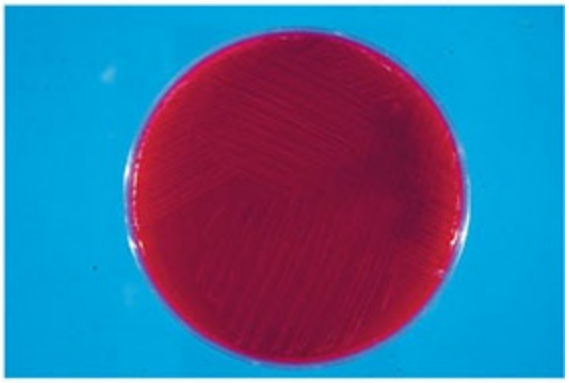
A

api CORYNE
NIT PYZ PyrA PAL βGUR βGAL αGLU βNAG ESC URE

B


IGEL O GLU RIB XYL MAN MAL LAC SAC GLYG

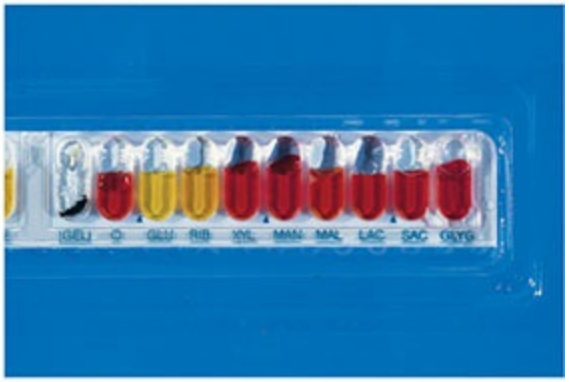
C



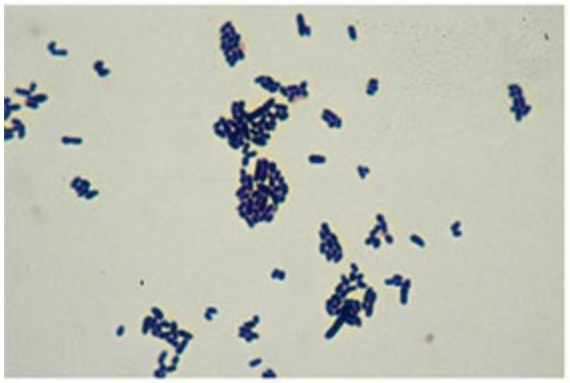
D

api CORYNE
NIT PYZ PyrA PAL βGUR βGAL αGLU βNAG ESC URE

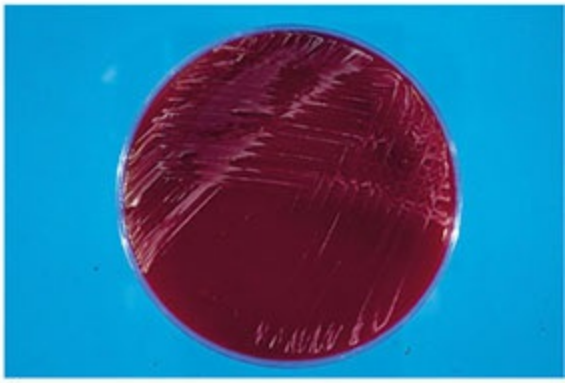
E


IGEL O GLU RIB XYL MAN MAL LAC SAC GLYG

F



G



H

IP-94

IMAGE PLATE 14-5

CORYNEBACTERIUM SPECIES

A. API Coryne inoculated with *C. glucuronolyticum*. This photograph shows the first half of the identification panel. The interpretations of the reactions shown are:

NIT	Negative	PYZ	Positive	PYRA Negative	Negative
PAL	Negative	β-GUR	Positive	β-GAL	Negative
α-GLU	Negative	β-NAG	Negative	ESC	Positive
URE	Positive				

B. API Coryne inoculated with *C. glucuronolyticum*. This photograph shows the second half of the identification panel. The interpretations of the reactions shown are:

GEL	Negative	CHO Control	Negative		
GLU	Positive	RIB	Negative	XYL	Negative
MAN	Negative	MAL	Positive	LAC	Negative
SAC	Positive	GLYG	Negative	CAT	Positive

The API biocode for these reactions is 2241125, which corresponds to an identification of *C. glucuronolyticum* (99.9% probability). This species has been recovered from semen specimens and is the only *Corynebacterium* species that is β-glucuronidase (β-GUR)-positive. This species was also described by another research group and was given the name "*C. seminale*." However, the name "*C. glucuronolyticum*" has taxonomic precedence.

C. Creamy, smooth, nonlipophilic, nonhemolytic colonies of *Corynebacterium pseudodiphtheriticum* growing on SBA after 24 hours at 35°C to 37°C.

D. API Coryne inoculated with *Corynebacterium pseudodiphtheriticu m*. The interpretations of the reactions shown are:

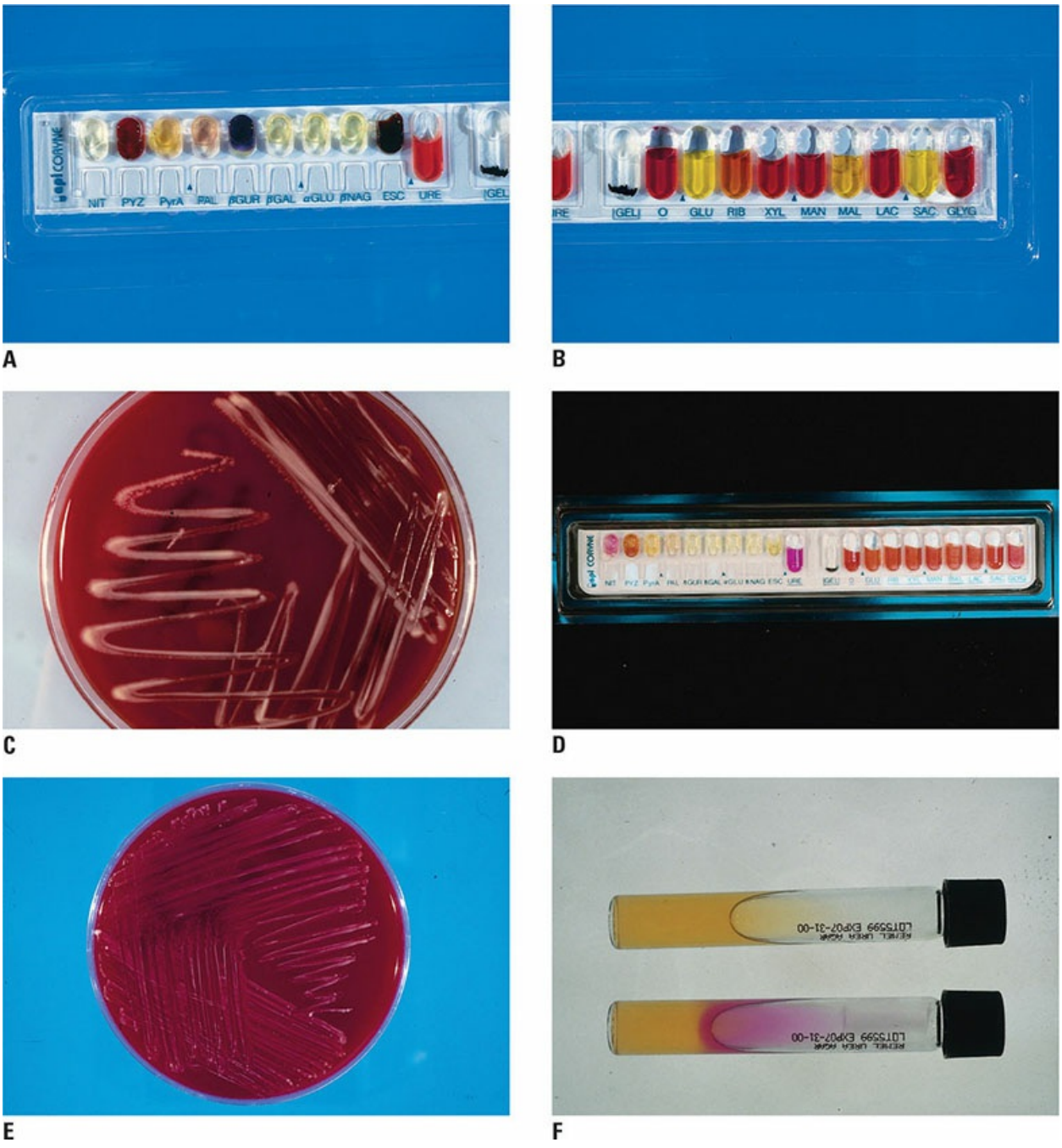
NIT	Positive	PYZ	Positive	PYRA	Negative
PAL	Negative	β-GUR	Negative	β-GAL	Negative
α-GLU	Negative	β-NAG	Negative	ESC	Negative
URE	Positive	GEL	Negative	CHO Control	Negative
GLU	Negative	RIB	Negative	XYL	Negative
MAN	Negative	MAL	Negative	LAC	Negative
SAC	Negative	GLYG	Negative	CAT	Positive

The API Coryne biocode for these reactions is 3001004, which corresponds to an identification of *C. pseudodiphtheriticum* (96.7% probability).

E. Creamy, nonlipophilic, nonhemolytic colonies of *C. riegelii* on SBA after 24 hours of incubation at 35°C to 37°C.

F. Rapid urease reaction of *C. riegelii*. *C. riegelii*, like *C. urealyticum*, is rapidly urease-positive. This photograph shows an uninoculated urea agar slant (**top**) and a urea agar slant inoculated with *C. riegelii* (**bottom**). This reaction was photographed after the slant on the right was incubated for 30 minutes at 35°C to 37°C.

IP-95



IP-96

G. API Coryne inoculated with *C. riegelii*. This photograph shows the first half of the identification panel. The interpretations of the reactions shown are:

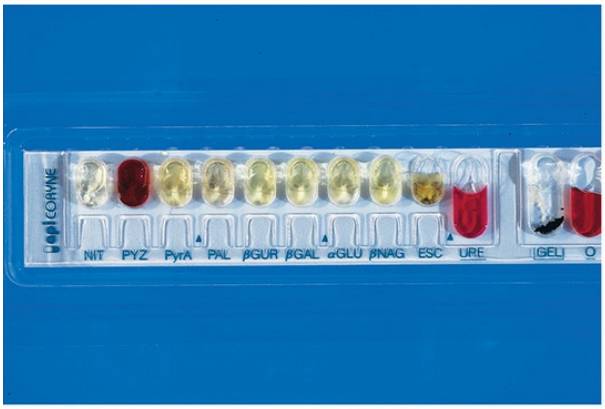
NIT	Negative	PYZ	Positive	PYRA	Negative
PAL	Negative	β-GUR	Negative	β-GAL	Negative
α-GLU	Negative	β-NAG	Negative	ESC	Negative
URE	Positive				

H. API Coryne inoculated with *C. riegelii*. This photograph shows the second half of the identification panel. The interpretations of the reactions shown are:

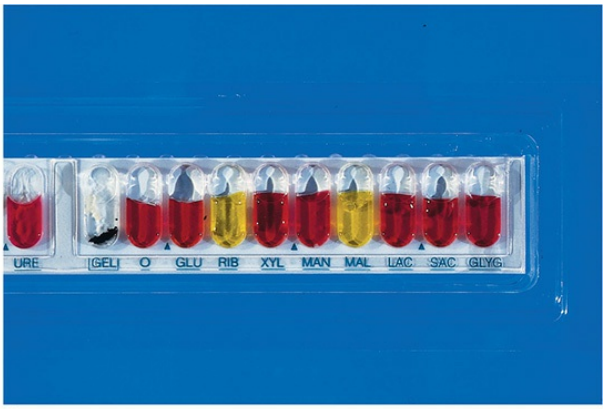
GEL	Negative	CHO Control	Negative		
GLU	Negative	RIB	Positive	XYL	Negative
MAN	Negative	MAL	Positive	LAC	Negative
SAC	Negative	GLYG	Negative	CAT	Positive

The API biocode for these reactions is 2001204. This biocode was not included in the API Coryne database at when this image was taken, but it is the same biocode that was published with the original description of this new species. *C. riegelii* is associated with urinary tract infections. Besides being rapidly urease-positive, this species is also atypical in that it produces acid from maltose and ribose, but not from glucose, as shown here.

IP-97



G



H

IP-98

IMAGE PLATE 14-6

Corynebacterium, *Arcanobacterium*, and *Brevibacterium* Species

A. Creamy, nonlipophilic, nonhemolytic colonies of *Corynebacterium striatum* on

SBA after 48 hours of incubation at 35°C to 37°C. Note the "target" morphology of the individual colonies.

B. API Coryne inoculated with *C. striatum*. The interpretations of the reactions shown are:

NIT	Positive	PYZ	Positive	PYRA	Negative
PAL	Positive	β-GUR	Negative	β-GAL	Negative
α-GLU	Negative	β-NAG	Negative	ESC	Negative
URE	Negative	GEL	Negative	CHO Control	Negative
GLU	Positive	RIB	Negative	XYL	Negative
MAN	Negative	MAL	Negative	LAC	Negative
SAC	Positive	GLYG	Negative	CAT	Positive

The API Coryne biocode for these reactions is 3100105, which corresponds to an identification of *Corynebacterium* group G, 55.5%/*C. striatum-amycolatum*, 43.6%. Since group G strains are lipophilic, this organism is either *C. striatum* or *C. amycolatum*. This isolate did not produce propionic acid from glucose on gas-liquid chromatographic analysis, and so this isolate is identified as *C. striatum*.

C. *Corynebacterium* group G after 24 hours of incubation at 35°C to 37°C. After 24 hours, individual colonies are extremely small, with good growth occurring only in the heavily inoculated areas. This lipophilic species has been isolated from vitreous, blood, cerebrospinal fluid, and urogenital tract specimens.

D. API Coryne inoculated with *Corynebacterium* group G. The interpretations of the reactions shown are as follows:

NIT	Negative	PYZ	Positive	PYRA	Negative
PAL	Positive	β-GUR	Negative	β-GAL	Negative
α-GLU	Negative	β-NAG	Negative	ESC	Negative
URE	Negative	GEL	Negative	CHO Control	Negative
GLU	Positive	RIB	Positive	XYL	Negative
MAN	Negative	MAL	Positive	LAC	Negative
SAC	Positive	GLYG	Negative	CAT	Positive

The API biocode for these reactions is 2100325. Consultation with the computer-assisted database provides an identification of *Corynebacterium* group G (76.6% likelihood)/*C. striatum/amycolatum* (22.7% likelihood). Since this organism is lipophilic and both *C. striatum* and *C. amycolatum* are nonlipophilic, this isolate can be identified as *Corynebacterium* group G.

E. Growth of *Arcanobacterium haemolyticum* on SBA after 48 hours of incubation at

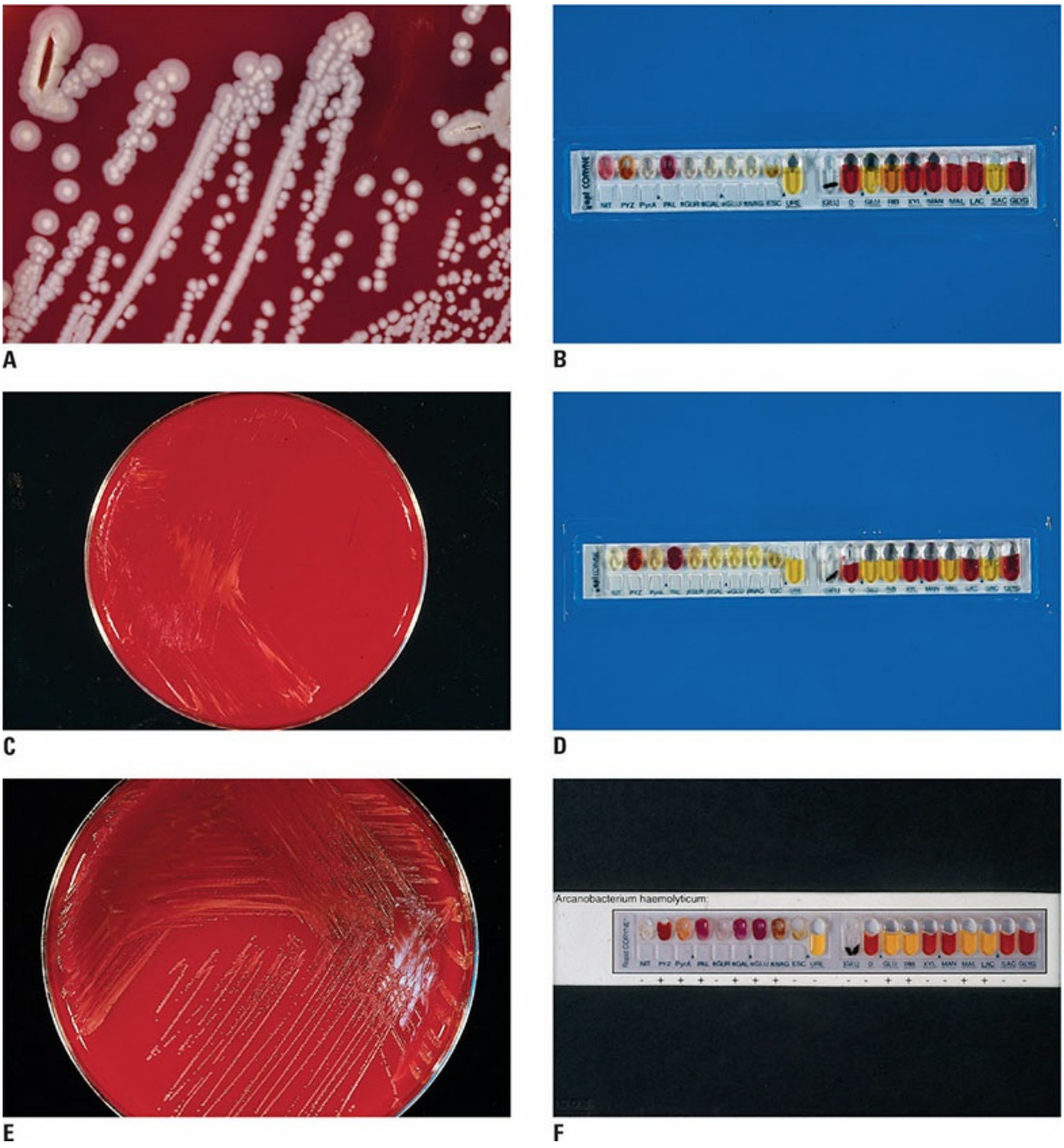
35°C to 37°C. Note the zone of β-hemolysis surrounding the colonies.

F. API Coryne inoculated with *A. haemolyticum*. The interpretations of the reactions shown are:

NIT	Negative	PYZ	Positive	PYRA	Positive
PAL	Positive	β-GUR	Negative	β-GAL	Positive
α-GLU	Positive	β-NAG	Positive	ESC	Negative
URE	Negative	GEL	Negative	CHO Control	Negative
GLU	Positive	RIB	Positive	XYL	Negative
MAN	Negative	MAL	Positive	LAC	Positive
SAC	Negative	GLYG	Negative	CAT	Negative

The API Coryne biocode for these reactions is 6530360, which corresponds to an identification of *A. haemolyticum* 99.9% (excellent identification)

IP-99



IP-100

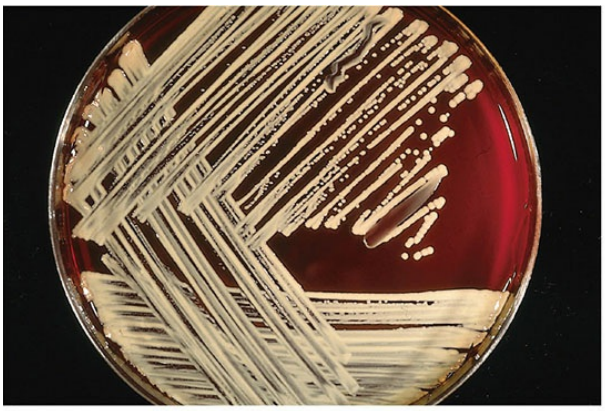
G. Growth of *Brevibacterium casei* on SBA after 24 hours of incubation at 35°C to 37°C. Colonies of this organism are pigmented pale yellow, which intensifies during incubation beyond 24 hours. These isolates also have a strong odor reminiscent of "stinky feet." This particular isolate was recovered from multiple blood culture of a patient with AIDS who had a central-line-associated infection.

H. API Coryne inoculated with *B. casei*. The interpretations of the reactions shown are:

NIT	Negative	PYZ	Positive	PYRA	Positive
PAL	Positive	β-GUR	Negative	β-GAL	Negative
α-GLU	Positive	β-NAG	Negative	ESC	Negative
URE	Negative	GEL	Positive	CHO Control	Negative
GLU	Negative	RIB	Negative	XYL	Negative
MAN	Negative	MAL	Negative	LAC	Negative
SAC	Negative	GLYG	Negative	CAT	Positive

The API Coryne biocode for these reactions is 6112004, which corresponds to an identification of *Brevibacterium* species. Additional testing confirmed that this blood culture isolate was indeed *B. casei*.

IP-101



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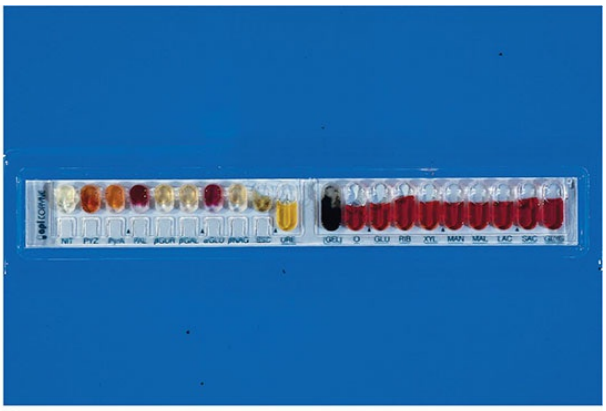


H

IP-102

IMAGE PLATE 14-7

Rothia, *Cellulosimicrobium*, *Cellulomonas/Microbacterium*, and *Lactobacillus* Species

A. Growth of *Rothia dentocariosa* on SBA after 48 hours of incubation at 35°C to 37°C. This gram-positive bacillary organism is related to the nocardioform organisms and forms dry, white, cerebriform colonies on agar media, as shown here.

B. API Coryne inoculated with *R. dentocariosa*. The interpretations of the reactions shown are:

NIT	Positive	PYZ	Positive	PYRA	Positive
PAL	Negative	β-GUR	Negative	β-GAL	Negative
α-GLU	Positive	β-NAG	Negative	ESC	Positive
URE	Negative	GEL	Negative	CHO Control	Negative
GLU	Positive	RIB	Negative	XYL	Negative
MAN	Negative	MAL	Positive	LAC	Negative
SAC	Positive	GLYG	Negative	CAT	Positive

The API biocode for these reactions is 7050125, which corresponds to an identification of *R. dentocariosa*, 99.5% likelihood.

C. Growth of *Cellulosimicrobium cellulans* on SBA after 24 hours at 35°C to 37°C. This bright yellow-pigmented organism was formerly called *Oerskovia xanthineolytica*.

D. API Coryne inoculated with *C. cellulans*. The interpretations of the reactions shown are:

NIT	Positive	PYZ	Positive	PYRA	Positive
PAL	Positive	β-GUR	Negative	β-GAL	Positive
α-GLU	Positive	β-NAG	Positive	ESC	Positive
URE	Negative	GEL	Positive	CHO Control	Negative
GLU	Positive	RIB	Positive	XYL	Positive
MAN	Negative	MAL	Positive	LAC	Negative
SAC	Positive	GLYG	Positive	CAT	Positive

The API biocode for these reactions is 7572727, which corresponds to an identification of *Oerskovia xanthineolytica* (*C. cellulans*), 99.9% probability.

E. Growth of a yellow-pigmented *Microbacterium* species on SBA after 24 hours at 35°C to 37°C.

F. API Coryne inoculated with *Microbacterium* species. The interpretations of the reactions shown are:

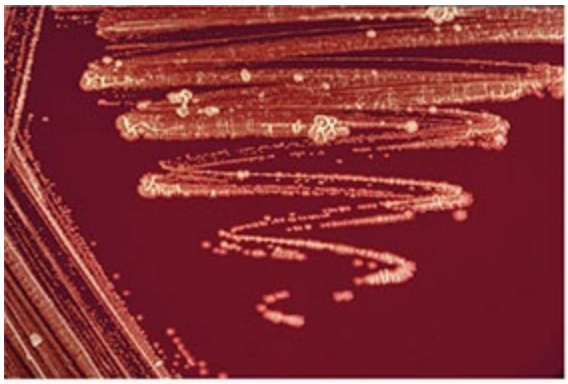
NIT	Negative	PYZ	Positive	PYRA	Negative
PAL	Positive	β-GUR	Negative	β-GAL	Positive
α-GLU	Positive	β-NAG	Positive	ESC	Positive
URE	Negative	GEL	Negative	CHO Control	Negative
GLU	Positive	RIB	Positive	XYL	Positive
MAN	Positive	MAL	Positive	LAC	Negative
SAC	Positive	GLYG	Positive	CAT	Positive

The API biocode for this organism is 2570737, which corresponds to an identification of *Cellulomonas* spp./*Microbacterium* spp. (99.9% likelihood). Additional tests are needed to separate these two genera.

G. Gram-stained smear of a blood culture that grew *Lactobacillus* species. These organisms are regular, gram-positive bacilli that occur singly, in pairs, or in short chains.

H. Small colonies of a nonhemolytic *Lactobacillus* species growing on SBA after 24 hours of incubation at 35°C to 37°C.

IP-103



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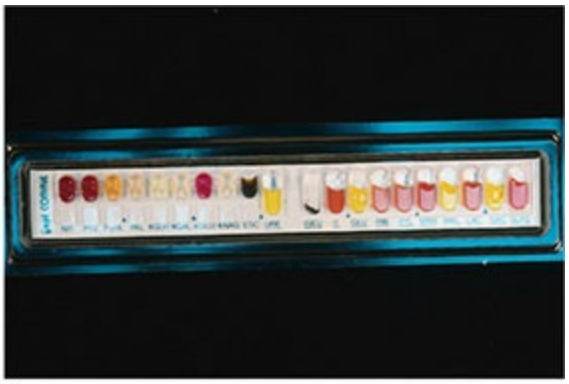

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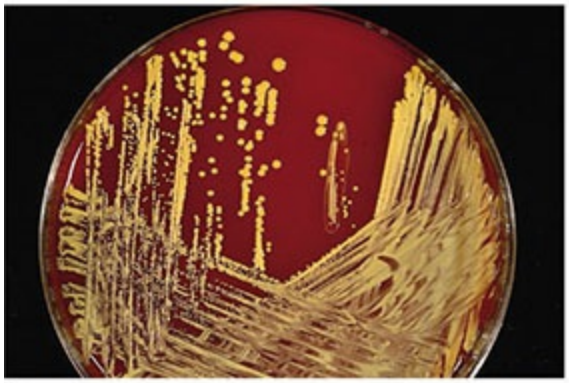

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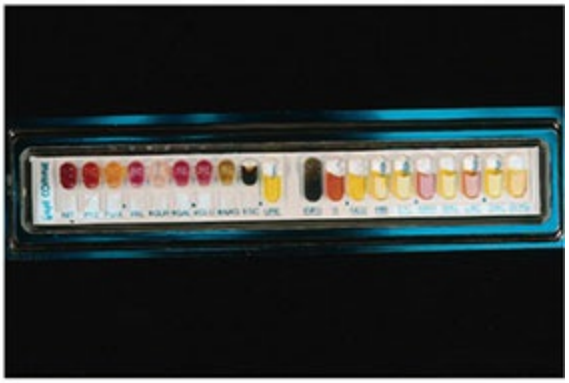

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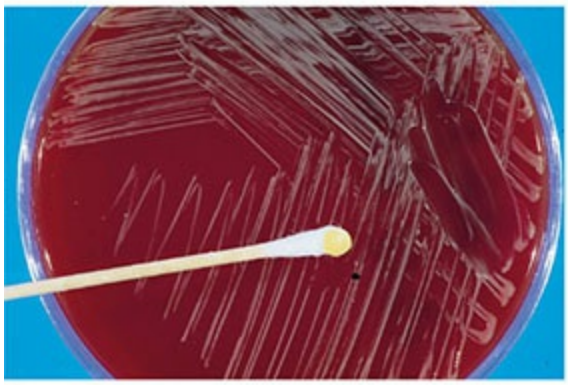

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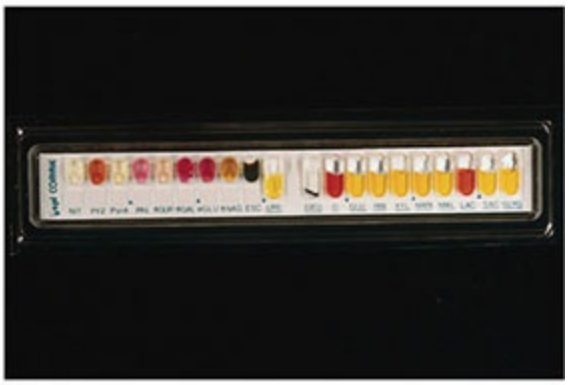

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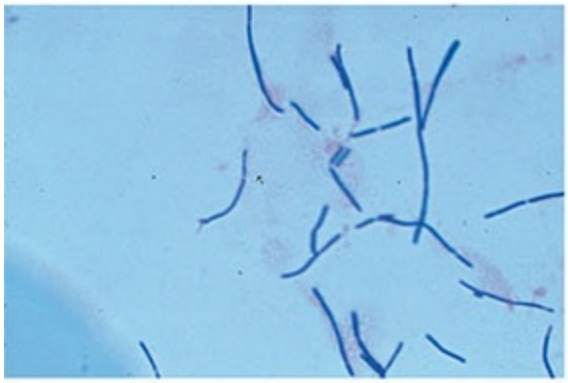

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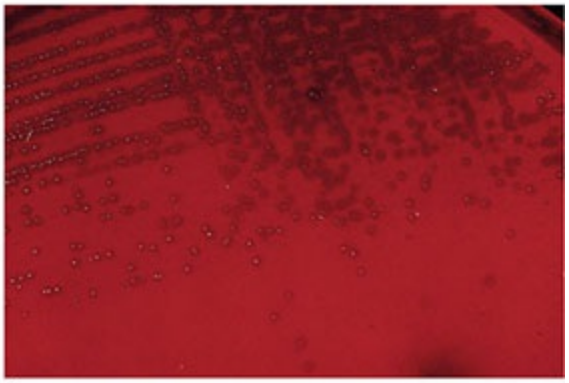


H

IP-104

IMAGE PLATE 14-8

Lactobacillus and *Gardnerella* Species

A. *Lactobacillus* species inoculated as a lawn on an SBA plate with a vancomycin disk. Some lactobacilli are resistant to the glycopeptide antibiotics vancomycin and teicoplanin, as indicated by growth of the organisms to the edge of the vancomycin disk, as shown here.

B. Gram-stained smear of a normal vaginal specimen. Note the presence of the large epithelial cells and the gram-positive bacilli that correspond to the "*Lactobacillus* morphotype."

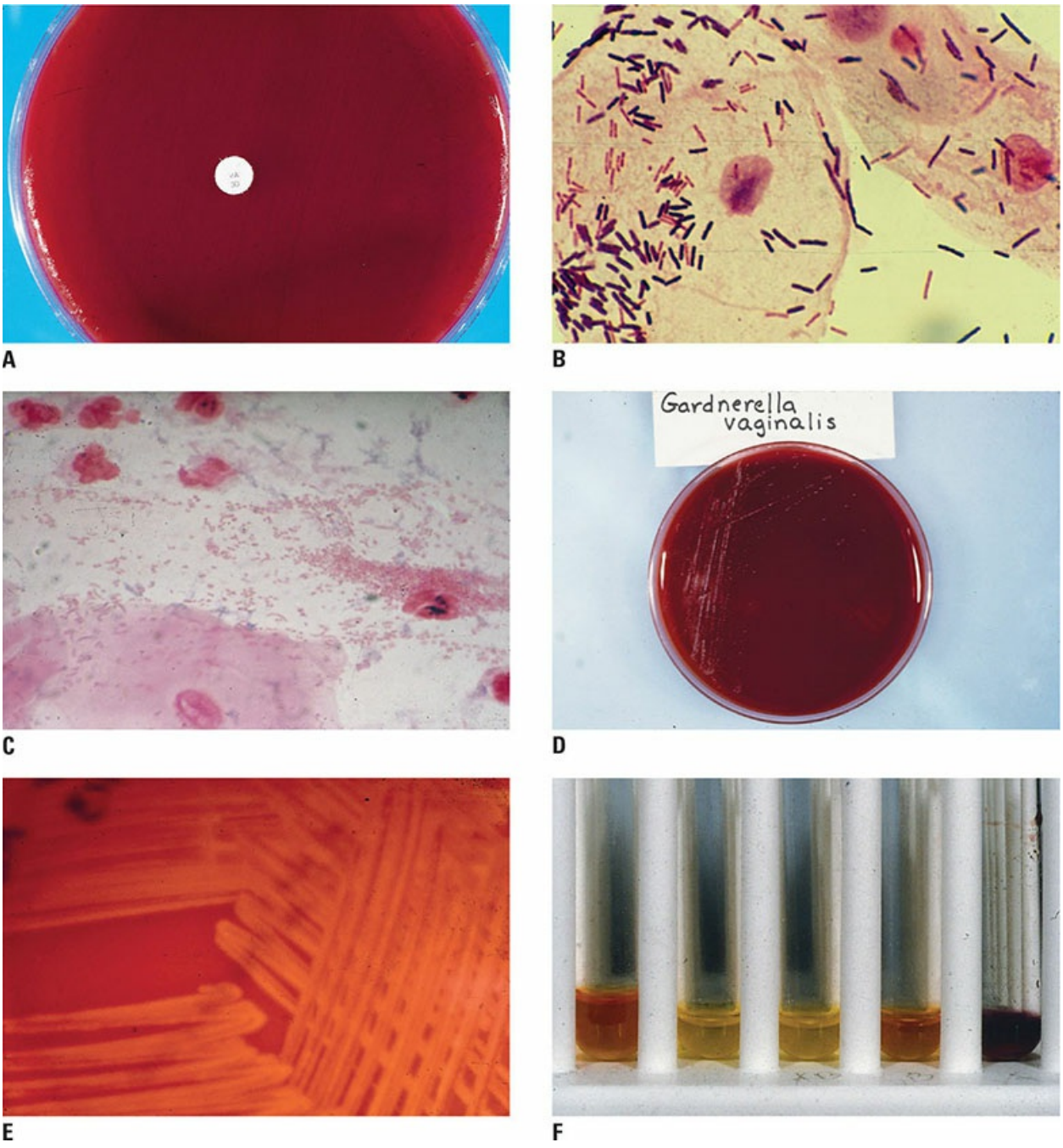
C. Gram-stained smear of material collected from the vagina of a patient with bacterial vaginosis. The epithelial cell shown in the slide is covered with a preponderance of gram-negative bacilli and coccobacilli, which represent anaerobic gram-negative bacilli and "*Gardnerella* morphotypes."

D. Growth of *G. vaginalis* on CNA blood agar after 48 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. After this time, colonies are very small and are best observed in areas of confluent growth.

E. Small, β-hemolytic colonies of *Gardnerella vaginalis* growing on HBT (human blood bilayer-Tween) medium after 72 hours of incubation at 35°C to 37°C in 5% to 7% CO_2. This organism is β-hemolytic on medium containing human blood (like HBT agar).

F. Rapid carbohydrate utilization technique for identification of *G. vaginalis*. This 4-hour identification procedure is similar to that used for identification of *Neisseria* species (Chapter 11). A balanced salt solution with phenol red and no carbohydrates (BSS) is dispensed in small tubes in 0.10-mL volumes, and single drops of 20% filter-sterilized carbohydrates are added to the BSS. A dense suspension of the organism is prepared in BSS with no carbohydrate (0.5 mL), and single drops of this suspension are added to the carbohydrate-containing tubes. In the photograph, the individual tubes are (left to right) the BSS organism suspension, BSS-glucose, BSS-maltose, and BSS-mannitol. The last tube (far right) is a hippurate-hydrolysis test. As shown, *G. vaginalis* produces acid from glucose and maltose, but not mannitol, and is strongly hippurate-hydrolysis–positive.

IP-105



IP-106

IMAGE PLATE 15-1

Identification of Aerobic and Facultatively Anaerobic Gram-Positive Bacilli

A. Close-up view of *Nocardia asteroides* complex after 7 days of incubation at 30°C on Middlebrook 7H11 agar. Seen here are the typical orange, glabrous, wavy colonies that tend to be adherent to the agar surface. Other species produce white, dry, chalky colonies (Image Plate 1-5J). It is noteworthy that *Nocardia* species will grow on the selective isolation media used for the recovery of *Mycobacterium* species; in fact, the laboratory diagnosis of nocardiosis is often first suspected in the mycobacteriology section of the laboratory. The detection of a distinct musty basement odor is an initial clue to the identification.

B. Middlebrook 7H11 agar plate on which a 48-hour culture of *Rhodococcus* species is growing. The colonies are relatively large, ranging from 2 to 4 mm in diameter, and are entire and smooth. Note the salmon-pink pigmentation, an initial clue to the identification of this species.

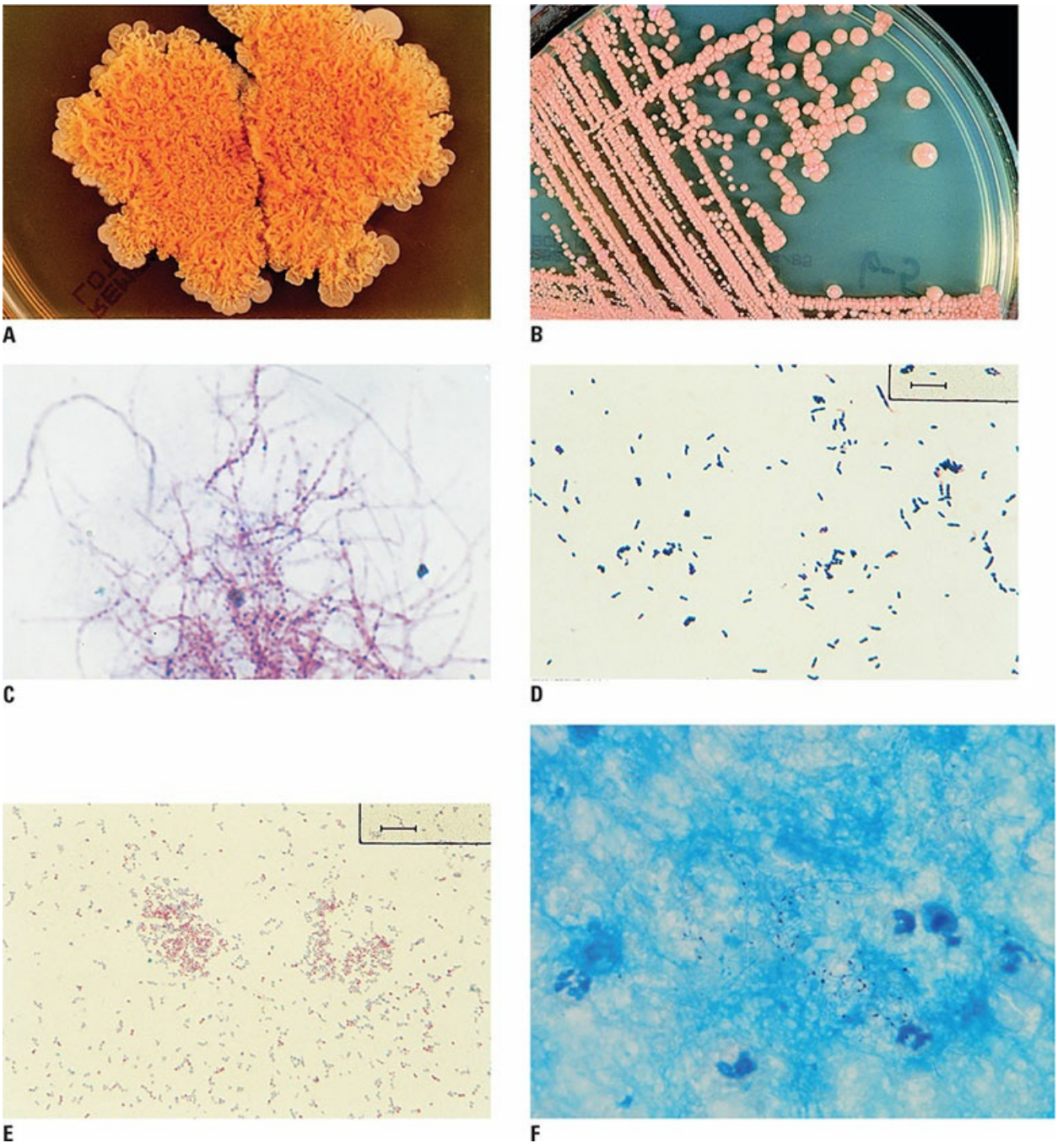
C. Modified or partial acid-fast stain of an exudate containing acid-fast, delicate, branching filaments characteristic of *Nocardia* species. *Nocardia asteroides* complex was recovered from the culture obtained from this specimen.

D. Gram's stain of *Rhodococcus* species illustrating short gram-positive coccobacilli arranged singly and in "Chinese letters" clusters.

E. Modified acid-fast stain of *Rhodococcus* species showing short coccobacilli, many of which are modified acid-fast-positive.

F. In some instances, when *Nocardia* is stained in direct clinical specimen, the filaments may not take up the stain and the organism stains in this "beaded" manner, wherein only the beaded foci are modified acid-fast-positive. Modified acid-fast stain, 1000× magnification.

IP-107



IP-108

IMAGE PLATE 16-1

IDENTIFICATION OF ANAEROBIC BACTERIA: GRAM-NEGATIVE BACILLI

A. *Bacteroides fragilis*. Gram's stain of cells in 48-hour thioglycolate broth culture.

B. *Bacteroides fragilis*. Colonies on anaerobic blood agar after 48 hours of incubation at 35°C.

C. *Prevotella melaninogenica*. Gram's stains of cells from a 48-hour colony on blood agar.

D. *Prevotella melaninogenica*. Black colonies on blood agar after 5 days of incubation at 35°C.

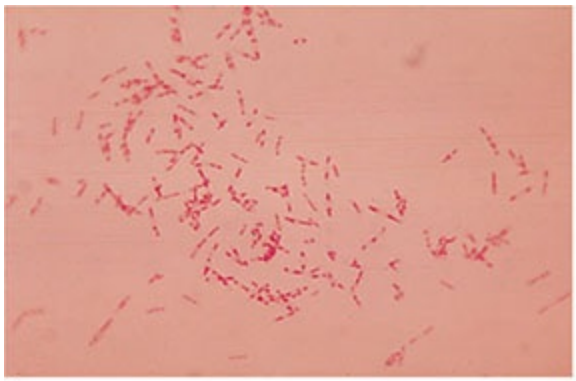
E. *Fusobacterium nucleatum*. Gram's stain of cells from a 48-hour colony on anaerobic blood agar. Note long, gram-negative bacilli with pointed ends.

F. *Fusobacterium nucleatum*. Characteristic colonies on anaerobic blood agar after 48 hours of incubation at 35°C, illustrating the opalescent effect.

G. *Fusobacterium necrophorum*. Gram's stain of cells from a 48-hour colony on anaerobic blood agar. Note pleomorphism.

H. *Fusobacterium necrophorum*. Colonies on anaerobic blood agar after 48 hours of incubation at 35°C.

IP-109



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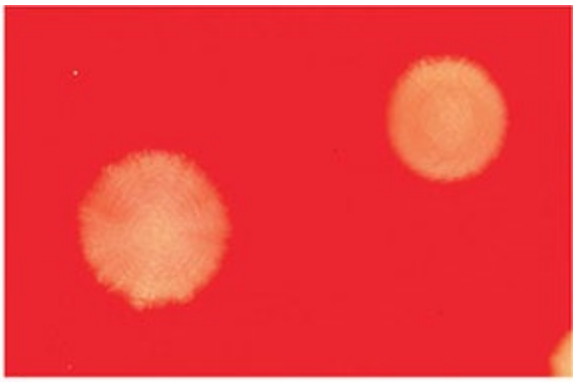

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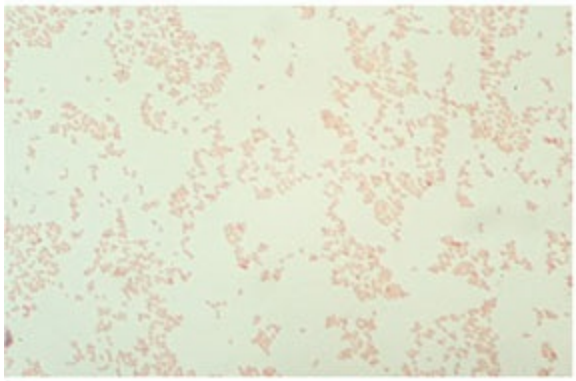

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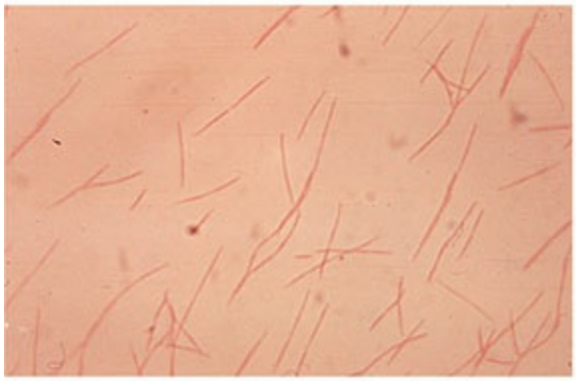

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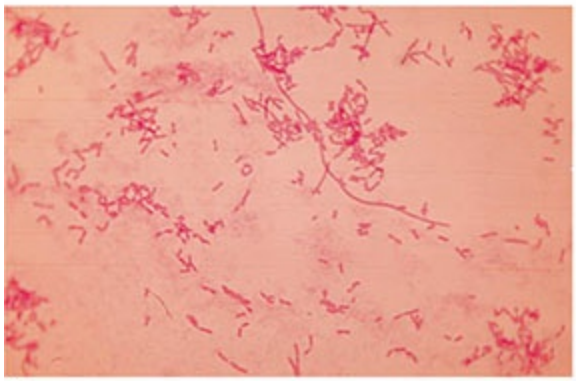

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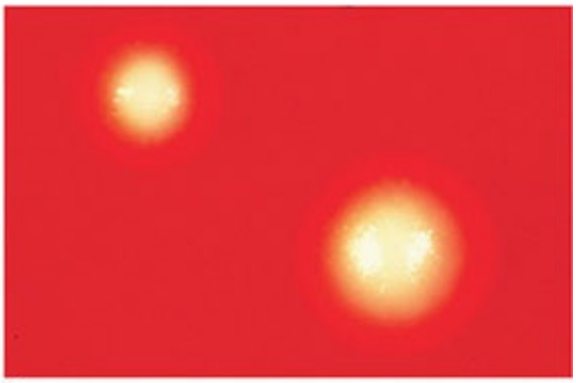


H

IP-110

IMAGE PLATE 16-2

IDENTIFICATION OF ANAEROBIC BACTERIA: GRAM-POSITIVE, NON–SPORE-FORMING ORGANISMS

A. Gram-stained direct smear of a purulent exudate showing degenerated neutrophils and a mixture of gram-negative bacilli of different sizes. The smallest coccobacilli suggest one of the pigmented *Prevotella–Porphyromonas* species; the larger, pleomorphic bacilli suggest a member of the *Bacteroides fragilis* group.

B. Gram-stained direct smear of a purulent exudate from an intra-abdominal abscess, showing segmented neutrophils, gram-positive cocci in pairs and short chains, and tiny gram-negative bacilli. Anaerobic infections usually contain a mixture of bacterial microbiota.

C. Dissecting microscope view of actinomycotic "sulfur granules." This exudate from an abdominal wound infection was photographed within a Petri dish.

D. Appearance of a Gram-stained smear from the same specimen as in C, showing a "sulfur granule" with thin, branching filaments of an *Actinomyces* species.

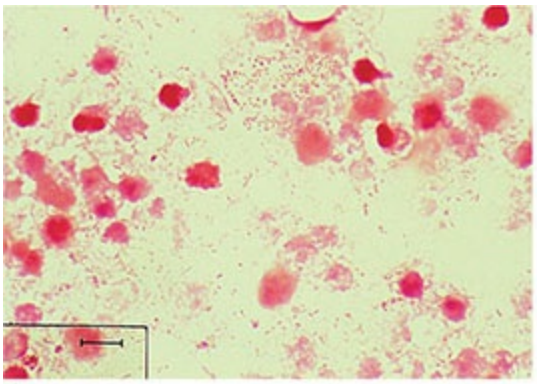
E. *Actinomyces israelii*. Gram-stained preparation of growth from a colony on blood agar. Note the branching of the cells.

F. The characteristic "molar tooth" colonies of *Actinomyces israelii* shown here were produced on brain–heart infusion agar after 7 days of anaerobic incubation at 35°C.

G. This Gram's stain demonstrates the cells of *Eubacterium alactolyticum* from growth in enriched thioglycolate broth after 48 hours of incubation at 35°C.

H. The colonies of *Eubacterium alactolyticum* on anaerobic blood agar are shown here after 48 hours of incubation.

IP-111



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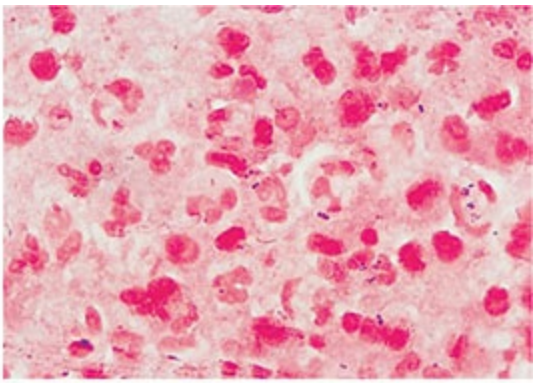

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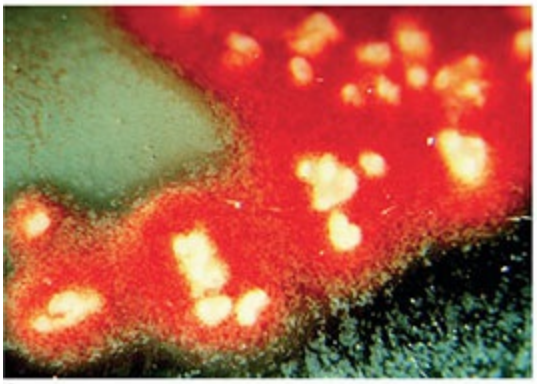

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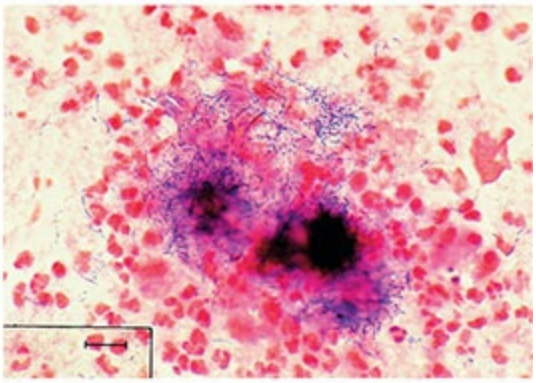

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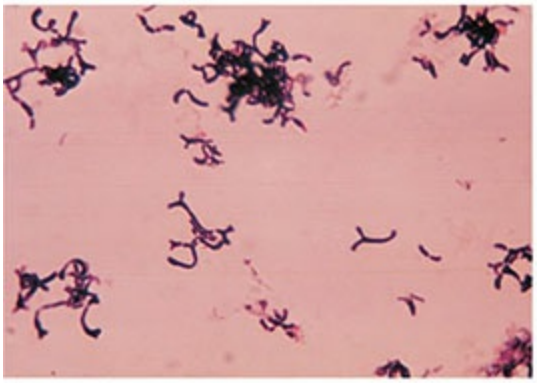

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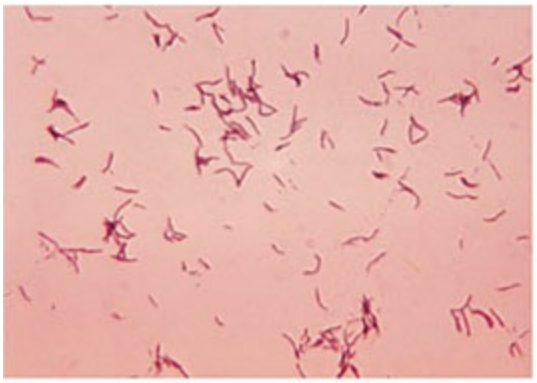

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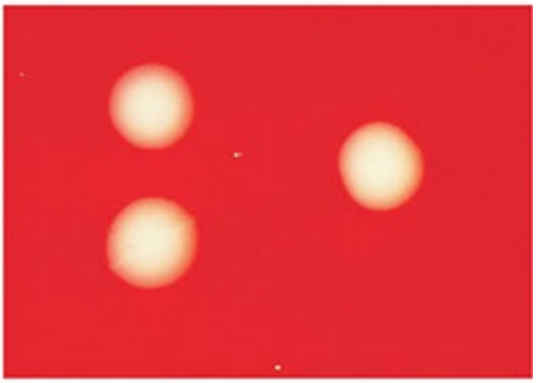


H

IP-112

IMAGE PLATE 16-3

IDENTIFICATION OF ANAEROBIC BACTERIA: CLOSTRIDIA

A. This is a Gram's stain of *Clostridium perfringens* from a 24-hour colony on blood agar. Note the lack of spores and that of some cells that tend to stain red (i.e., gram-negative).

B. This is a Gram's stain of *Clostridium perfringens* from a 24-hour thioglycolate broth culture. Note the lack of spores and presence of a few filamentous forms.

C. This is the typical appearance of *C. perfringens* on blood agar after 24 hours of incubation at 35°C. Note the double zone of hemolysis. The inner zone of complete hemolysis is due to theta-toxin and the outer zone of incomplete hemolysis to alpha-toxin (i.e., lecithinase activity).

D. *C. perfringens*. This is the direct Gram's stain of a muscle tissue aspirate from a patient with gas gangrene myonecrosis. There is a necrotic background, without intact inflammatory cells or muscle cells, and relatively large "box car"-shaped gram-positive bacilli and a gram-negative bacillus that is either a gram-variable *C. perfringens* cell or another organism.

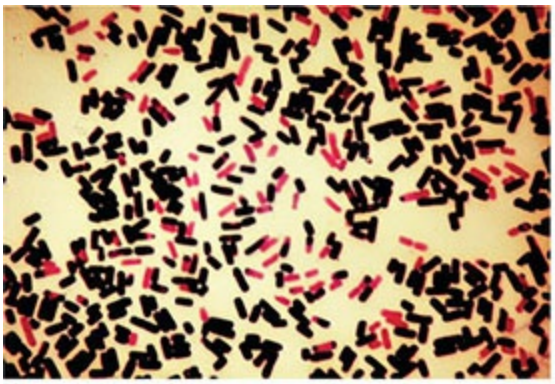
E. Colonies of *C. perfringens* on modified McClung egg yolk agar. The precipitate surrounding the colonies indicates lecithinase activity of alpha-toxin produced by the organism.

F. Lipase production on egg yolk agar. A few clostridia, such as *C. botulinum*, *C. sporogenes*, and *C. novyi* type A, exhibit lipase activity on egg yolk agar, as shown here. Note the iridescent pearly layer on the surface of the colonies extending onto the surface of the medium immediately surrounding them.

G. These rough, irregular, flat, rhizoid, spreading colony on a 48-hour anaerobic blood agar plate is characteristic of *C. septicum*.

H. This is a direct Gram-stained smear of a positive blood culture that contained *C. septicum*. Numerous ovoid or citron-shaped, subterminal spores are present.

IP-113



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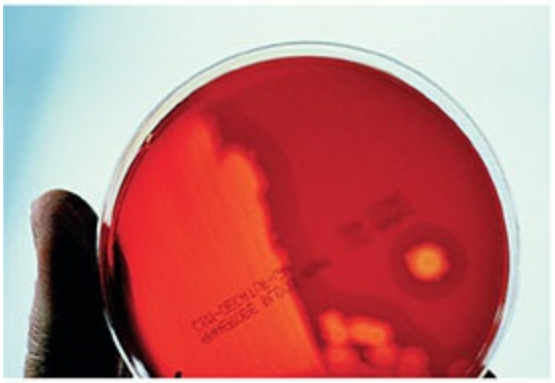

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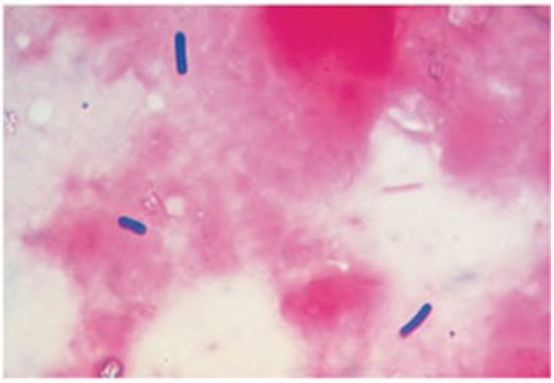

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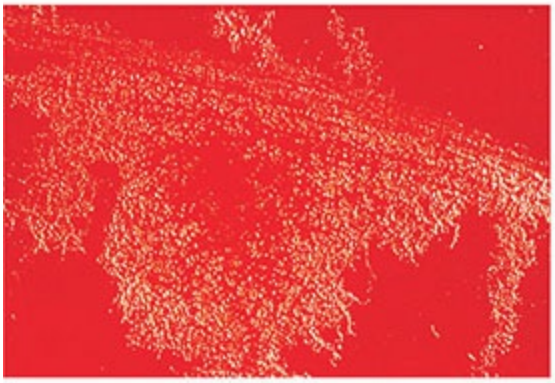

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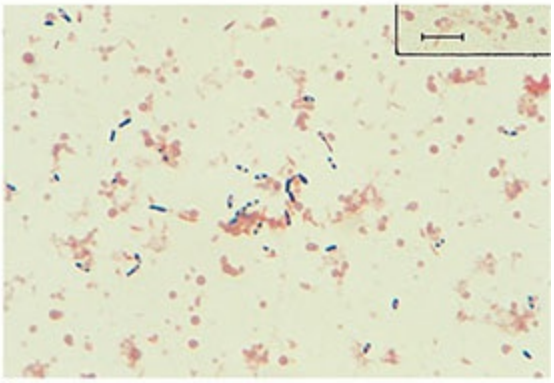


H

IP-114

IMAGE PLATE 16-4

IDENTIFICATION OF ANAEROBIC BACTERIA: CLOSTRIDIA (CONTINUED)

A. These are colonies of *C. tetani* on stiff blood agar (4% agar), which is used to inhibit the swarming of the microorganism so that it can be isolated from other bacteria present in mixed cultures.

B. This is a Gram's stain of *C. tetani* from a chopped meat glucose broth culture. Some of the cells have round, terminal spores, which are characteristic of *C. tetani*.

C. The "Medusa-head" colonies on this anaerobic blood agar after 48 hours of incubation are characteristic of *C. sporogenes*.

D. This Gram's stain of *C. ramosum* is from a thioglycolate broth culture after 48 hours of incubation.

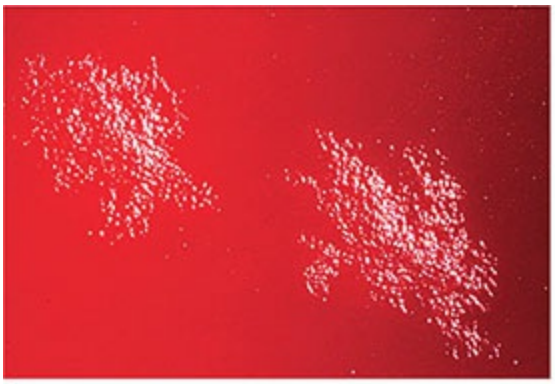
E. These are colonies of *C. difficile* on anaerobe blood agar after 48 hours of incubation.

F. This is *C. difficile* on cycloserine-cefoxitin fructose agar after 48 hours of incubation.

G. This is *C. sordellii* on an anaerobe blood agar following prolonged anaerobic incubation. Colony is 5 × 10 mm.

H. This Gram's stain of *C. sordellii* is from a 2-day-old blood agar plate. It reveals clumps of free spores and bacteria distended with ovoid subterminal spores.

IP-115



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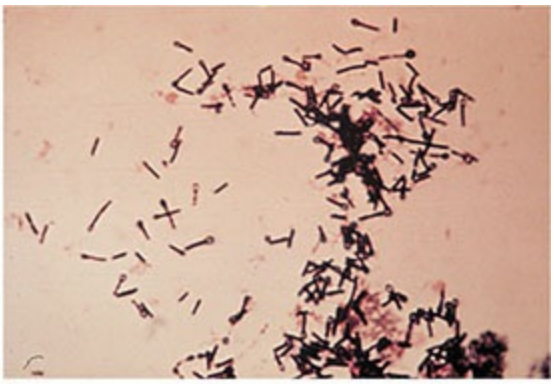

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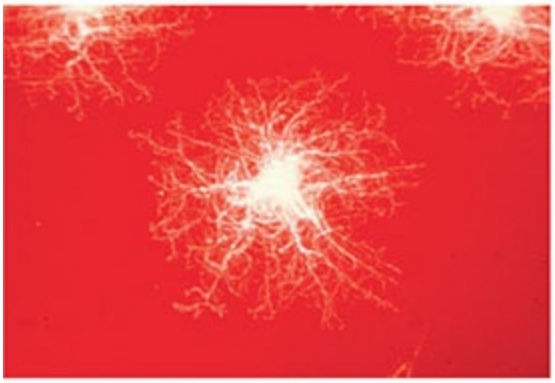

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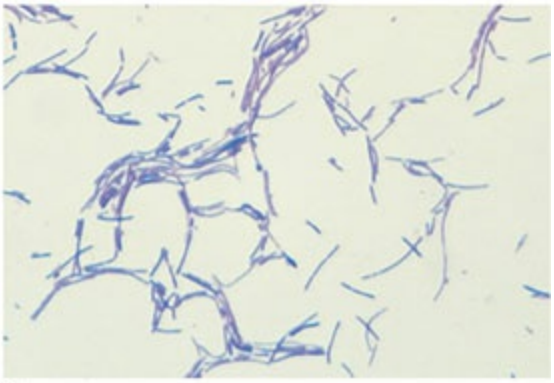

D

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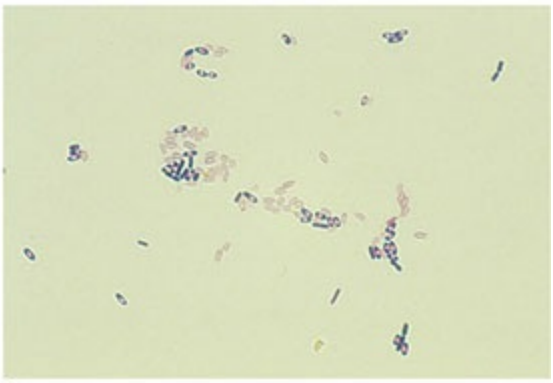
G



H

IMAGE PLATE 16-5

Identification of Anaerobic Bacteria: Use of Presumpto Quadrant Plates and Disks on Anaerobe Blood Agar

A. Overview appearance of three quadrant plates (described in detail in the text).

B. A Presumpto 1 quadrant plate and a plate of CDC anaerobe blood agar. After inoculation with an active broth culture or cell suspension of the isolate to be identified, antibiotic disks (penicillin, 2 units; rifampin, 15 g; kanamycin, 1,000 g) are placed on the blood agar medium, and a blank filter-paper disk is placed on the LD agar portion of the quadrant plate for use in detection of indole production. The Presumpto 1 plate contains the following media: LD agar, LD esculin, LD egg yolk agar, and LD bile agar.

C. Growth of *Bacteroides fragilis* on Presumpto 1 plate after 48 hours of incubation at 35°C. On the first quadrant (top, far right), LD agar shows moderate growth. Indole production can be detected by adding a drop of para-dimethylaminocinnamaldehyde to the paper disk (see **F**). The LD esculin agar to the left of the LD agar is diffusely dark because of the hydrolysis of the esculin. The LD egg yolk agar underneath the esculin agar shows good growth but no lecithinase, lipase, or proteolytic activity. There is abundant growth in the LD bile agar (bottom, far right), and a characteristic precipitate was produced in the medium by this strain.

D. Antibiotic disk tests. A zone of growth inhibition is seen around the 15-g rifampin disk, but no growth inhibition is seen around the 2-unit penicillin disk or the 1,000-g kanamycin disk. This pattern is characteristic of the *B. fragilis* group.

E. Reactions of *B. thetaiotaomicron* of the Presumpto 1 quadrant plate. The first quadrant (top, far right) shows a weak, positive indole reaction, as indicated by the pale blue color of the disk on LD agar after addition of para-dimethylaminocinnamaldehyde reagent. Black (amber) appearance of esculin agar (left of first quadrant) indicates esculin hydrolysis. There is adequate growth but no lecithinase, lipase, or proteolysis on LD egg yolk agar (bottom, far left). There is good growth on LD bile agar but no precipitate as exhibited by *B. fragilis* (bottom, far right).

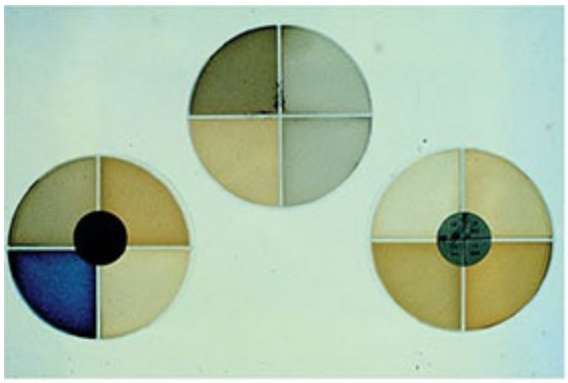
F. Reactions of *Fusobacterium necrophorum* on the Presumpto 1 quadrant plate after 48 hours of incubation at 35°C. The first quadrant (bottom, far right) shows strong

indole reaction as evidenced by the dark blue color of the paper disk on LD agar after the addition of para-dimethylaminocinnamaldehyde reagent. Growth is inhibited on LD bile agar (top, right). Although not visible in this photograph because of the lighting arrangement, there is good growth in LD egg yolk agar (top, far left) and characteristic lipase activity as evidenced by an iridescent sheen—a pearly layer—on the surface of the colonies and in the medium immediately surrounding the bacterial growth. This is best demonstrated with reflected light. On LD esculin agar (bottom, left), the *F. necrophorum* shows good growth and hydrogen sulfide production, as evidenced by the black appearance of the colonies, but no darkening of the medium to suggest esculin hydrolysis.

G. Presumpto 2 quadrant plate (uninoculated). The plate contains the following quadrants (clockwise, starting at 12 o'clock): LD starch agar, LD glucose agar, LD-DNA agar, and LD milk agar.

H. Presumpto 2 quadrant plate following inoculation, incubation, and addition of reagents. Note positive reactions for hydrolysis, deoxyribonuclease activity, and glucose fermentation.

IP-117



A

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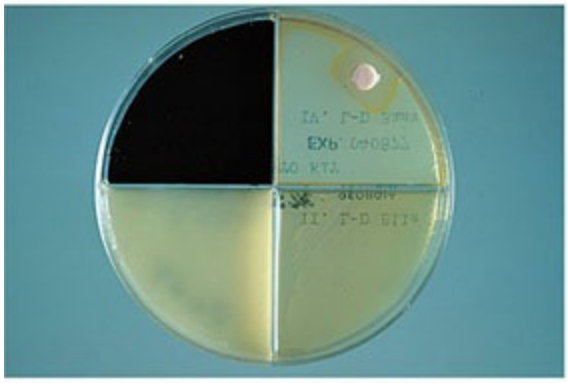

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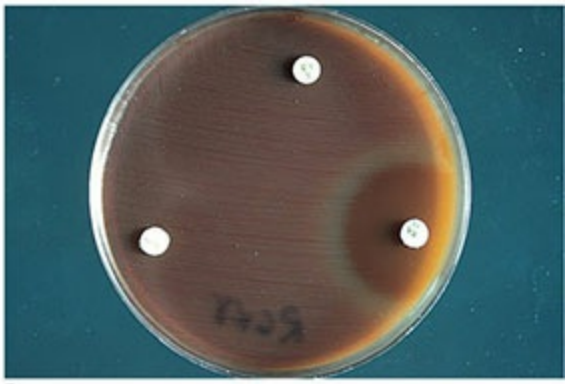

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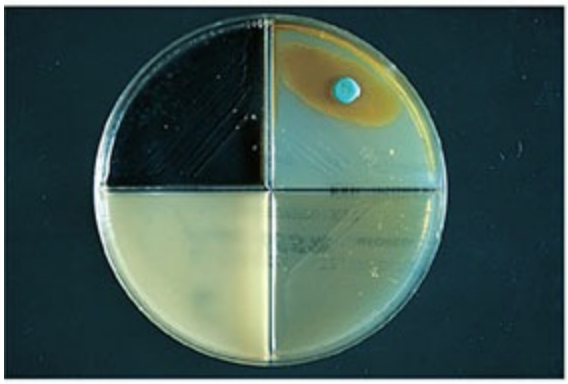

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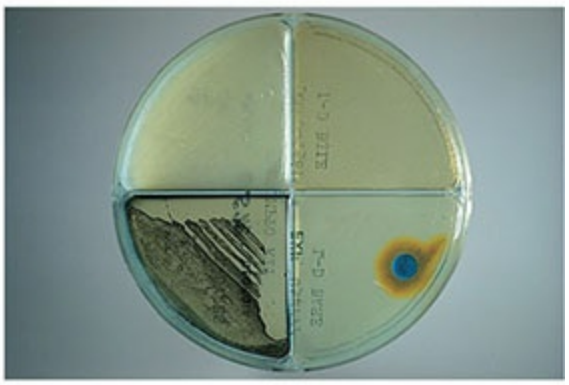

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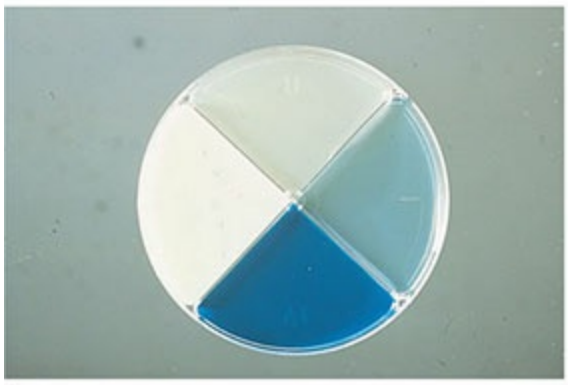

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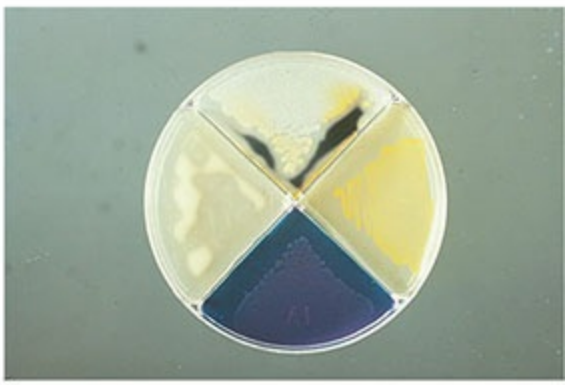


H

IP-118

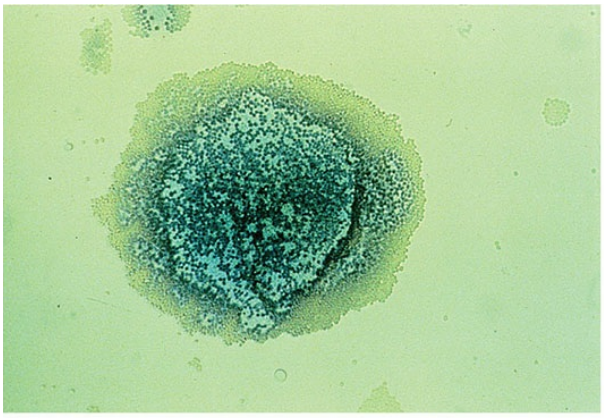
IMAGE PLATE 18-1

MYCOPLASMAS AND UREAPLASMAS

A. Colony of *M. pneumoniae* on mycoplasma glucose agar. This photograph shows a single colony of the organism to which guinea pig erythrocytes have become adsorbed. *M. pneumoniae* is the only species that demonstrates this hemadsorbing property. (Photograph courtesy of Health and Education Resources, Bethesda, MD.)

B. Colonies of *M. hominis* and *U. urealyticum* growing on Shepard's A7B differential agar. *M. hominis* colonies have the typical "fried egg" morphology; the blue color is due to the Diene's stain. *U. urealyticum* colonies are smaller, denser, and have a brownish pigmentation because of precipitation of manganese oxide in the colony due to the action of urease enzymes. (Photograph courtesy of Health and Education Resources, Bethesda, MD.)

IP-119



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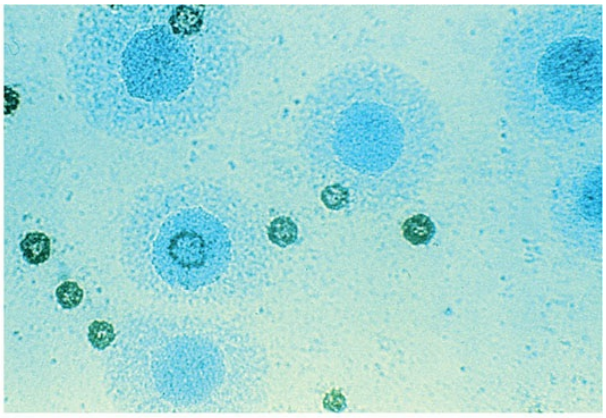


B

IP-120

IMAGE PLATE 19-1

THE LABORATORY IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS*

A. This photomicrograph is of a smear prepared from a colony similar to that

illustrated in Image Plate 19-1C, stained with auramine–rhodamine. Note the bright yellow fluorescence of the very short, slightly curved bacilli.

B. This photomicrograph is of a smear prepared from material obtained from a necrotic tuberculoma of the lung stained with the Kinyoun acid-fast stain. Note the relatively short, thin, beaded, slightly curved, red-staining acid-fast bacilli.

C. A microcolony growing on the surface of Middlebrook 7H10 agar, observed microscopically under low-power optics. Note that the bacterial cells are arranged in an undulating, serpiginous pattern. This indicates the production of cording factor, as illustrated in the acid-fast stain shown in Image Plate 19-1I.

D. A niacin-accumulation test. The tubes contain a filter paper strip impregnated with cyanogen bromide. The fluid in the tube on the right had been extracted from the surface of Löwenstein–Jensen culture medium after colonial growth of an unknown *Mycobacterium* species, compared with a negative water control on the left. The development of a yellow color on the strip and in the underlying fluid indicates the presence of niacin. *M. tuberculosis* has the uncommon property of accumulating niacin when colonies are grown on Löwenstein–Jensen medium, a property that is shared by the less commonly encountered species, such as *M. simiae* and occasional strains of *M. marinum*.

E. Tubes illustrating the nitrate-reduction test. The tube on the right had been inoculated with an unknown *Mycobacterium* species, which is compared with an uninoculated control on the left. The development of a red color after addition of sulfonamide and α-naphthylethylenediamine reagents indicates the presence of nitrites and a positive test. *M. tuberculosis* is an active nitrate reducer.

F. A quadrant plate containing Middlebrook 7H10 agar in which thiophene-2-carboxylic acid hydrazide (T2H) had been incorporated. The three quadrants in which growth is observed are different strains of *M. tuberculosis* (i.e., resistant to T2H); the quadrant without growth was inoculated with a strain of *M. bovis*. The ability to grow or not grow on medium containing T2H is helpful in separating *M. tuberculosis* from *M. bovis*, respectively.

G. A flask of Löwenstein–Jensen medium on which are growing colonies of *M. tuberculosis* after a 22-day incubation at 35°C in a 10% CO_2 incubator. The colonies seen here are distinctly rough in consistency. Although this isolate was determined to be *M. tuberculosis,* it should be considered unusual, as it has more of a yellow pigmentation than the buff-colored colonies usually observed (**H**).

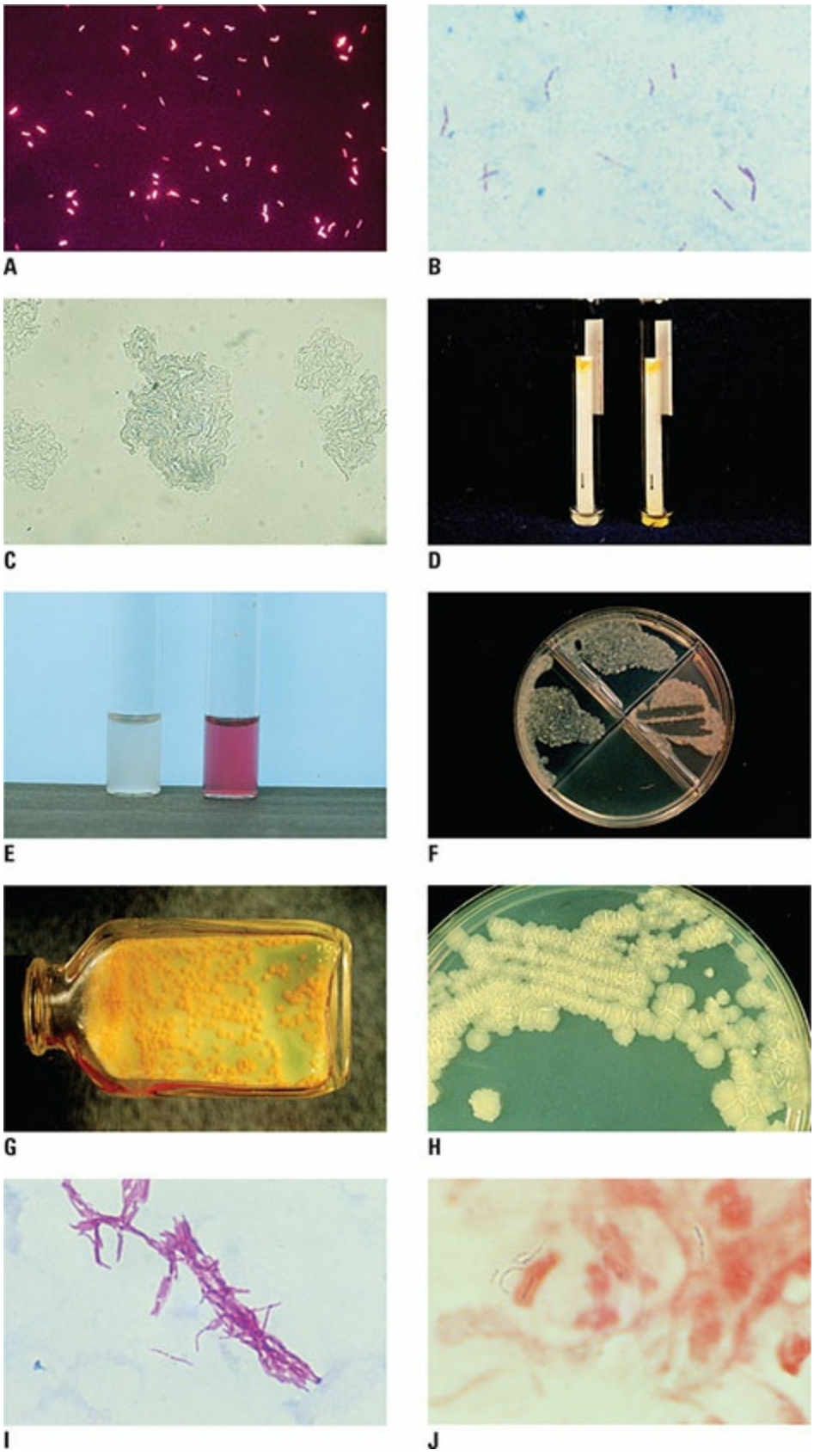
H. These are typical colonies of a subculture of *M. tuberculosis* growing on Middlebrook 7H10 agar after a 25-day incubation at 35°C in 10% CO_2 incubator.

The colonies have a rough, buff appearance, characteristic for the species.

I. This photomicrograph is of a Kinyoun acid-fast stain prepared from the broth of a BACTEC 12A blood culture vial after a 10-day incubation at 35°C. Numerous acid-fast bacilli are seen, arranged in parallel sheaths. When the aggregate of bacterial cells, as illustrated here, is observed in smears prepared from a broth preparation, the isolate is likely *M. tuberculosis*, because the phenomenon is due to the production of cording factor, thought to be a virulence factor.

J. This photomicrograph is of a Gram's stain prepared from a smear of pleural fluid from a patient with AIDS who developed an empyema. Note the relatively long, slender, delicately beaded, poorly staining gram-positive bacilli. Because of their thick waxy outer coating, the bacterial cells of mycobacteria do not stain well with crystal violet, the primary stain used in the Gram's stain. *M. tuberculosis* grew out in the culture from this patient specimen.

IP-121



A B C D E F G H I J

IMAGE PLATE 19-2

LABORATORY IDENTIFICATION OF *MYCOBACTERIUM* SPECIES OTHER THAN *M. TUBERCULOSIS*

A. Photomicrograph of microcolonies of *M. avium* growing on Middlebrook 7H10 agar after a 6-day incubation at 35°C in 10% CO_2. The flat, thin, translucent microcolonies, with a central darker umbonate elevation, suggestive of a fried egg, are characteristic of the smooth strains of *M. avium* commonly recovered from patients with AIDS. The observation of this type of microcolony may be helpful in directing one to perform an *M. avium*/*M. intracellulare* complex nucleic acid probe in the rapid culture confirmation of an unknown isolate.

B. Close-up photomicrograph of a single microcolony of *M. avium*/*M. intracellulare* complex, again illustrating the thin, translucent colony described in Image Plate 19-2A. The rhizoid-like peripheral extensions, as illustrated here, are often seen and provide an added clue to the presumptive identification of *M. avium*/*M. intracellulare* complex organisms.

C. Two Middlebrook 7H10 agar plates, each inoculated with the same subculture of *M. kansasii*. The plate on the left was exposed to light; the one on the right remained in the dark until just before the photograph was taken. The yellow pigment of the colonies in the light-exposed plate is characteristic of a photochromogen.

D. Two Middlebrook 7H10 agar plates each inoculated with the same subculture of *M. scrofulaceum*. The plate on the left was exposed to light; the one on the right remained in the dark until just before the photograph was taken. The yellow pigment of the colonies in both the light-exposed and non–light-exposed plates is characteristic of a scotochromogen.

E. A Middlebrook 7H10 agar plate previously inoculated with a strain of *M. gordonae*. This organism is a scotochromogen and characteristically produces a deep yellow pigment, as illustrated here.

F. Several tubes containing varying concentrations of buffered sodium nitrate inoculated with various strains of *Mycobacterium* species to illustrate 1+ (second tube from the left) through 4+ (rightmost tube) reactions. The red color develops after the addition of sulfanilamide and *N*-naphthylethylenediamine reagents; the intensity of color reflects the concentration of nitrites produced. The quantitative

nitrate-reduction test may be helpful in differentiating strains of *M. tuberculosis* (4+ positive) from *M. bovis* (1+ positive), and *M. kansasii* (4+ positive) from other photochromogens (negative to 1+ positive).

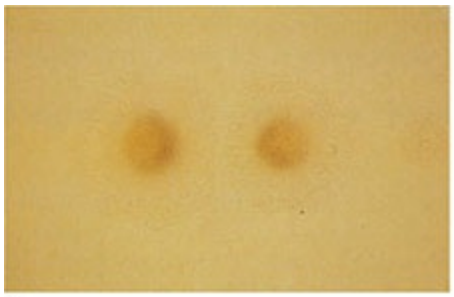
G. Two tubes containing Tween 80 reagent. The tube on the left had been inoculated with a negative control strain, whereas the tube on the right with a subculture of *M. kansasii*. The development of a red color (right tube) after incubation for 3 to 10 days is a positive test, indicating the ability of the test strain to hydrolyze Tween 80. The color change from yellow to red in this assay is not due to a shift in pH; rather, it results from a change in optical density as oleic acid and polyoxyethylated sorbitol are produced as breakdown products of Tween hydrolysis.

H. Two tubes of Löwenstein–Jensen deeps previously inoculated with a catalase-negative control (left tube) and a subculture of *M. kansasii* (right tube). Note the tall column of bubbles formed in the right tube after the addition of hydrogen peroxide to the surface of the agar. A column exceeding 45 mm is indicative of a positive test and strong catalase activity.

I. A positive catalase test. Immediately before the photomicrograph was taken, a few drops of hydrogen peroxide were placed on isolated colonies of the photochromogenic colonies of *M. kansasii*. The evolution of rapid bubbles, as illustrated here, indicates a positive test, indicating active catalase activity.

J. Two tubes of Dubos broth base. The right tube contains the tripotassium phenolphthalein disulfate reagent (arylsulfatase reagent) compared with a negative control tube on the left devoid of reagent. Both tubes had been previously inoculated with a subculture of a strain

IP-123



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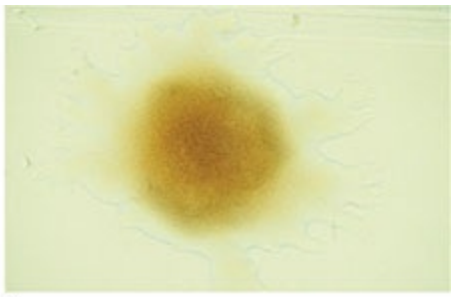

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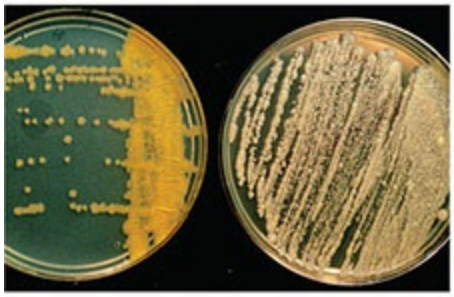

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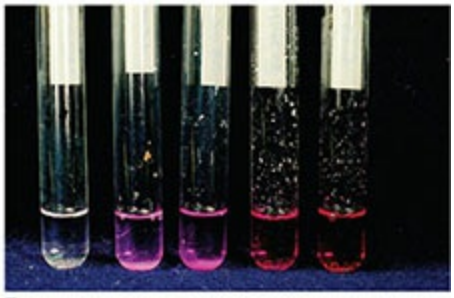

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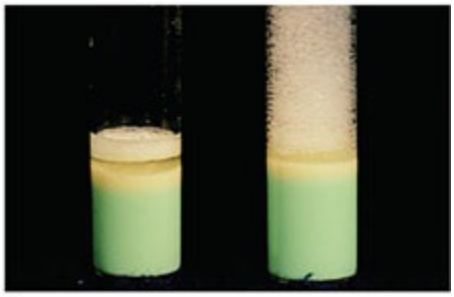

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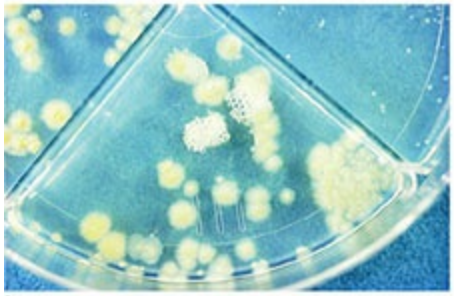


I

J

of *M. fortuitum*. The development of a pink color after the addition of sodium carbonate reagent in the right tube indicates a positive test and arylsulfatase activity of the test strain.

K. Two tubes of urea broth previously inoculated with a negative control (left tube) and a subculture of *M. gordonae* (right). Mycobacterial strains having urease activity will turn the broth pink within several hours (strong positive) to a few days, owing to the release of ammonia and alkalinization of the medium. Assessment of urease activity may be helpful in separating *M. gordonae* (strong positive) from other scotochromogens, and *M. fortuitum*/*M. chelonae* from other rapidly growing mycobacteria.

L. Two tubes of Dubos broth base. The tube on the left contains pyrazinamide, whereas the tube on the right is a negative control devoid of reagent. Both tubes had previously been inoculated with a subculture of a strain of *M. avium*. The development of a pink-red band at the reagent layer toward the surface of the agar in the left tube, after addition of ferrous ammonium sulfate reagent, indicates a positive test and the ability of this strain to deaminate pyrazinamide.

M. Photomicrograph of an acid-fast smear prepared from a subculture of *M. kansasii*. The bacterial cells of *M. kansasii* are described as rectangular, straight, and banded, which is reasonably represented here. Because of variation between strains and between other species, a presumptive identification of *Mycobacterium* species based on acid-fast stain morphology is not recommended; the banding of *M. kansasii,* however, is the best indicator of this particular species to the experienced mycobacteriologist.

N. A Middlebrook 7H10 agar plate previously inoculated with a subculture of a classical strain of *M. avium*. The classical strains of *M. avium* were originally included in the group III non-photochromogens in the Runyon classification. Note that the colonies here are relatively small, smooth, and gray-white, without evidence of pigmentation.

O. A Middlebrook 7H10 agar plate previously inoculated with a strain of the *M. avium*/*M. intracellulare* (MAI) complex recovered from a patient with AIDS. Many of the MAI strains recovered from patients with AIDS have this distinct yellow pigmentation. The peculiar doughnut-shaped colonies are a well-described colonial morphology, but not so for all strains.

P. Low-power photomicrograph of a liver biopsy from a patient with AIDS who was

also infected with a member of the *M. avium*/*M. intracellulare* complex. Note the tight intracellular clusters of short, acid-fast bacilli in the central part of the photograph.

Q. A Middlebrook 7H10 agar plate inoculated 3 days before with a strain of *M. fortuitum* and incubated at 35°C in 10% CO_2. The colonies average 1 to 2 mm in diameter and are entire, smooth, moist, and devoid of pigmentation. *Mycobacterium fortuitum* is one of the rapidly growing mycobacteria that were classified by Runyon as group IV.

R. A modified MacConkey agar plate, which is devoid of crystal violet, demonstrates colonies of *M. fortuitum*. Strains of *M. fortuitum*-chelonae have the unique ability to grow on modified MacConkey agar. The colonies average 2 mm in diameter and are entire, moist, and have a slight pink-yellow tinge due to pigment derived from the agar.

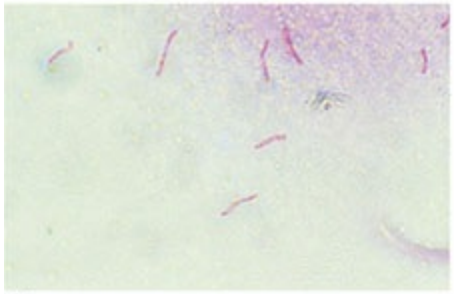
S. A 5% sheep blood agar plate with a 48-hour growth on *M. fortuitum*. Rapidly growing mycobacteria such as this will grow on routine 5% sheep blood agar within 48 to 72 hours and may cause confusion with other gram-positive bacillary organisms if a partial acid-fast stain is not performed.

T. Photomicrograph of a Gram's stain of a smear prepared from an isolated colony illustrated in Image Plate 19-2S. The bacterial cells appear as short, slender, almost filamentous, faintly staining gram-positive coccobacilli. Microscopists must be alerted to these microscopic findings and not make a presumptive misidentification of an *Actinomyces* species or *Corynebacterium*. An acid-fast stain of a second smear will be helpful in making the correct identification.

IP-125

K

L



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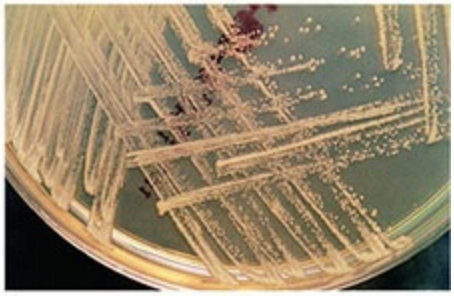

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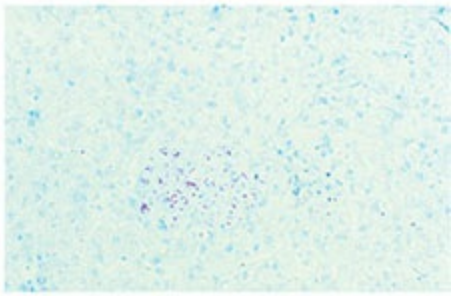

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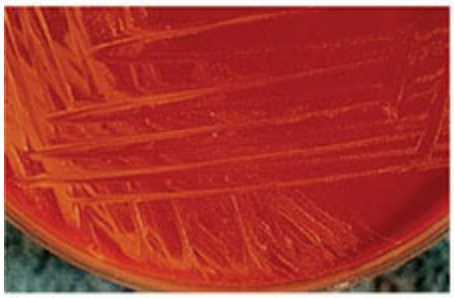

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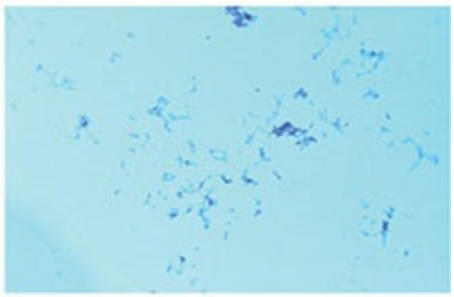


T

IP-126

IMAGE PLATE 19-3

Clinical Manifestations of Select Mycobacterial Diseases

A. Chest roentgenogram from a patient with pulmonary tuberculosis. Note the extensive area of dense infiltration of the right upper lobe.

B. A solitary nodular lesion with well-defined margins is seen in the upper segment of the left lower lung field. A coin lesion of pulmonary coccidioidomycosis, histoplasmosis, and cryptococcosis must be included in the differential diagnosis of such a lesion, as well as malignancy. This lesion turned out to be a tuberculoma.

C. A resected lobe of lung illustrating centrally a well-circumscribed, 2.5-cm-diameter, white, firm-appearing nodule. This histologically was a tuberculoma, similar to what may have been removed from the patient whose chest radiograph is shown in Image Plate 19-3B.

D. This photomicrograph of a hematoxylin and eosin-stained section taken from a tuberculoma illustrates varying degrees of background fibrosis and a cellular infiltration composed of lymphocytes and macrophages, many of the latter forming characteristic Langhans giant cells.

E. The base of the brain in a patient dying from tuberculous meningitis. The pia arachnoid covering the base of the brain, particularly in the area of the optic chiasm, appears cloudy, as a manifestation of the chronic inflammatory reaction.

F. A patient with cutaneous tuberculosis of the fifth digit. The lesion shown here appears active and inflammatory. The radiograph revealed bone and joint involvement of the middle and distal phalanx. Lesions of the fingers similar to that shown here were, at one time, common among individuals who milked cows.

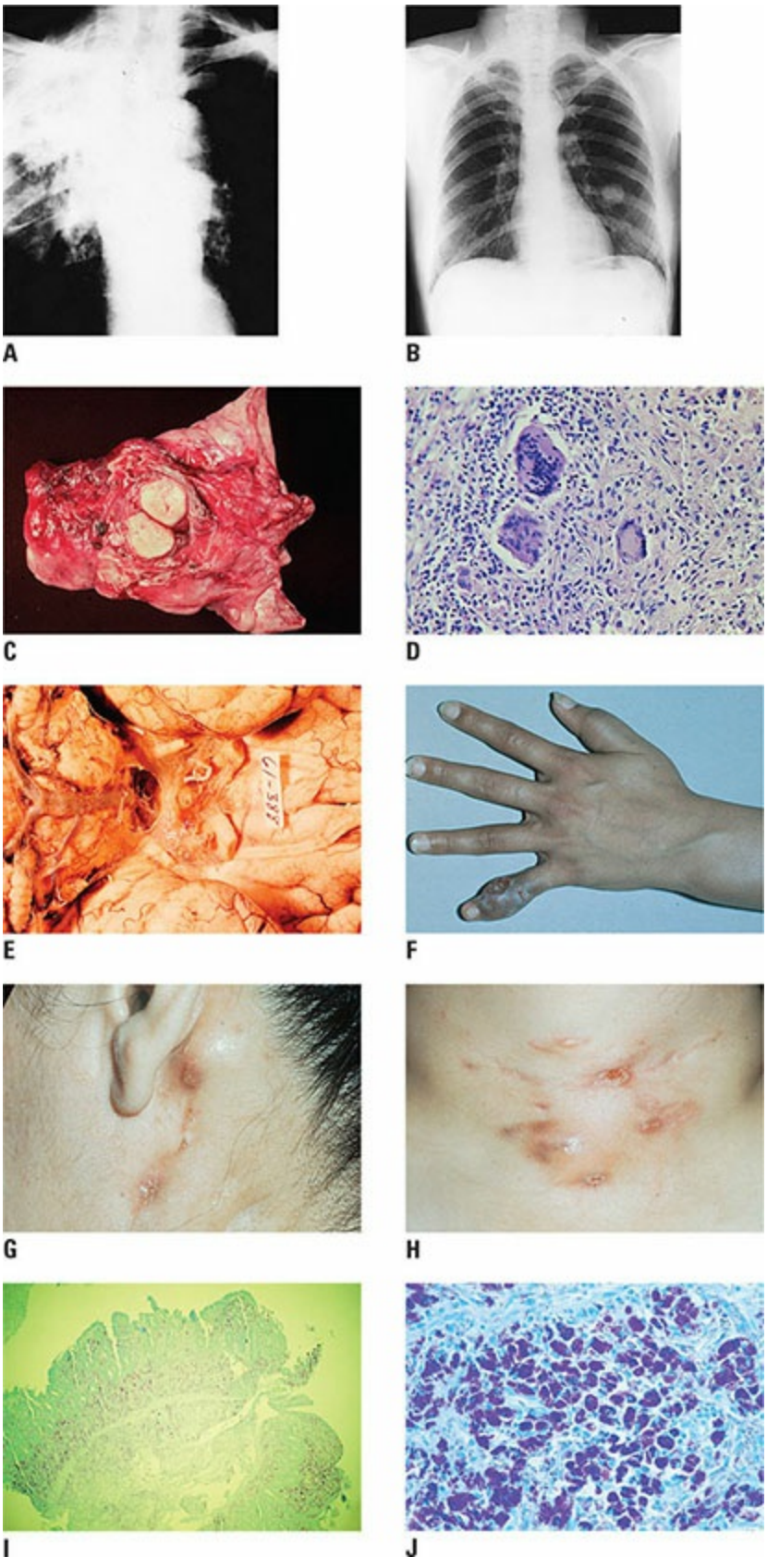
G. The posterior neck of this patient demonstrates an enlarged, postauricular lymph node with healed draining sinuses along the route of the lymphatic drainage. This is a classical picture of "scrofula" or mycobacterial lymphadenitis, typically caused by *M. scrofulaceum* and mycobacteria other than *M. tuberculosis*, such as *M. avium*. The route of infection is oral, and commonly occurs in two age groups during periods of teething—in children 3 to 7 years of age, when secondary teeth are coming in, and in persons in their late teen years, when molars are being cut. The causative agent of mycobacterial lymphadenitis in areas with a high prevalence of tuberculosis is *M. tuberculosis*.

H. The anterior portion of the neck illustrates multiple healed sinus tracts in this patient with chronic tuberculous lymphadenitis.

I. Low-power photomicrograph of a section of small bowel stained with a carbol-fuchsin-based acid-fast stain. The pink mottling seen in the submucosa is dense aggregates of acid-fast bacilli from a patient with AIDS with an intestinal infection caused by *M. avium*/*M. intracellulare* complex.

J. High-power photomicrograph of an area shown in Image Plate 19-3I, better illustrating the dense intracellular aggregates of acid-fast bacilli.

IP-127



IP-128

IMAGE PLATE 20-1

Laboratory Diagnosis of Spirochetal Diseases

A. *Treponema pallidum*. This photomicrograph shows a Warthin–Starry stain of a histologic section of placenta from a patient with congenital syphilis. The tight spiral coils of the spirochete are clearly evident (original magnification, ×1,000).

B. *Treponema pallidum*. This control for the FTA-ABS test demonstrates 4+ staining of the spirochetes. The helical shape is evident, and the tightly coiled spirals can be visualized in some of the bacterial cells. This photomicrograph is an indirect immunofluorescence preparation of *T. pallidum* using fluorescein-conjugated antihuman globulin (original magnification, ×600). (Courtesy of Burton Wilcke, PhD, and Mary Celotti, Vermont Department of Public Health, Burlington, VT.)

C. Wright-stained peripheral blood smear from a case of *Borrelia* infection. The loose coils of the *Borrelia* organisms are evident, but the thin structure is easy to overlook. These spirochetes may be detected when examining smears for malaria or for cellular morphology, even if the physician does not suspect the diagnosis (original magnification, ×1,000). (Courtesy of Thomas Fritsche, MD, PhD.)

D. An acridine orange stain of peripheral blood demonstrates *Borrelia* spp. dramatically. If the diagnosis is suspected, this technique, which may be more sensitive than Giemsa or Wright's stains, may be used (original magnification, ×1000). (Courtesy of Brian Lauer, MD.)

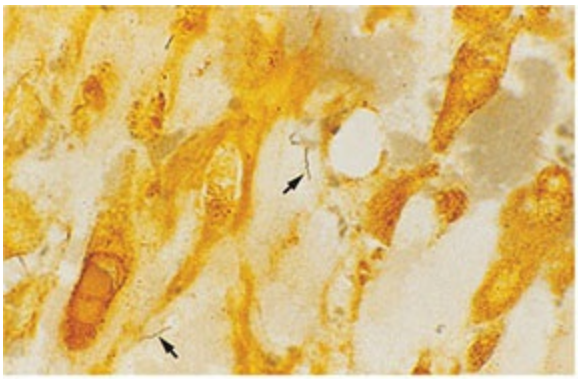
E. *Ixodes scapularis* (*dammini*) ticks. These dark-colored hard ticks, also known as black-legged ticks or deer ticks, contain a dorsal scutum that covers the whole back on the male (M) but only a portion of the back on the female (F), nymph (N), and larva (L). It is the principal vector of *Borrelia burgdorferi*, the agent of Lyme disease, in the eastern United States. *Ixodes pacificus*, the vector in the Pacific region, looks very similar. The primary vectors are nymphs and adult females. Adult males bite only rarely, and the larval stage has not usually been infected when it bites humans. Nymphal ticks are a particular problem, because their small size makes it difficult to detect their presence.

F. *Borrelia burgdorferi* in a culture pellet stained with a monoclonal antibody to OspA protein conjugated to streptavidin-alkaline phosphatase. The typical polymorphism of *B. burgdorferi* is demonstrated. (Courtesy of Paul Duray, MD.)

G. A histologic section of dog kidney stained with a silver impregnation stain (Warthin–Starry) demonstrates several renal tubules in longitudinal orientation. Even at low power, the masses of *Leptospira* organisms are evident as blackening of the surface of the tubular epithelium. It is clear that this privileged site, where the leptospires are protected from host defenses, can serve as a productive source for spread of infection as the leptospira-contaminated urine is passed onto the ground (original magnification, ×1000). (Courtesy of David Miller, DVM, MS.)

H. Tubes of polysorbate 80-bovine albumin (PSO-BA) semisolid medium. An uninoculated tube is shown on the left. The tube on the right was inoculated with *Leptospira interrogans*. The subsurface growth in semisolid culture media is typical of leptospires, such as *L. interrogans* sv. *pomona*. Growth appears as a gray-white horizontal band about 1 cm below the medium surface after incubation for 2 to 6 weeks at room temperature. The band grows denser with continued incubation, but may be very scant with fastidious isolates, such as with *L. interrogans* sv. *hardjo*. (Courtesy of David Miller, DVM, MS.)

IP-129



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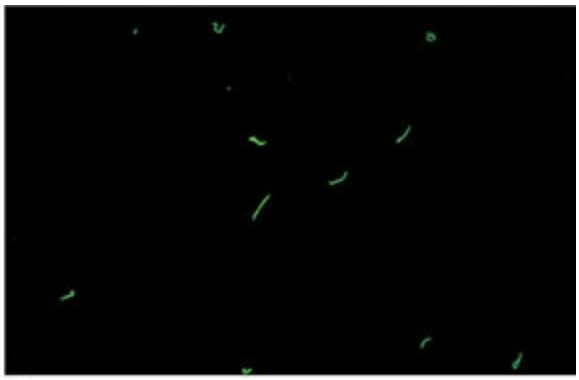

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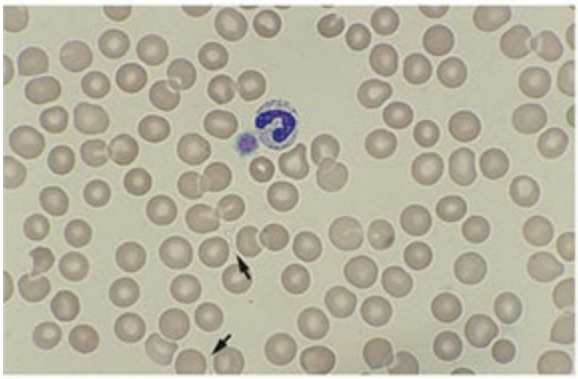

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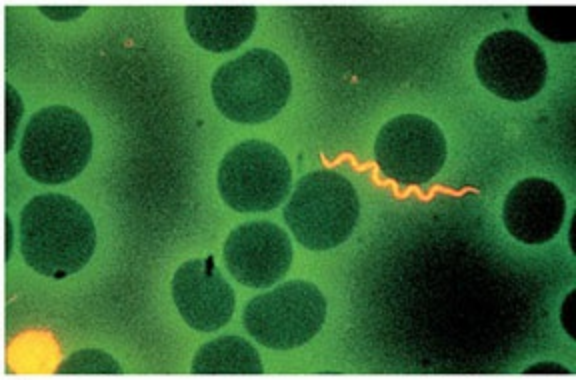

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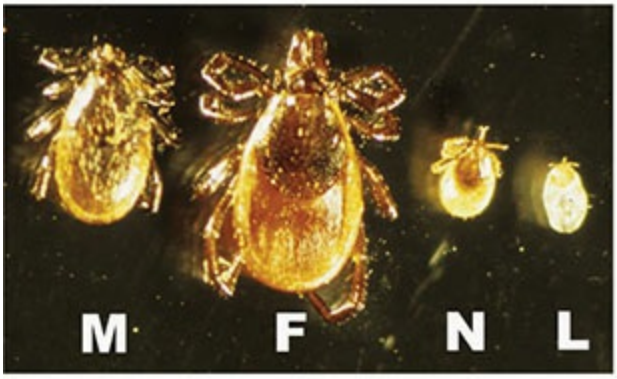

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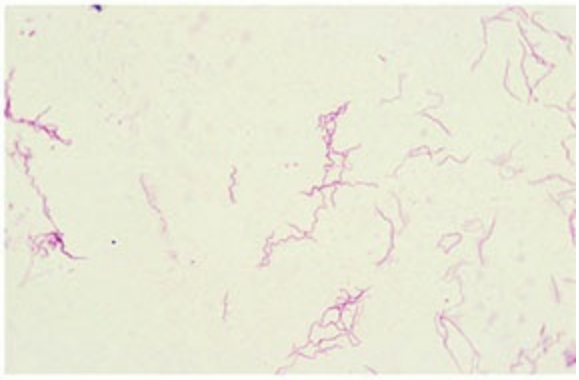

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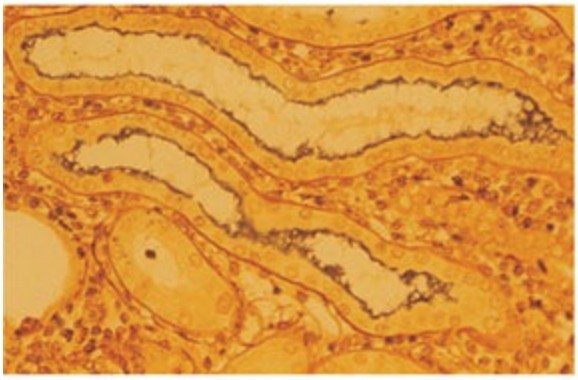

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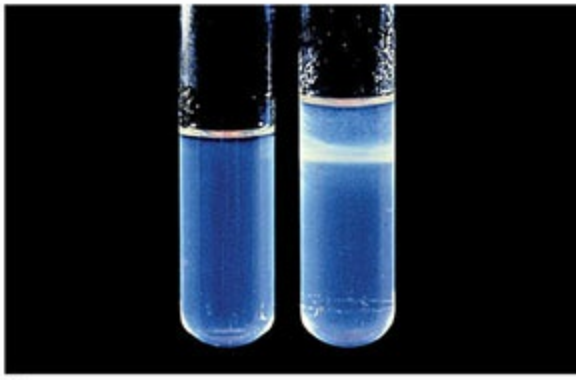

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IP-130

IMAGE PLATE 21-1

COLONY MORPHOLOGY OF *ZYGOMYCETES* SPECIES AND SELECT *ASPERGILLUS* SPECIES

Members of the *Zygomycetes* and certain *Aspergillus* species are commonly encountered in clinical laboratories as agents of opportunistic infections. These fungi grow rapidly in culture, particularly the *Zygomycetes* species, which can fill a culture plate within 48 hours. Of the aspergilli, *A. fumigatus*, *A. flavus*, *A. niger,* and *A. terreus* are the most commonly recovered from specimens obtained from patients with aspergillosis. Other species, when recovered, are usually not identified to the species level. *A nidulans* and *A. glaucus* are two uncommonly encountered species that often produce cleistothecia, which are sexual reproductive structures that contain ascospores.

A. Sabouraud's dextrose agar plate inoculated with a *Zygomycetes* species. Notice that the entire plate is filled with a wooly, gray-white surface mycelium.

B. Close-up view of a colony of a *Zygomycetes* species after 96 hours of incubation. As this fungus colony matures, it takes on a gray-brown color, often with black, pepper-like stipples, indicating the production of sporangia.

C. The surface of a colony of *Aspergillus fumigatus* after a 5-day incubation on Sabouraud's dextrose agar. Mature colonies of *A. fumigatus* are generally powdery or granular, and have some shade of green-blue from the production of pigmented conidia. The growing margin often appears as a white apron, as illustrated here, which represents the outward growth of new mycelium.

D. The surface of a colony of *Aspergillus flavus* growing on Sabouraud's dextrose agar. The texture of mature colonies is usually granular from the production of conidia, and as the species name suggests, some shade of yellow pigmentation is characteristic, which becomes mixed with dark green with time.

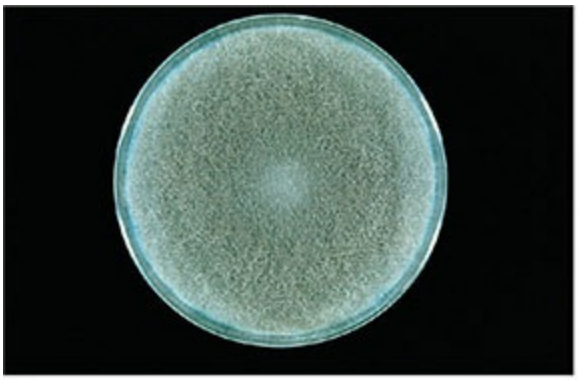
E. The surface of a colony of *Aspergillus niger* after a 4-day incubation on Sabouraud's dextrose agar. The deep black, densely stippled surface is characteristic. The black surface pigmentation of *A. niger* can be distinguished from one of the dematiaceous fungi by observing the reverse side. The reverse of *A. niger* is a light gray or buff color, as the pigmentation is caused by surface conidia; in contrast, the reverse of dematiaceous fungi is jet black, as the vegetative hyphae contain the pigment.

F. The surface of a colony of *Aspergillus terreus* after a 6-day incubation on Sabouraud's dextrose agar. *A. terreus* characteristically has a granular surface and some shade of yellow or brown pigmentation, often described as cinnamon. Rugae radiating from the center are often seen, and the concentric zones of light and dark pigmentation as seen here are not unusual.

G. The surface of an albino strain of *Aspergillus nidulans*. Although most strains have some light pigmentation, albino strains of most *Aspergillus* species may occasionally be encountered and must be kept in the differential identification. The granular surface, with radiating rugae, is also common.

H. The surface of a colony of *Aspergillus glaucus*. The green and yellow variegated appearance seen here is not specific, because other *Aspergillus* species may have a similar appearance. Microscopic examination is necessary to make a species identification.

IP-131



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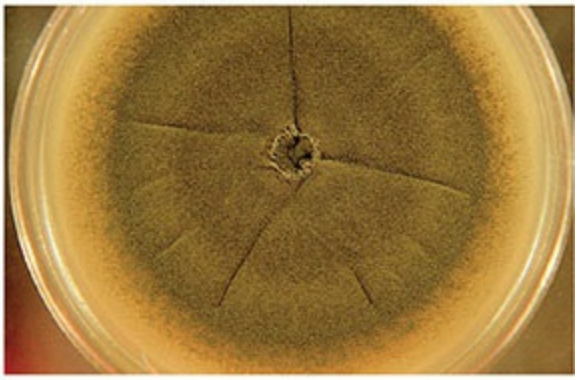

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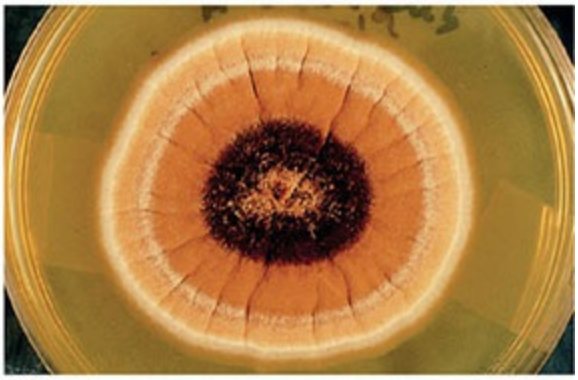

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IP-132

IMAGE PLATE 21-2

Colony Morphology of Other Commonly Encountered Hyaline Molds

The colonies of the hyaline fungi have a varied appearance. Most display light pastel or deeper pigmentation, with combinations of green, yellow, orange, and brown hues commonly observed. The texture of the colonies may be smooth (glabrous), granular, cottony, or wooly, depending on their maturity and degree of sporulation. Microscopic studies must be performed to confirm the genus and species identification.

A. The surface of a colony of *Penicillium* species after a 5-day incubation on Sabouraud's dextrose agar, illustrating the distinctive green color, granular surface, radial rugae, and white apron at the periphery. Most strains of *Penicillium* species display some shade of green; colony colors, as well as microscopic features, are useful for differentiating *Penicillum* species from *Paecilomyces*.

B. The surface of a colony of *Penicillium marneffei,* a dimorphic fungus, after a 4-day incubation on Sabouraud's dextrose agar, illustrating the distinctive red pigmentation of a portion of the colony. Note that the pigment has diffused out into the agar, which has a light wine-red coloration. The surface tends to be granular to fluffy, depending on the degree of sporulation; note the distinct white apron at the margin of peripheral new growth.

C. The surface of a colony of *Paecilomyces variotii*. The colonies of *Paecilomyces* species are usually granular and often have a yellow-brown pigmentation, as illustrated here.

D. The surface of a colony of *Scopulariopsis brevicaulis* after 5 days of growth on Sabouraud's dextrose agar. The yellow pigmentation, granular texture, and radiating rugae, as illustrated here, are characteristic.

E. The surface of a colony of an *Acremonium* species after 5 days of incubation on Sabouraud's dextrose agar. The colonies of *Acremonium* may have a smooth, almost yeast-like appearance, as illustrated here, in the early phase of growth. These will become more mold-like, with production of delicate hyphae and fruiting bodies. Light pastel yellow, light green, and peach-red hues may be observed.

F. The surface of a typical colony of a *Fusarium* species after 6 days of growth on Sabouraud's dextrose agar. *Fusarium* species can be suspected when a rapidly

growing granular or fluffy colony, with a distinct rose-red, lavender, or purple pigmentation, is observed.

G. The surface of a typical fluffy, "house mouse gray" colony of a member of the *Pseudallescheria boydii* complex after 5 days of incubation on Sabouraud's dextrose agar. A rapidly growing colony with the "mouse gray" appearance, as illustrated here, always suggests this fungus. The unique pigmentation is caused by darkly pigmented conidia.

H. A colony of a *Gliocladium* species after 5 days of growth on Sabouraud's dextrose agar. The green pigment, extension of the colony from border to border without a well-defined growing margin, and granular texture (i.e., a so-called green lawn) are unique for *Gliocladium* and *Trichoderma* species. The latter more frequently may show a yellow, rather than a green, pigmentation.

IP-133

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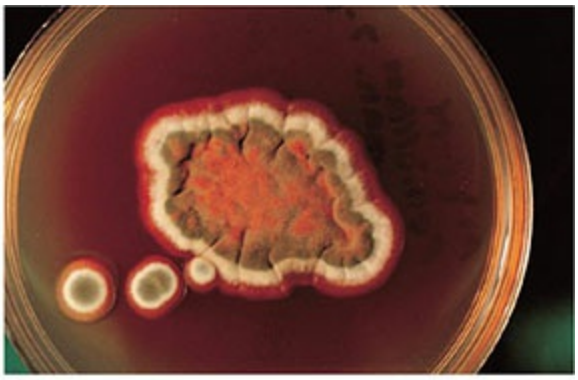

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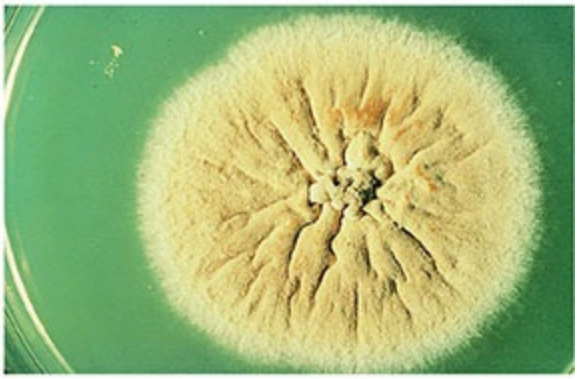

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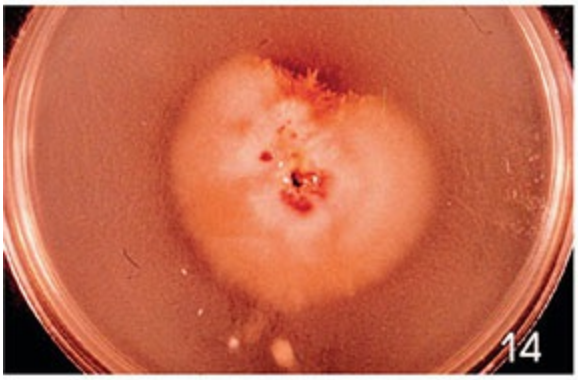

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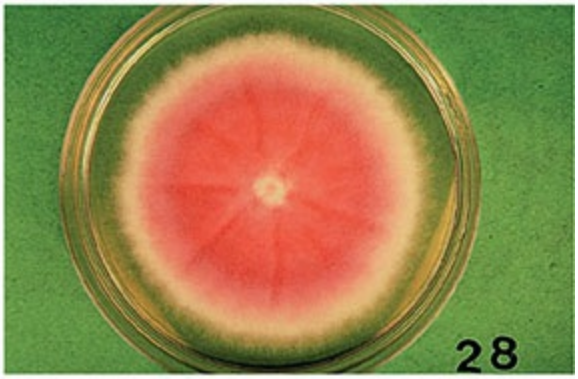

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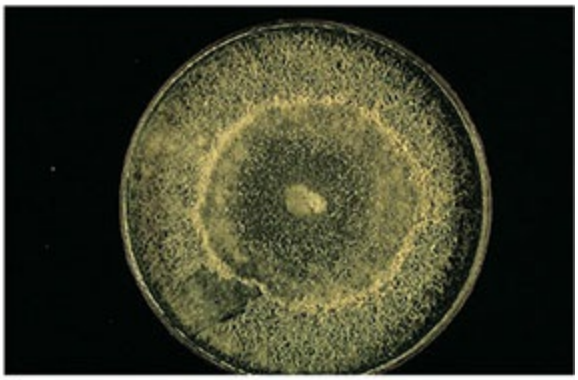

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IP-134

IMAGE PLATE 21-3

COLONY MORPHOLOGY OF COMMONLY ENCOUNTERED DEMATIACEOUS MOLDS

The dematiaceous (dark) molds are characterized by the growth of deep green, brown, or black colonies, with black pigmentation on the reverse surface. Most environmental species grow rapidly, producing mature colonies within 5 days. Slower-growing species, at times requiring 2 weeks or more before mature colonies are observed, are often recovered from clinical specimens taken from patients with phaeohyphomycosis, mycetomas, or chromomycosis. Photographs of representative colonies of select dematiaceous molds growing on Sabouraud's dextrose agar are illustrated here.

A. Fluffy, gray-black surface of a colony of an *Alternaria* species after 6 days of growth. The appearance shown here is characteristic, but not distinctive for an *Alternaria* species, but may also be observed with other species of environmental dematiaceous molds.

B. Reverse surface of a dematiaceous mold, illustrating the dark appearance caused by the dark brown pigmentation of the vegetative hyphae.

C. Surface view of a subculture of an *Ulocladium* species, illustrating a wooly, dark brown-to-black pigmentation. Although frequently seen with certain strains of dematiaceous fungi, the light and darker concentric rings observed here are not distinctive for any given species.

D. Surface view of a colony of *Phialophora verrucosum* after 14 days of incubation. This particular strain has a dark gray-black hue, with a hair-like texture. Microscopic study is necessary, because other slow-growing species may produce similar-appearing colonies.

E. Surface view of a colony of *Fonsecaea pedrosoi* after 16 days of incubation. The colony is quite small, typical for the species, and the typical dark brown-black pigmentation is evident. This strain has a flatter, almost suede-like or velvet-like consistency.

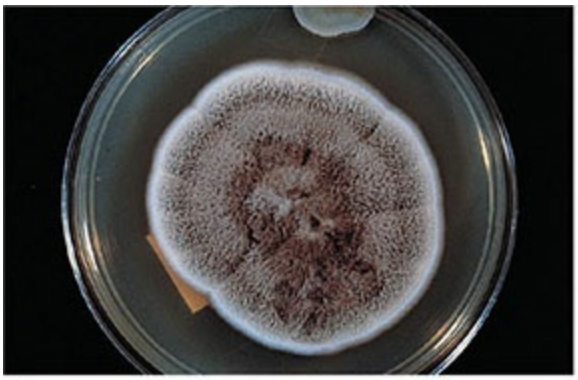
F. Surface view of a colony of a more rapidly growing (5 days) environmental strain of *Cladosporium* species, illustrating a dark green, suede-like surface interrupted by irregular rugae. Environmental strains of *Cladosporium* species may appear dark green, as shown here, or may be gray, gray-brown, or brown-black,

simulating other dematiaceous fungi.

G. Surface view of another variant of an environmental *Cladosporium* species showing a dark gray, leather-like consistency. Colony texture among the dematiaceous molds range from wooly, to downy or hair-like, to suede-like, to leathery.

H. Appearance of the flat, smooth, yeast-like surface of *Aureobasidium pullulans* after 6 days of incubation. *Aureobasidium pullulans* and, morphologically similar, *Hormonema dematioides* should be considered when a tan-to-white yeast-like colony is isolated that darkens into a dematiaceous fungus with time. When the yeast-like colony is brown-to-black from first isolation, then an *Exophiala* species should be considered.

IP-135



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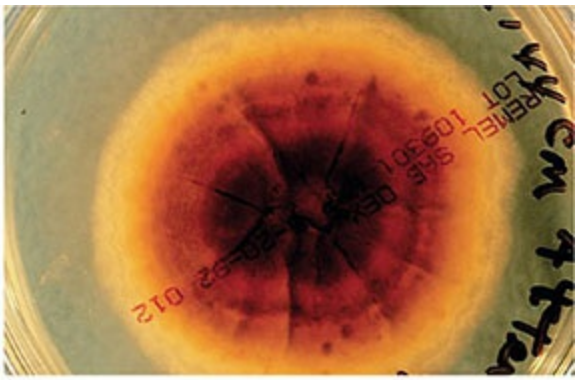

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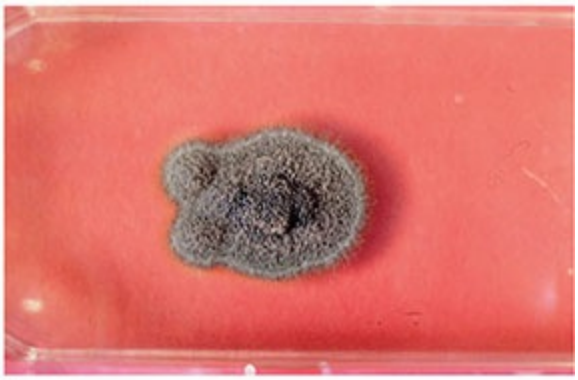

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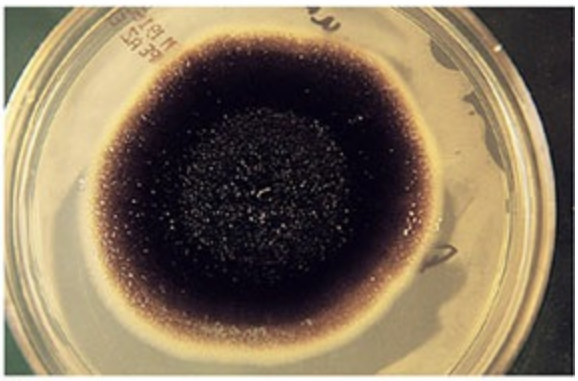

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IP-136

IMAGE PLATE 21-4

COLONY MORPHOLOGY OF DERMATOPHYTES

The colonies representative of the dermatophytic molds on culture media are not distinctive. There is sufficient strain variation and differences in appearance of the same strain, depending on the type of culture medium used and the environmental conditions during incubation, that colonial morphology is not a reliable criterion for making presumptive species-level identifications in most instances. Microscopic studies are almost always necessary to derive a definitive identification. Illustrated here are photographs of the colonies of select dermatophytes growing on Sabouraud's dextrose agar at 30°C, chosen to show certain characteristics that may offer clues to making a presumptive identification.

A. A colony of *Microsporum canis* after 5 days of incubation. The colonies tend to be fluffy or, at times, granular if conidiation is heavy. The lemon-yellow apron seen here, with pigment also extending to the reverse surface, is one clue suggesting *M. canis*.

B. Surface of a powdery, off-yellow colony of *Microsporum gypseum* after 6 days of incubation. *M. gypseum* tends to sporulate heavily; therefore, it is more prone to produce granular colonies than *M. canis*.

C. The surface of a smooth, white variant of *Trichophyton mentagrophytes*, with no evidence of background pigmentation of the agar. The colony illustrated here is not distinctive, and microscopic study is required to make an identification.

D. Surface of a fluffy variant of *Trichophyton mentagrophytes* demonstrating a reddish pigmentation of the background agar. Wine-red pigmentation is one feature that suggests *T. rubrum*; however, certain strains of *T. mentagrophytes* may also produce reddish pigment when growing on Sabouraud's dextrose agar. The pigmentation of these isolates, however, is never as intense as with *T. rubrum* when colonies are grown on cornmeal or potato dextrose agar.

E. The reverse of a colony of *Trichophyton rubrum* growing on potato dextrose agar. The deep wine-red pigmentation is characteristic of the species, and is particularly intense when the colony is grown on potato dextrose or cornmeal agar.

F. Comparative growth of *T. mentagrophytes* (bottom) and *T. rubrum* (top) on cornmeal agar, illustrating the distinct difference in pigmentation between the two

species.

G. The surface of a small colony of *Trichophyton tonsurans* after 14 days of incubation is illustrated. *T. tonsurans* grows slower on Sabouraud's dextrose agar. Most strains produce some shade of yellow-brown pigment. The surface tends to be granular and radial rugae are frequently seen.

H. The surface of *Epidermophyton floccosum* after 6 days of incubation. This colony appears more fluffy than granular and has an off-yellow pigmentation. Classical colonies of *E. floccosum* are described as khaki green. The colony texture tends to be more fluffy than granular, as microconidia are not produced by this genus.

IP-137

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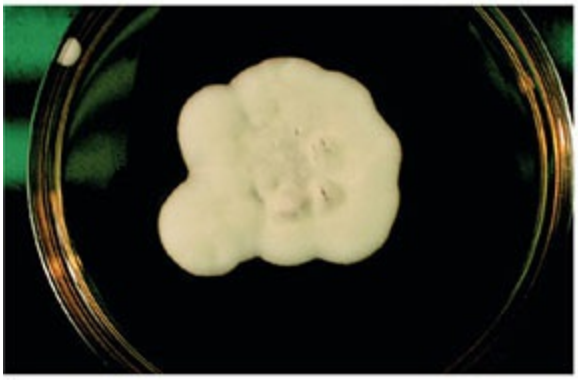

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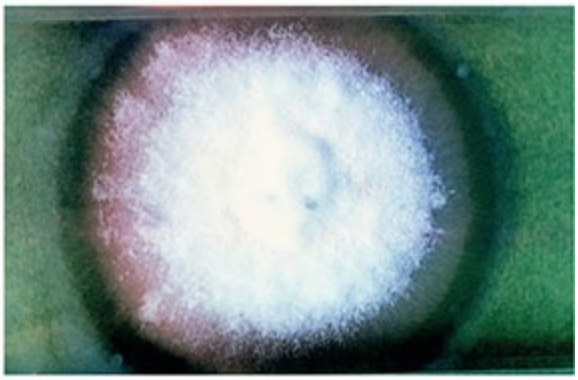

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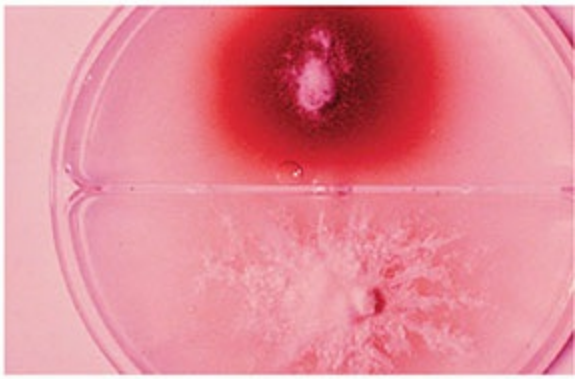

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IMAGE PLATE 21-5

COLONY MORPHOLOGY OF DIMORPHIC FUNGI

The dimorphic fungi, so-called because they grow in a mold form at 25°C to 30°C (i.e., room temperature) and a yeast or yeast-like form within the human body and, with the exception of *Coccidioides* species, at 37°C. These fungi are pathogenic in humans and are the agents of the deep-seated mycoses. Although definitive species identification depends on microscopic study, confirmation that a given species belongs to the dimorphic group depends on either molecular confirmation or by demonstrating both the mold and yeast forms in laboratory isolates. The mold form may be converted to the yeast form by incubating subcultures on enriched media at 37°C and observing microscopically for the appearance of yeast forms typical of the species. *Coccidioides* species are the exception and are not thermally dimorphic. In the past, the exoantigen technique was used to more rapidly confirm the identification of a dimorphic fungus, but this has largely been replaced by commercially-available nucleic-acid probe assays.

A. The surface colony of a dimorphic mold, illustrating evidence of the residual yeast form centrally, which has not yet converted to the mold form, which is seen at the periphery. This colony was incubated for 14 days at 30°C. When this mixture of yeast and mold texture is observed, one of the dimorphic fungi can be suspected, particularly if the colony is relatively slow-growing and the mold appears more hair-like or cobweb-like in consistency.

B. Colonies of *Blastomyces dermatitidis* growing on 5% sheep blood agar after 5 days of incubation. Note the cobweb-like appearance of these colonies, a clue that the isolate may be a dimorphic fungus. All molds should be handled within appropriate biologic safety cabinets to avoid a laboratory-acquired infection.

C. Appearance of colonies of *Blastomyces dermatitidis* incubated at 37°C, during a mold-to-yeast conversion. There is an intermediate form, shown here, of the colonies between mold and yeast, known as the "prickly stage." Note the rather coarse spicules formed by these white colonies. A microscopic preparation would show a mixture of hyphae, budding yeasts, and forms intermediated between the two.

D. Colonies of *Histoplasma capsulatum* on brain–heart infusion agar after 25 days of incubation at 30°C. The colonies appear white and cobweb-like in consistency.

These colonies are nondescript and could represent a variety of filamentous fungi. Microscopic examination is necessary to make an identification.

E. Surface of a 5% sheep blood agar plate incubated at 37°C on which are growing small, yellowish yeast colonies of *Histoplasma capsulatum* after a successful conversion from the mold form. The yeast forms are similar to other true yeasts, except that growth is very slow-growing and the colonies remain quite small.

F. The surface of colonies of *Coccidioides immitis* growing on 5% sheep blood agar after 7 days of incubation. The colonies are gray-white and have a delicate hair-like texture. Colonies of *C. immitis* growing on blood agar often show a red discoloration from leaching of hemoglobin from the culture medium (not shown here).

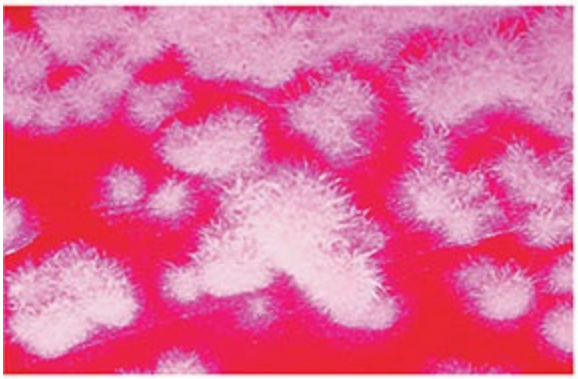
G. The surface of a smooth, gray-brown yeast colony of *Sporothrix schenckii*. Colonies of *S. schenckii* often appear more yeast-like than mold-like in the early phase of recovery, even when incubated at 25°C to 30°C, then these become more filamentous with extended incubation. After prolonged incubation, colonies of *S. schenckii* also tend to darken considerably, becoming almost jet black with some strains (see Image Plate 21-5H).

H. Tubes of brain–heart infusion agar that contrast the yeast (top) and mold (bottom) forms of *S. schenckii*. The mold form is after extended incubation wherein the colonies tend to darken with maturity, becoming distinctly black in some cases, as illustrated here.

IP-139

A

B



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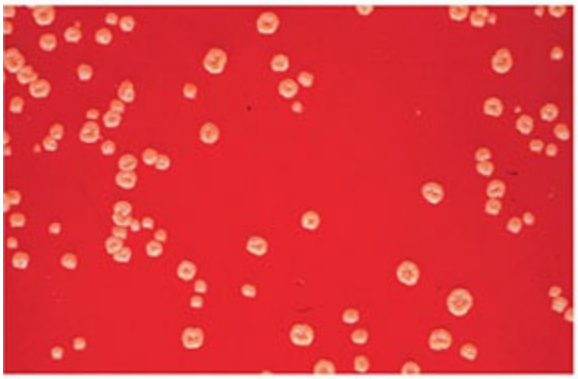

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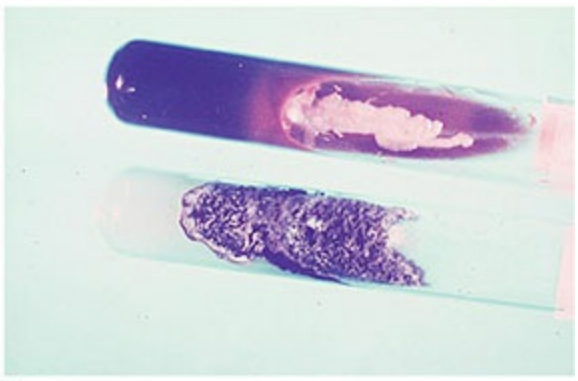


H

IP-140

IMAGE PLATE 21-6

MORPHOLOGY OF COMMONLY RECOVERED YEASTS

The laboratory identification of medically important yeasts involves a visual assessment of colony characteristics and the interpretation of supplemental results. Traditionally, these have included carbohydrate fermentation and assimilation tests, which are still used as needed. More commonly, MALDI-TOF and, in some instances, molecular assays are being used to identify these fungi.

A. The surface of a smooth, off-white yeast colony, obtained from a subculture of *Candida albicans* after 3 days of incubation, is demonstrated. The colony shown here is not distinctive for *C. albicans*, but may be seen with other *Candida* species and yeasts of other species. Additional studies are required when a yeast-like colony, as illustrated here, is recovered.

B. Surface of 5% sheep blood agar plate on which are growing white colonies of a yeast. The foot-like extensions from the margins of the colonies are typically produced by *C. albicans*.

C. Colonies of *Cryptococcus neoformans* on Sabouraud's dextrose agar after 4 days of incubation. The distinct mucoid consistency of the colonies is indicative of a capsule-forming yeast, suggesting a *Cryptococcus* species. Additional tests are required to identify *C. neoformans*.

D. Colonies of *Cryptococcus neoformans* growing on niger seed (i.e., birdseed) agar. The darkly pigmented colonies are characteristic of *C. neoformans*.

E. Colonies of *Candida glabrata* growing on Sabouraud's dextrose agar after 3 days of incubation at 30°C. *C. glabrata* is suggested when small, more translucent yeast colonies are observed, although additional studies are necessary to confirm the identification.

F. Colonies of a *Rhodotorula* species, illustrating the distinctive orange-red pigmentation characteristic of this genus. Additional studies are necessary to make a definitive species-level identification.

G. Surface of a blood agar plate on which are growing the colonies of *Malassezia furfur* only in the area where olive oil had been spread. *M. furfur* is a lipophilic yeast commonly found on human skin. A medium rich in long-chain fatty acids,

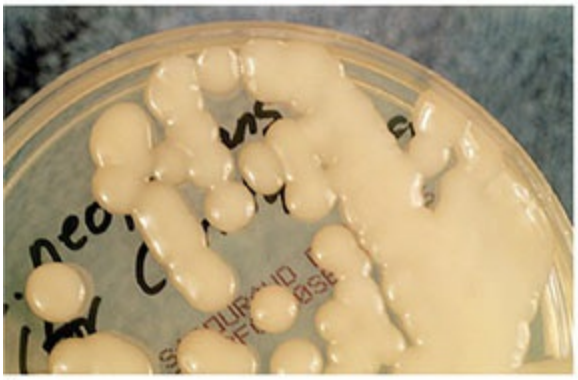
which can be supplied by an olive oil overlay on the surface, as shown here, or using Tween, is necessary for the recovery of this yeast. Laboratory personnel must be notified if an infection by a *Malassezia* species is suspected, so that an oil overlay preparation can be made.

H. A photomicrograph of an acid-fast-stained smear prepared from an isolated colony of *Saccharomyces cerevisiae* growing on the surface of ascospore agar. The spherical, red-staining (acid-fast) bodies seen here are ascospores. *Wickerhamomyces* (*Hansenula*) *anomalus* also produces acid-fast-staining ascospores when grown on ascospore agar; however, they are cap-shaped, rather than spherical, in outline.

IP-141

A

B



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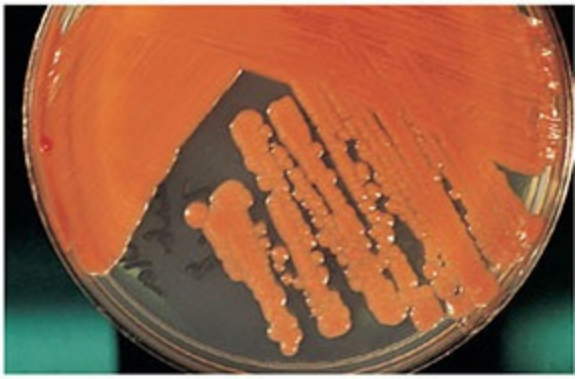

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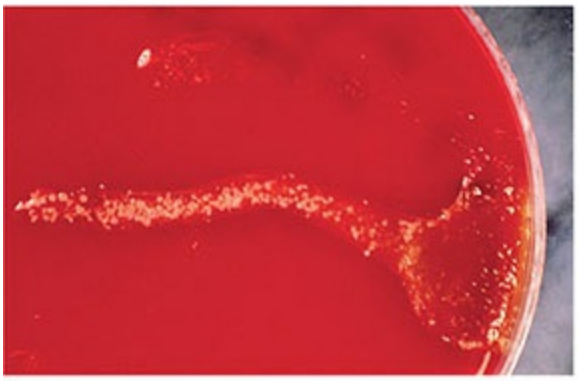

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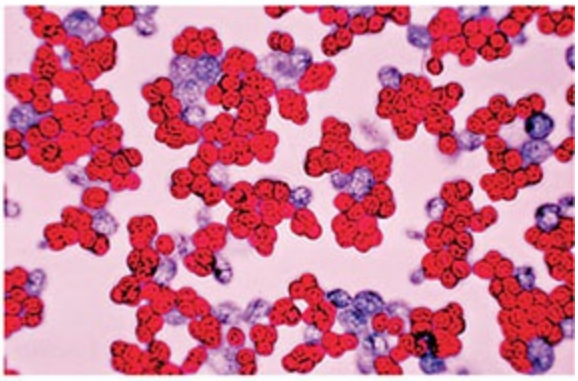


H

IP-142

IMAGE PLATE 22-1

ARTIFACTS: "NOBODY KNOWS THE RUBBLE I HAVE SEEN"

A. Vegetable matter simulating a decorticate *Ascaris lumbricoides* egg.

B. Skeletal muscle fiber from ingested red meat simulating a parasitic larval form.

C. Talc powder fragments, often contaminating specimens collected while wearing rubber gloves, simulating amebic cysts.

D. Crystals simulating parasite eggs.

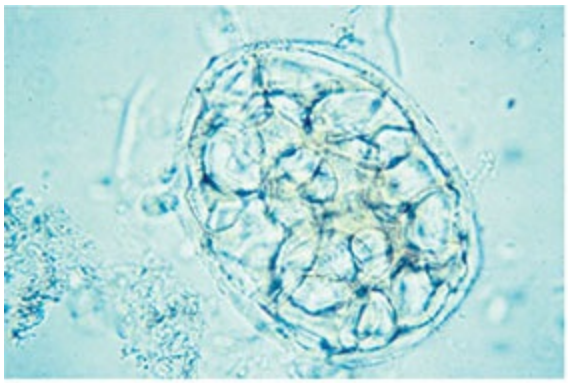
E. Vegetable strand simulating rhabditiform larva.

F. Echinulate pollen grain simulating egg of *Taenia* species.

G. Pollen grain simulating hookworm, *Strongyloides*, or decorticate *Ascaris* egg.

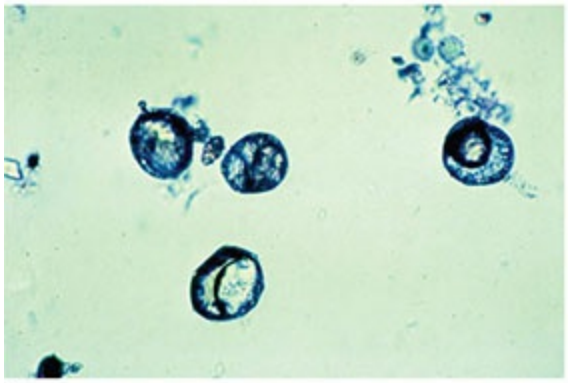
H. Peach hair simulating filariform larva.

IP-143



A

B



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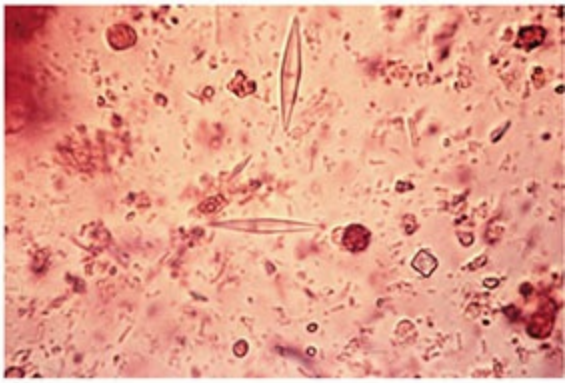

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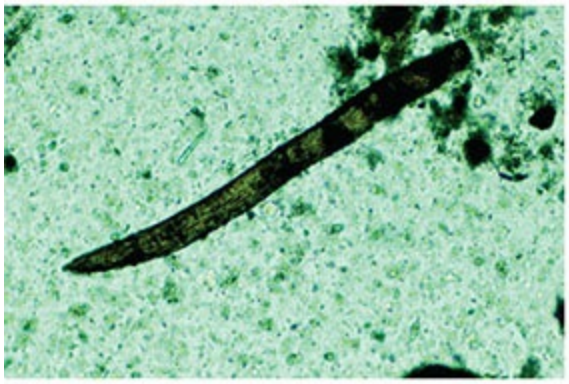

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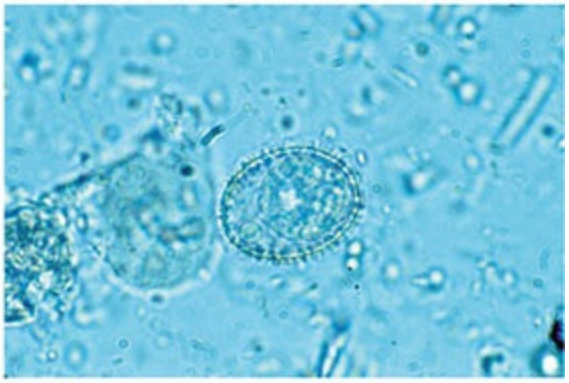

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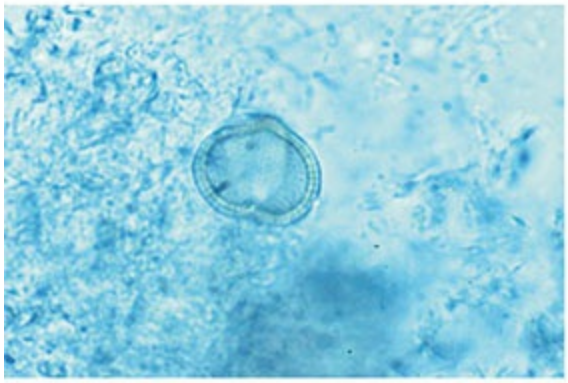

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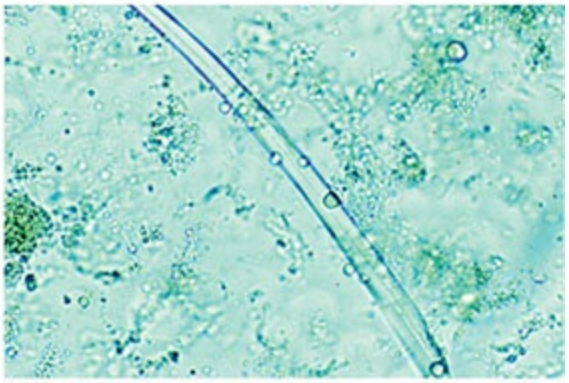


H

IP-144

IMAGE PLATE 22-2

INTESTINAL AMEBA/FLAGELLATES

A. Photomicrograph of fecal smear stained with trichrome. In the center of the field is a two-lobed segmented neutrophil simulating an *Entamoeba*-type cyst. Care must always be taken to be sure that polymorphonuclear leukocytes in fecal specimens are not mistaken for amebic cysts.

B. Photomicrograph of a fecal smear stained with iron hematoxylin, illustrating a single trophozoite of *Entamoeba histolytica*, measuring 10 µm in greatest dimension. Note the spherical nucleus containing a tiny, compact, centrally placed karyosome, and even, beadlike distribution of chromatin along the nuclear membrane (the *Entamoeba*-type nucleus). The extension of a single pseudopod reflects a purposeful, directional motility.

C. Photomicrograph of a direct iodine mount of a cyst of *Entamoeba histolytica*, measuring 12 µm in diameter. Four nuclei are observed, each of which contains a small centrally placed karyosome and a fine, even band of nuclear chromatin around the nuclear membrane.

D. Hematoxylin- and eosin-stained section of colon mucosa illustrating many infiltrating *Entamoeba histolytica* trophozoites. The trophozoites are the light-staining, irregularly spherical bodies, some of which show an *Entamoeba*-type nucleus. (×400)

E. Iron hematoxylin-stained fecal smear illustrating a single trophozoite of *Entamoeba coli*. Note the large karyosome, the uneven distribution of the chromatin along the nuclear membrane, and the coarse "junky" cytoplasm containing undigested debris and several irregular-sized vacuoles. The trophozoites of *E. coli* tend to be larger than those of *E. histolytica*, ranging, on average, between 14 and 25 µm in diameter.

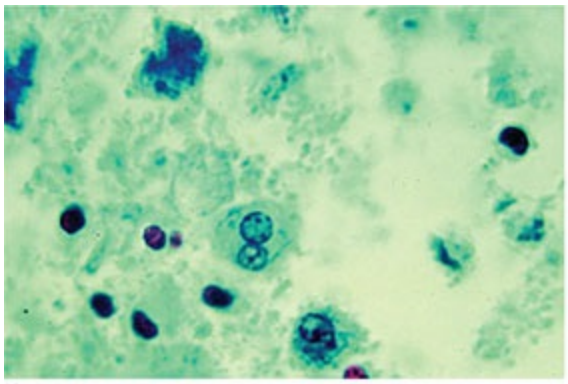
F. Trichrome-stained cyst of *Entamoeba coli* illustrating five nuclei, each containing a relatively large centrally or slightly eccentrically placed karyosome. The presence of more than four nuclei rules out *Entamoeba histolytica*. A single red-staining intracytoplasmic chromatoidal bar with pointed ends is seen in the 8 o'clock position.

G. Iron hematoxylin stain of a precyst of *Entamoeba histolytica*, measuring 8 µm in

diameter. Precysts often have a single vacuole, simulating a cyst of *Iodamoeba bütschlii*. Note in the photograph the distinct "*Entamoeba*-type" distribution of chromatin around the nuclear membrane, ruling out *I. bütschlii*.

H. Iron hematoxylin stain of a small (6 µm in diameter) cyst of *Entamoeba hartmanni*. The size is in the range of *Endolimax nana*; however, the *Entamoeba*-type nucleus and the chromatoidal bars with rounded ends rule out that possibility.

IP-145



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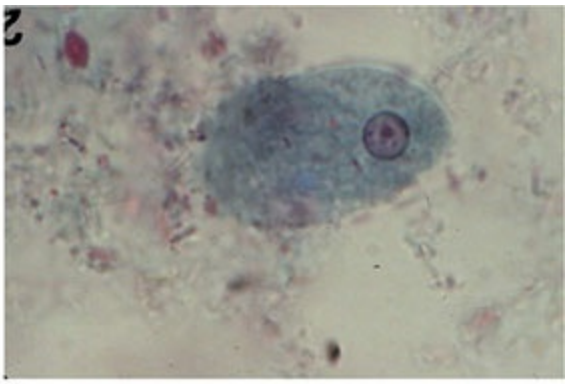

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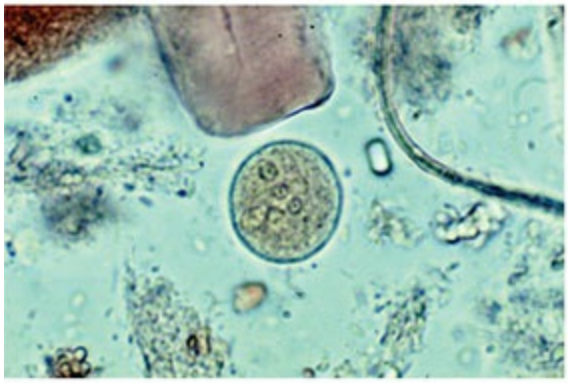

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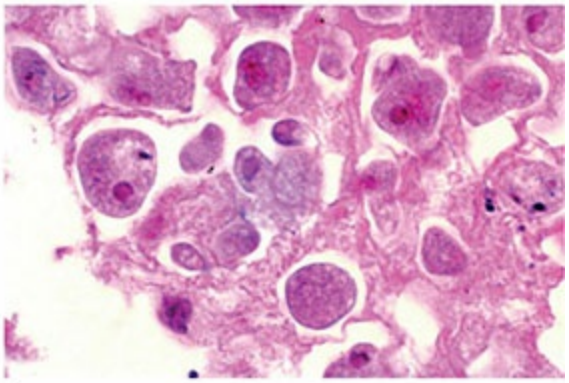

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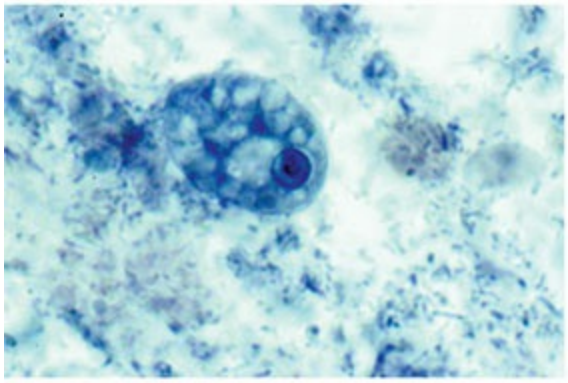

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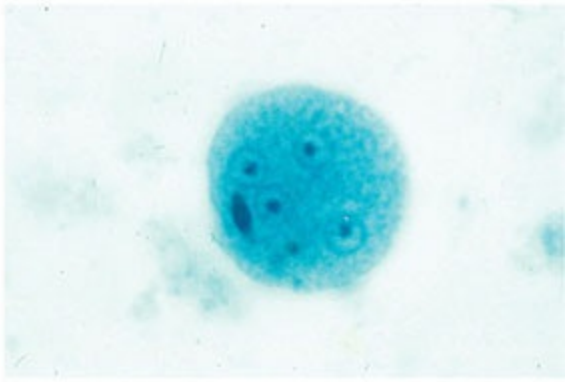

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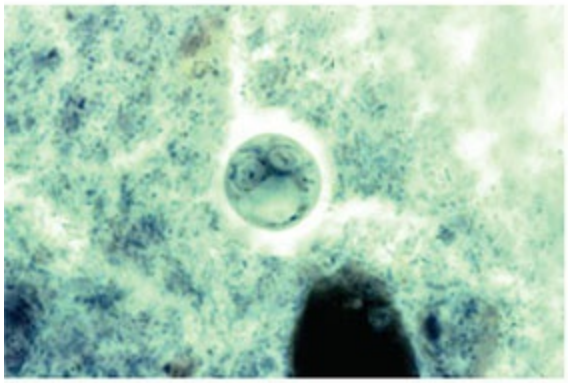

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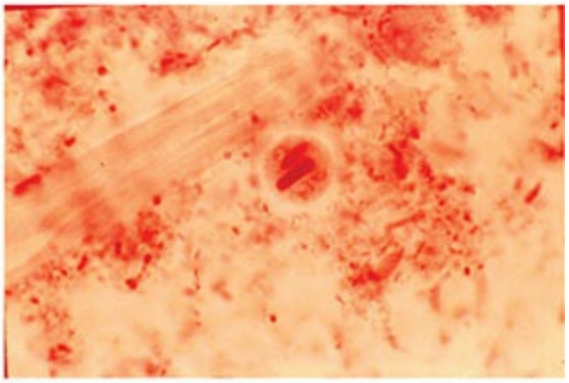


H

IP-146

I. Trichrome-stained fecal smear illustrating a trophozoite of *Iodamoeba bütschlii*. This form measures approximately 14 µm in diameter; the size range is 6 to 25 µm. Note the nucleus with a large karyosome surrounded by an empty space, with no visible nuclear membrane (so-called ball-in-socket nucleus). The smaller trophozoites are difficult to distinguish from those of *Endolimax nana*; other fields must be examined to detect the more characteristic cysts in making a final diagnosis.

J. Trichrome-stained fecal smear illustrating a cyst of *Iodamoeba bütschlii*, measuring 10 µm in diameter. The nucleus has a large central karyosome surrounded by a clear area with absence of peripheral chromatin (ball-in-socket nucleus). Note the single large vacuole in the cytoplasm, which stains positive with an iodine stain, indicating glycogen content.

K. Trichome-stained fecal smear illustrating a small trophozoite of *Endolimax nana*, measuring 8 µm in diameter. Note the small central nucleus with a prominent karyosome, and a narrow surrounding clear space and absence of nuclear chromatin.

L. Methylene blue–stained preparation illustrating a cyst of *Endolimax nana*, measuring 6 to 8 µm in diameter. Note the four tiny nuclei, each of which contains a minute karyosome, surrounded by a clear space.

M. Trichrome-stained fecal smear illustrating a single, spherical form of *Blastocystis hominis*, with a prominent central body surrounded by nuclear material that is plastered in clumps against the inner membrane of the cyst wall. The form here measures 10 µm; however, they vary considerably in size between 5 and 20 µm, with a mixture within any given preparation.

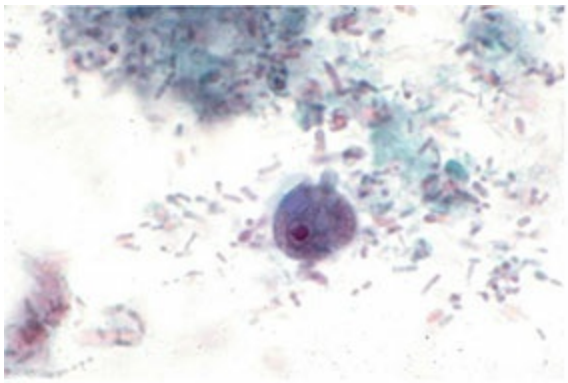
N. Photomicrograph of a hematoxylin- and eosin-stained section of small bowel epithelium. Note the small, pear-shaped trophozoite of *Giardia intestinalis*, with its familiar "face." *Giardia* trophozoites attach to the mucosa, where they cause local damage, leading to the characteristic symptoms of diarrhea and flatulence.

O. Trichrome-stained smear illustrating a *G. intestinalis* (formerly *G. lamblia*) trophozoite recognized by its distinctly oval to elliptical shape, the two nuclei, with prominent central karyosomes, lying anteriorly on either side of the rodlike axostyle, which runs the length of the organism. Note also the eosinophilic staining parabasal body (the "mustache") lying posteriorly on the axostyle. These features provide for the so-called monkey face appearance. *G. intestinalis* trophozoites

have six flagella, two each at anterior, posterior, and caudal positions; only three of these are visible in the photograph.

P. Trichrome-stained smear of intestinal debris including a *Giardia intestinalis* cyst. The cyst is typically oval and has a thin, smooth outer wall. The cyst ranges from 9 to 12 µm in diameter, and typically includes double the number of organelles seen in the trophozoite. Each cyst typically has four nuclei, only two or three of which are usually seen in a single plane of focus, two axostyles, multiple parabasal bodies, and flagella that are wrapped beneath the smooth-walled cyst, all of which may not be observed in a single plane of focus.

IP-147



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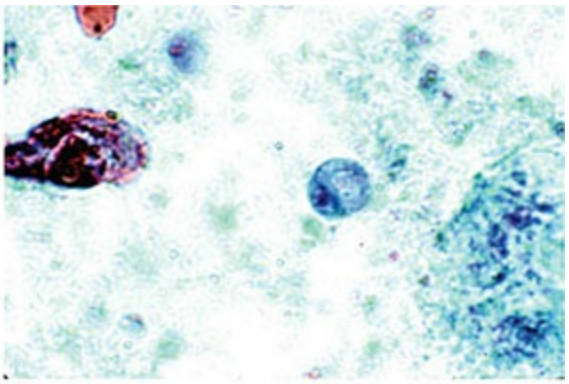

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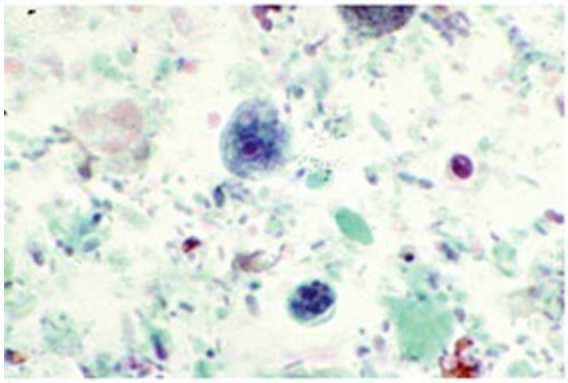

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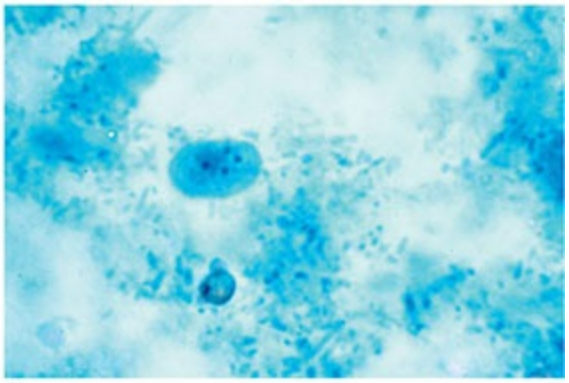

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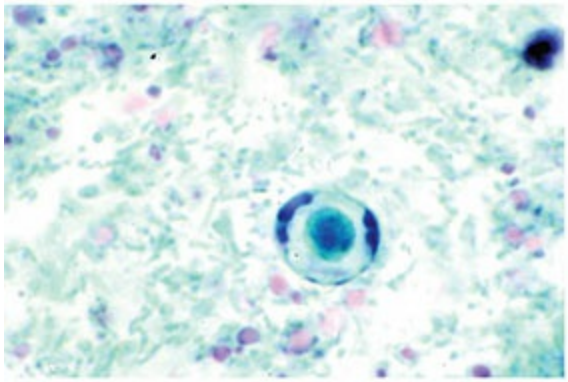

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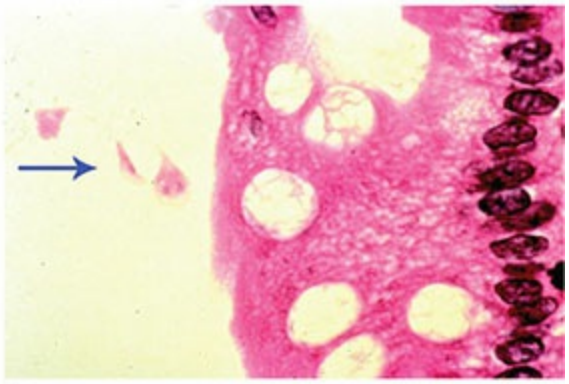

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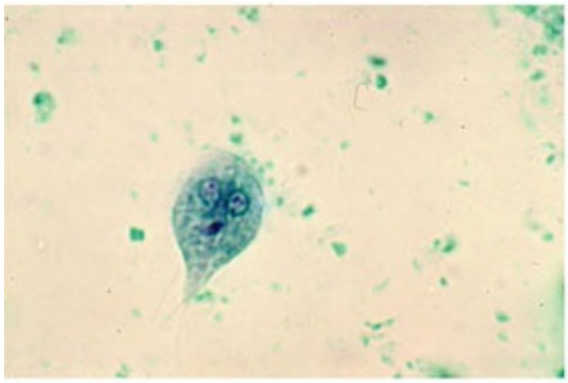

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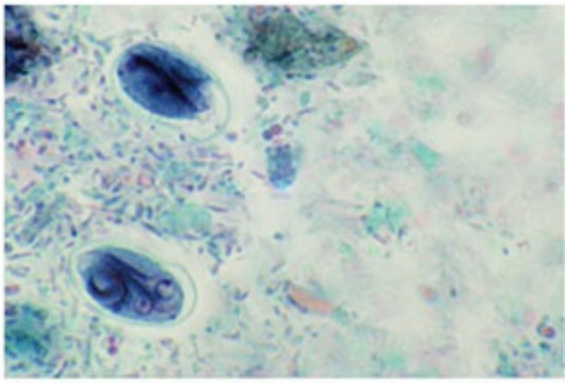


P

IMAGE PLATE 22-3

FLAGELLATES

A. Photomicrograph of two trophozoites of *Chilomastix mesnili*, one seen on end and the other from a lateral view. The trophozoite is typically pear-shaped and can be readily identified by observing the extreme anterior placement of a single nucleus, the posterior protrusion of the axostyle, and a spiral groove, which is difficult to observe in most preparations. A tuft of three anterior flagella, adjacent to the nucleus, is an additional identifying feature that is difficult to observe in most microscopic mounts.

B. Photomicrograph of a cyst of *C. mesnili*. A single nucleus, with central karyosome and a distinct cytostome, appearing as a well-outlined clear area in the cyst shown here, is easily observed. The outline of the cytostome, described as appearing as a shepherd's crook, is seen in this photomicrograph. Cysts typically are lemon-shaped, have a smooth cell wall, range from 6 to 70 µm in diameter and, in addition to the cytostome, often have an anterior knob that, when seen, is helpful in making an identification (see Image Plate 22-3C).

C. View of another cyst of *Chilomastix mesnili*, in which the anterior hyaline knob is better seen *(arrow)*. The nucleus and cytostome are also visible.

D. Trichrome-stained fecal smear highlighting a single trophozoite of *Dientamoeba fragilis*. This cyst measures 8 to 12 µm, and one can readily observe the characteristic double nucleus, each with a large karyosome and indistinct nuclear membrane. The cytoplasm of this trophozoite is finely granular and shows early evidence of disintegration, as reflected by the species name.

E. Iron hematoxylin preparation illustrating a single trophozoite of *Pentatrichomonas* (*Trichomonas*) *hominis*. Note the typical tear-drop shape and single nucleus placed anteriorly, but not against the cell wall, as is characteristic of the trophozoite of *Chilomastix mesnili* (Image Plate 22-3A). The longitudinally running axostyle is visible in this photomicrograph, as is a single posterior flagellum. Typically *P. hominis* has three to five anterior flagella and one posterior flagellum and an undulating membrane that runs along the full length of the body, a structure that is readily seen only in preparations made from cell cultures.

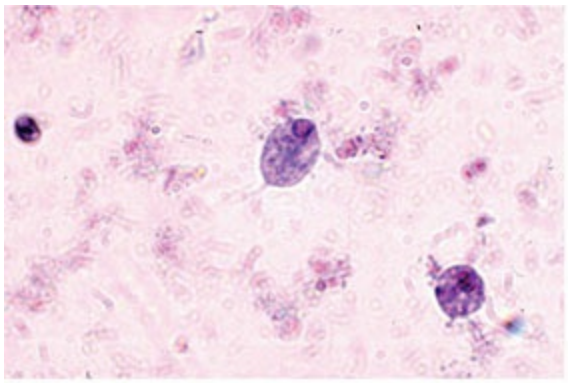
F. Photomicrograph of a hematoxylin- and eosin-stained section of an intestinal biopsy illustrating many invading trophozoites of *Balantidium coli*. Note the distinct rod

or horseshoe-shaped macronucleus, a feature that, along with its large size (80 µm in diameter), is helpful in making an identification. The identification of cilia is key, as this is the only ciliated human parasite.

G. View of a trophozoite of *Balantidium coli* illustrating an oval outline with a smooth wall that is covered throughout the circumference with short, delicate cilia. These trophozoites range between 40 and 70 µm wide by 50 and 100 µm long. The presence of an anterior cytostome and the circumference of cilia and large rod or horseshoe-shaped macronucleus are the key characteristics leading to an identification.

H. A cyst of *Balantidium coli* is illustrated in this trichrome-stained mount. These cysts range from 50 to 75 µm in diameter, are spherical to ellipsoid, and have a characteristic dumbbell-shaped macronucleus, clearly observed in this photograph. A single indistinct micronucleus is observed adjacent to the macronucleus, and the cytoplasm includes a metabolic vacuole.

IP-149



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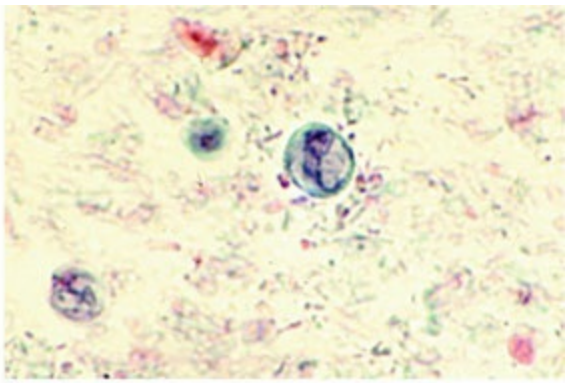

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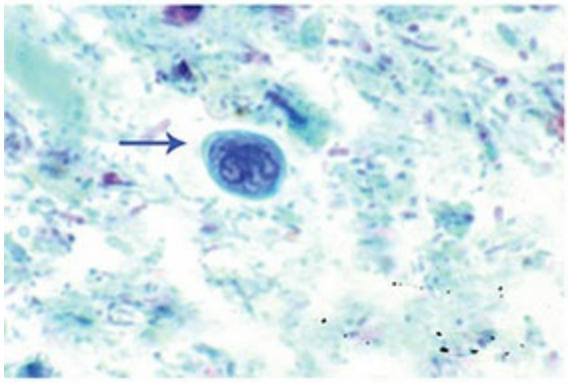

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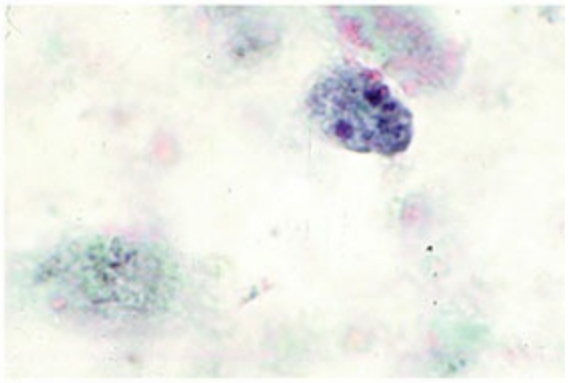

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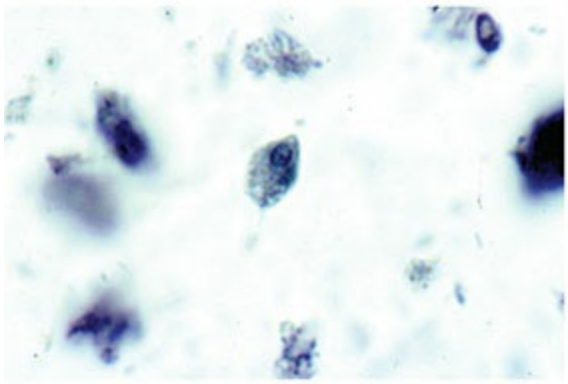

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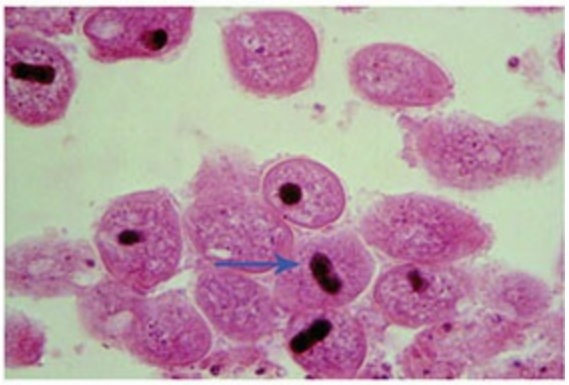

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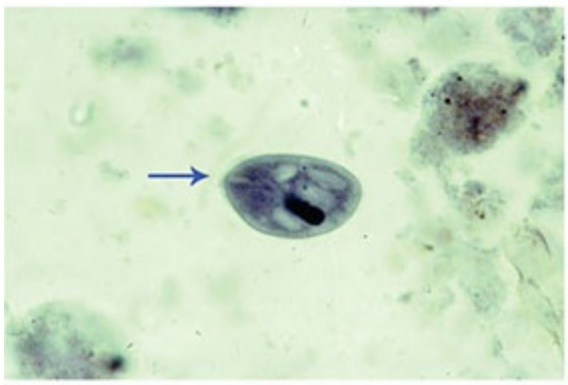

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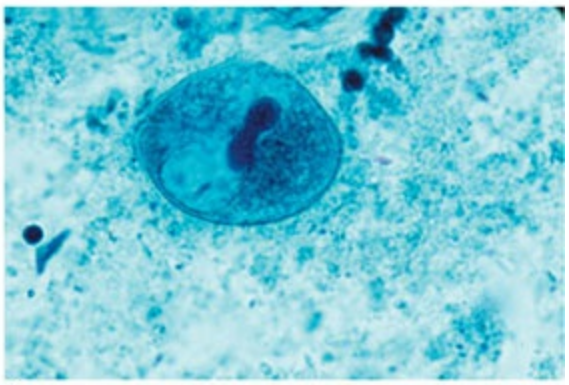


H

IMAGE PLATE 22-4

COCCIDIA

A. Photomicrograph of a hematoxylin- and eosin-stained section of bowel mucosa along the brush border of which are attached many tiny, 2- to 3-µm-diameter oocysts of *Cryptosporidium* species. A histologic diagnosis is possible when these aggregates of oocysts are observed in this location.

B. Acid-fast stain of fecal smear illustrating two red-staining, acid-fast, spherical, homogeneous staining, 4- to 5-µm-diameter oocysts of *Cryptosporidium* species (arrows). These forms are diagnostic when seen in modified acid-fast smear preparations.

C. Photomicrograph of an acid-fast-stained fecal smear illustrating a single spherical, acid-fast oocyst of *Cyclospora* species. These oocysts are about two times the size of the oocysts of *Cryptosporidium* species, ranging from 8 to 10 µm in diameter, ergo the need for a calibrated ocular micrometer. Also in contrast, the *Cyclospora* oocysts have an ill-defined internal structure and lack the homogeneous staining of *Cryptosporidium* oocysts. It is common to have well-stained *Cyclospora* oocysts adjacent to unstained oocysts; this heterogeneous staining is characteristic of *Cyclospora.*

D. Low-power (×250) view of an acid-fast-stained spindle-shaped oocyst of *Cystoisospora belli* located in the center field of view. These oocysts measure 25 to 30 µm in length, a relatively large size that cannot be appreciated by this low-power view, except when compared with the very tiny, almost dot-like, 5-µm-diameter oocysts of *Cryptosporidium* species that are scattered in this field. The coexistence of these two species in the same stool specimen is highly suggestive of a person with HIV infection, which should always be ruled out in such a clinical scenario.

E. Photomicrograph of a direct mount preparation illustrating an elliptical-shaped, immature oocyst of *Cystoisospora belli*. These oocysts measure 25 to 30 µm in the long axis, and when immature, enclose only a single spherical sporocyst, as illustrated in the photograph. Compare with the mature oocyst illustrated in Image Plate 22-4F.

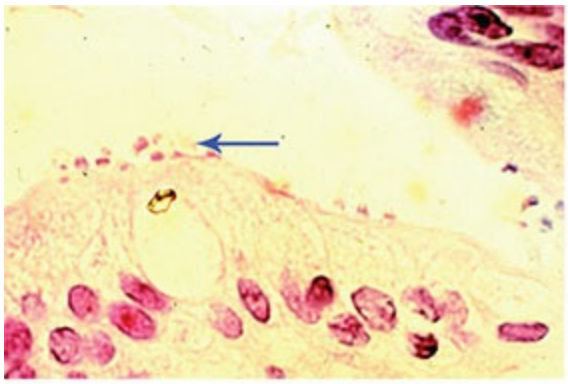
F. View of a direct mount of fecal material illustrating a mature oocyst of *Cystoisospora belli*, enclosing two spherical sporocysts. The sporocysts, now

mature, are infective for a second host, and ready to release sporozoites into the intestine on ingestion.

G. Hematoxylin- and eosin-stained section of skeletal muscle within which are two cysts of *Sarcocystis* spp. These cysts measure as large as 300 µm in diameter, and are filled with inactive, encysted bradyzoites. Humans are infected with this form if, as accidental intermediate hosts, infective oocysts are ingested in contaminated food or water.

H. Weber stain of a stool preparation illustrating many small, 2- to 3-µm, rod-shaped spores of a Microsporidium. With this stain, the spores stain a salmon-yellow. Note the characteristic thin, transverse septum that divides some of the spores *(arrow)* seen in this field of view, a helpful feature in making a genus identification. These organisms are now classified as Fungi, but testing often remains in the parasitology section of the laboratory for practical purposes.

IP-151



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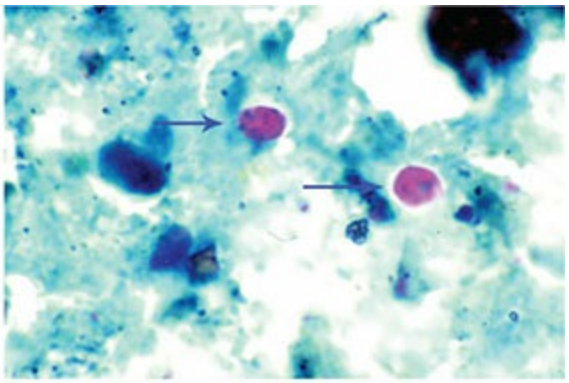

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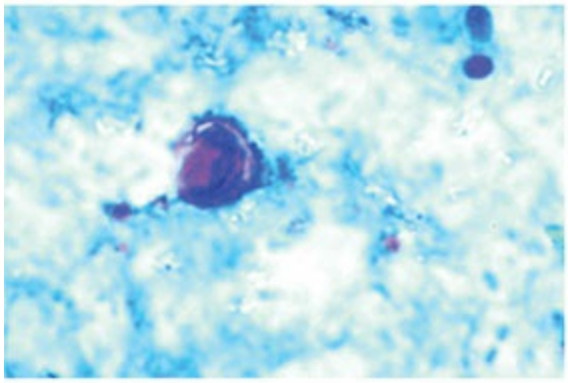

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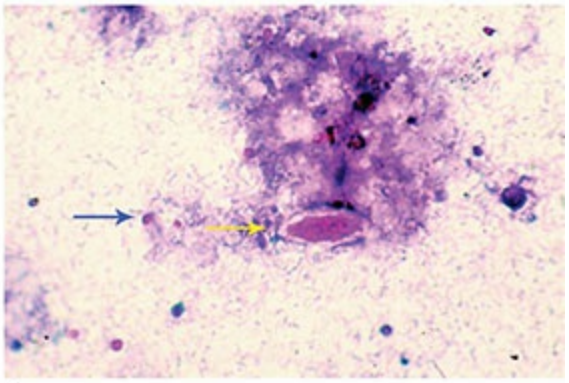

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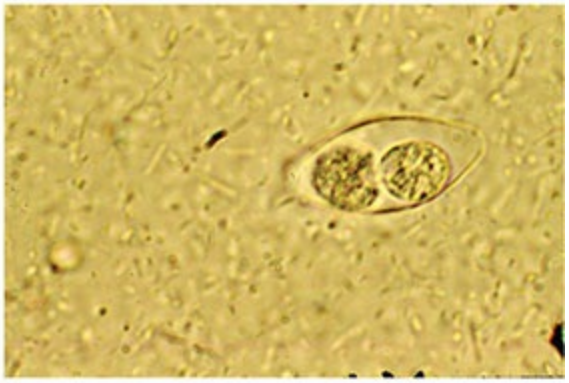

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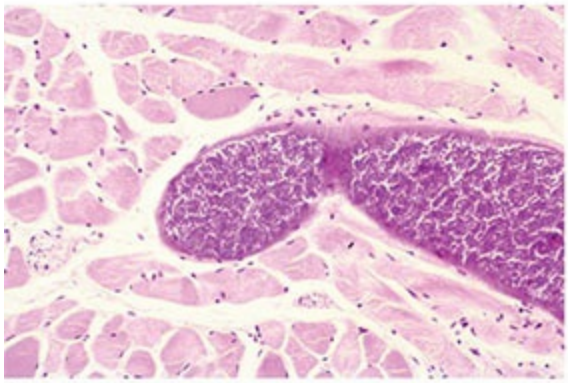

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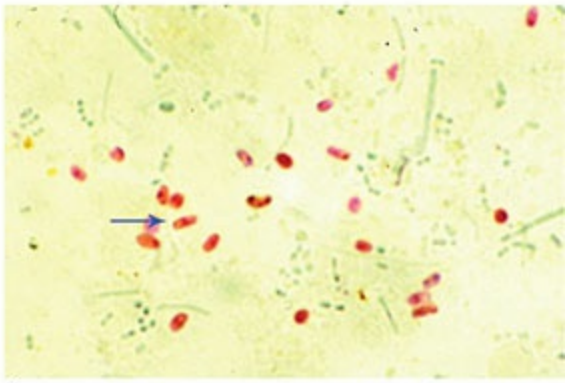


H

IP-152

IMAGE PLATE 22-5

NEMATODES

A. Adult male and female worms of *Ascaris lumbricoides*. The adult males are generally shorter (20 to 25 cm) than the females (25 to 30 cm) and have a curved tail (lower worm in the photograph). The smooth, nonsegmented cuticle is a helpful feature in distinguishing adult ascarids from common earthworms, which on occasion may find their way into toilets and sewage systems, where they may be mistaken for worms passed in the feces.

B. Bile-stained, yellow-brown, slightly ovoid egg of *Ascaris lumbricoides*, which range in size from 45 to 60 µm in diameter. They possess a thick shell covered by a characteristic undulating albuminous coat. The retraction and development of the internal cleavage indicates fertilization and potential infectivity.

C. Illustrated is a spherical, decorticate egg of *Ascaris lumbricoides* featuring a well-developed larva that is at the stage of near hatching. This egg obviously is highly infective.

D. Fertilized egg of *Ascaris lumbricoides* showing a smooth outer shell. When exposed to bile within the intestinal lumen, the albuminous outer coat is dissolved, leaving a decorticate surface as seen here.

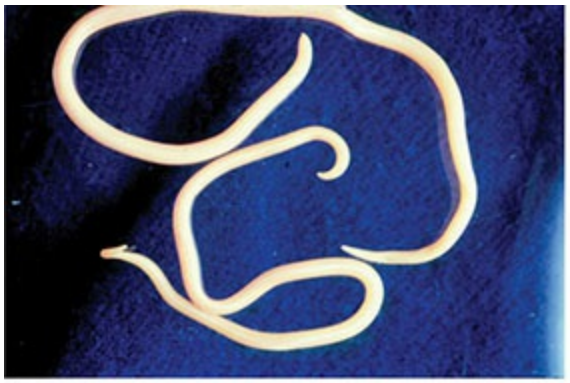
E. Composite photograph illustrating an adult worm of *Enterobius vermicularis* in the left frame. These worms, measuring 0.3 to 0.5 mm in diameter and between 8 and 1.3 mm long, have a well-developed intestinal tract and internal reproductive organs. The pointed tail and paraoral alae (arrow in the left photograph) are diagnostic. The close-up view in the right frame better demonstrates the paraoral alae.

F. Split-frame photograph illustrating gravid *Enterobius vermicularis* eggs, the one to the left possessing a well-developed larva. These eggs have a thin, smooth wall, and measure approximately 55 × 25 µm. Each egg is elongated, asymmetrical, and flattened on one side, resembling an underinflated football.

G. Photograph of adult *Trichuris trichiura* worms demonstrating the characteristic long, attenuated whip-like anterior segment and the shorter, thicker more handle-like posterior body. These worms are relatively small, measuring 35 to 45 mm in length.

H. Photomicrograph of a typical bile-stained, yellow-brown egg of *Trichuris trichiura* with a relatively thick, smooth wall. Note the characteristic barrel-shape and the distinctive, dome-shaped, hyaline plug at each polar extremity. (From "A Pictorial Presentation of Parasites" by H. Zaiman.)

IP-153



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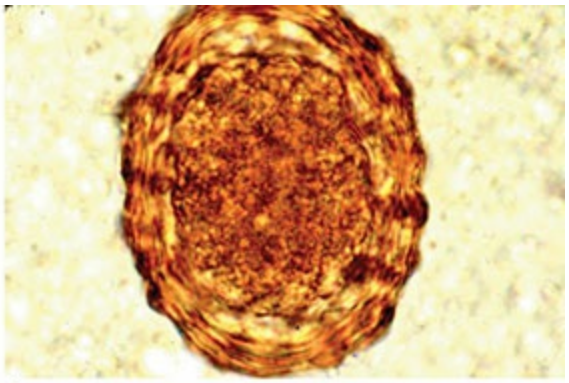

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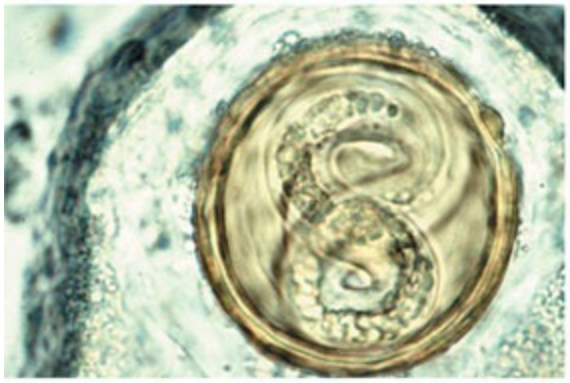

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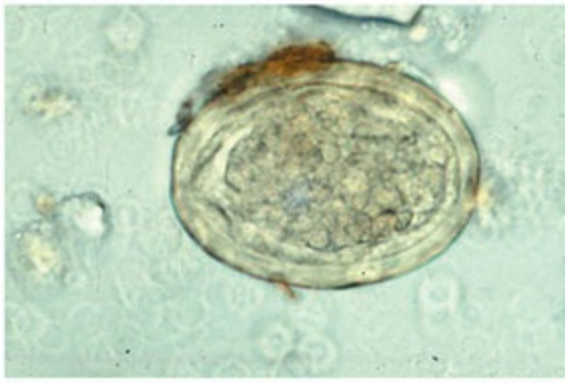

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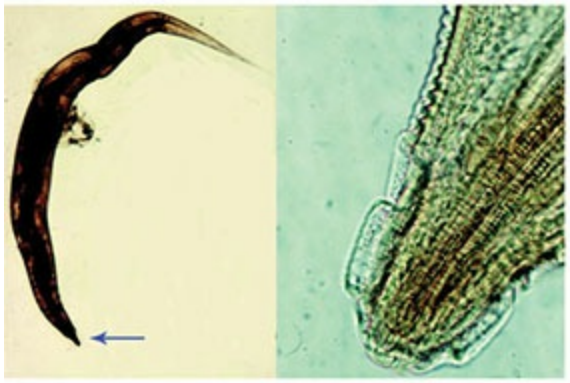

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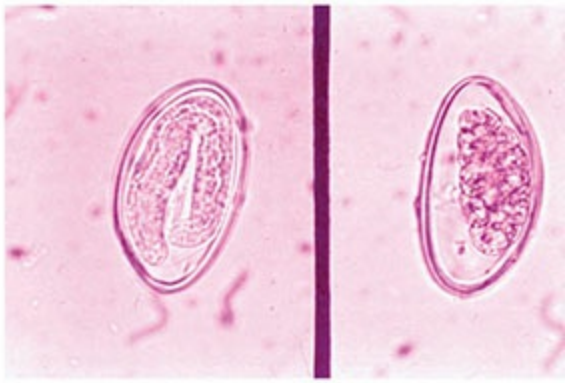

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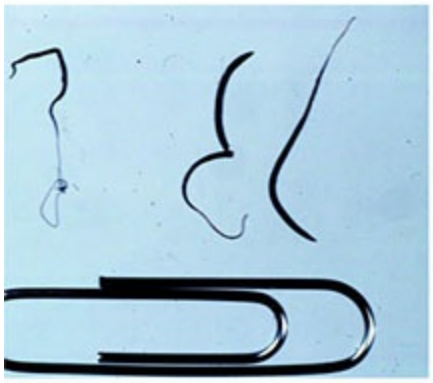

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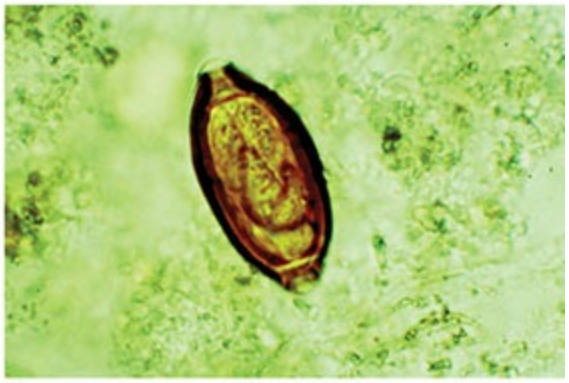

H

IP-154

I. Photograph of the mouthpart of an adult hookworm. The chitinous teeth observed here are characteristic of the old-world hookworm, *Ancylostoma duodenale*. This mouthpart is well suited for the deep attachment of the worm to the intestinal mucosa and the cutting of capillaries enabling the ingestion of blood from the circulation.

J. Similar view of the mouthpart of the new-world hookworm, *Necator americanus*, illustrating the characteristic cutting plates *(arrows)*. As with *Ancylostoma duodenale*, this mouthpart is also well suited for the deep attachment of the worm to the intestinal mucosa, the laceration of capillaries, and the ingestion of blood.

K. Photomicrograph of a direct iodine mount of fecal material containing a typical oval-shaped hookworm egg measuring approximately 40 to 60 µm, with the characteristic thin, smooth wall, and a clear space beneath the shell. These eggs cannot be distinguished from those of *Strongyloides stercoralis*. This differential identification is generally not necessary as *S. stercoralis* rarely passes eggs in the feces and only in instances of hyperinfection, when larvae are abundant.

L. Split-frame photomicrograph in which the buccal cavities of the rhabditiform larvae of hookworm (left) and *Strongyloides* (right) are compared. The buccal cavity of the hookworm rhabditiform larva is characteristically long *(arrows)* compared with the shorter *(arrows)* distance observed with *Strongyloides*. Hookworm rhabditiform larvae are rarely seen in fecal specimens.

M. Microscopic field of view of a direct mount of fecal material, within which a small, coiled rhabditiform larva of *Strongyloides stercoralis* is present, as it would be observed under low magnification (×100). These larvae are highly motile when observed in direct mounts. Observation of the buccal cavity as described above is necessary to confirm the identification. These larvae average 15 µm in diameter and over 200 µm long.

N. Rhabditiform larvae of *S. stercoralis*, after a short feeding period of 2 to 3 days, develop into long, slender, nonfeeding, infective filariform larvae, that can reach 700 µm in length. These filariform larvae can penetrate directly into the skin of a human host on direct contact.

O. Hematoxylin-and-eosin-stained section of small intestine in which round, smooth filariform larvae of *Strongyloides stercoralis*, characteristic of the histologic findings of individuals with *Strongyloides* hyperinfection syndrome. These are observed burrowing into the lamina propria. In patients with *Strongyloides* in

whom a compromised immune system develops, the life cycle from oviposition to rhabditiform larva to filariform larva formation may be sufficiently short that autoinfection may occur within the intestine before stools are formed, as observed in this photograph.

P. Photomicrograph of a yellow-brown, bile-stained egg of *Capillaria philippinensis*. These eggs are lemon-shaped or elongated, measure approximately 60 × 35 µm, and resemble those of *Trichuris trichiura*, except that the shell is striate and the polar protuberances are broad and flat.

IP-155

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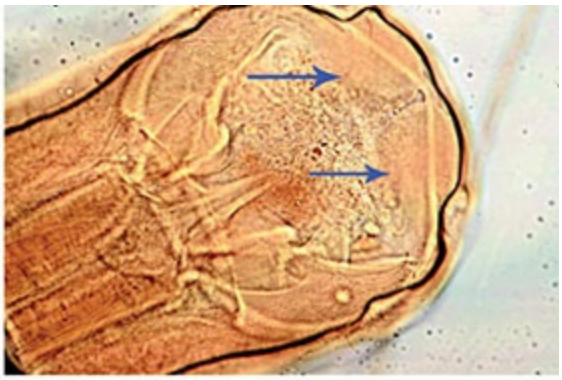

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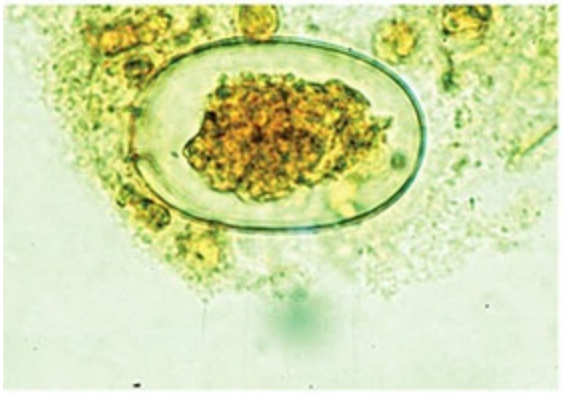

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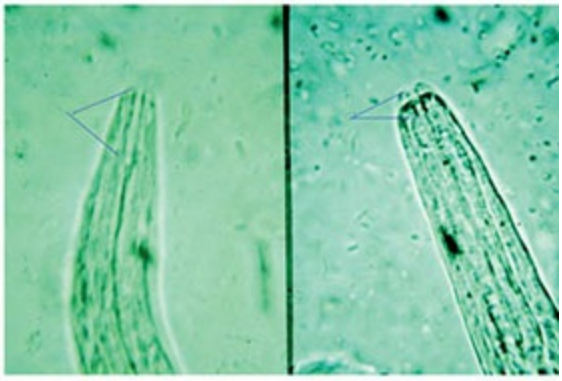

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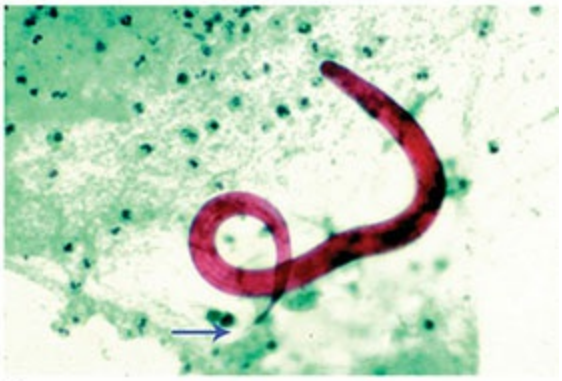

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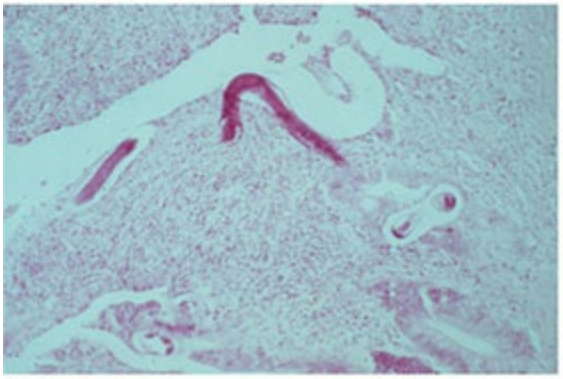


O

P

IP-156

IMAGE PLATE 22-6

CESTODES

A. An adult *Taenia saginata* tapeworm illustrating the small scolex *(arrow)* and a strobila extending several feet long, and composed of a long chain of proglottids.

B. Close-up view of the scolex of a *Taenia solium* tapeworm demonstrating the four suckers characteristic of the genus. The protruding, rostellum armed with a circular row of hooklets, permits the identification of *Taenia solium*. The scolex of *Taenia saginata* is not armed.

C. Close-up view of a single *Taenia saginata* proglottid that has been injected with India ink through the genital pore to outline the branching uterine segments. The proglottids of *T. saginata* possess more than 13 lateral uterine branches, as shown in the photograph, to distinguish them from the proglottids of *Taenia solium*, that typically have fewer than 13 branches. This observation permits a species identification based on an examination of proglottids that may have been passed intact in the feces.

D. Split-frame photographs of a piece of measly pork (left) including several small cystic spaces, each inhabited by a cysticercus larva of *Taenia solium*. A cysticercus is illustrated in more detail in the right frame. Within the fluid-filled sac is contained an invaginated scolex. The suckers and hooklets of which are obscured by the stain.

E. Hematoxylin-and-eosin-stained section of brain illustrating a cystic space occupied by a *Taenia solium* cysticercus. The cyst has two walls, the outer composed of reactive fibrous tissue produced by the host tissue, and the second an inner thin membrane produced by the worm (bladder worm). The invaginated scolex is cut in cross section in this photograph and demonstrates internal structures.

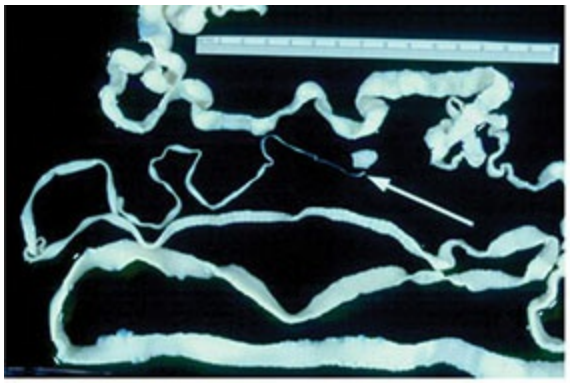
F. Photograph of a cysticercus of the rat tapeworm, *Hymenolepis diminuta*. The invaginated scolex is better visualized in this field of view. These cysticerci form in an intermediate insect host, including fleas, cockroaches, and beetles. Humans are infected through ingestion of insect-contaminated foods, and the adult worms develop in the intestine after evagination and attachment of the scolex to the mucosa.

G. Photograph of the uterus of a gravid female of the *Taenia* species, illustrating the

dense aggregation of thick-walled, spherical eggs, some of which show the characteristic striations.

H. Close-up view of *Taenia* species eggs as they would be observed microscopically in direct mounts of infected material. These eggs average 30 to 45 µm in diameter and have a thick, smooth wall, interrupted by distinctive radial striations. Many eggs also enclose three pairs of hooklets (hexacanth embryo) not observed in this photograph, but similar in appearance to those seen within the inner membrane of the *Hymenolepis diminuta* egg included in Image Plate 22-6N. The species of *Taenia* cannot be determined by the morphology of the eggs.

IP-157



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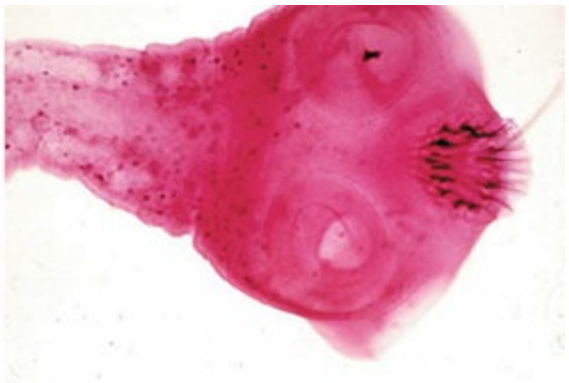

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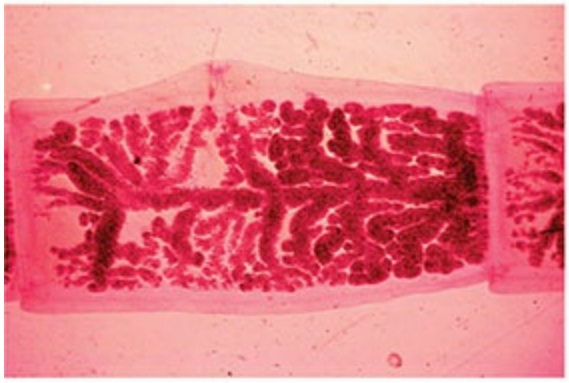

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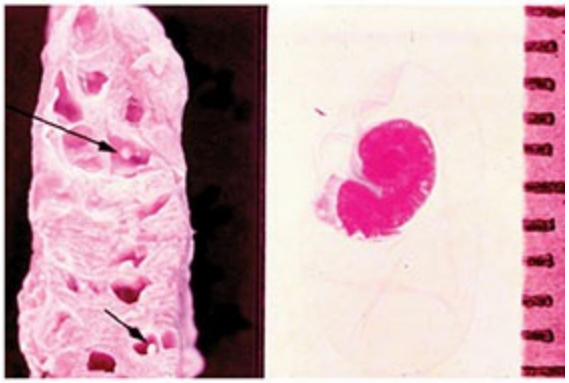

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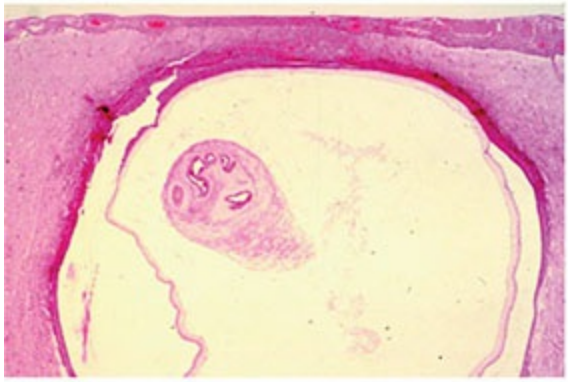

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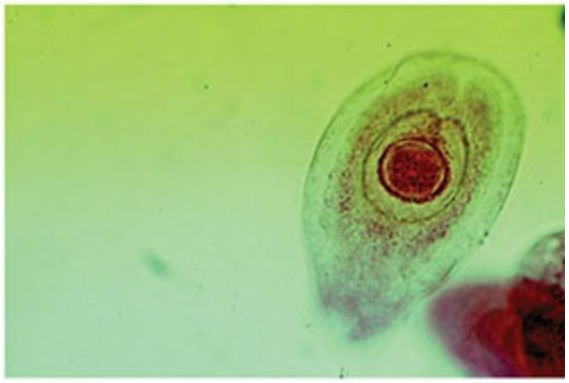

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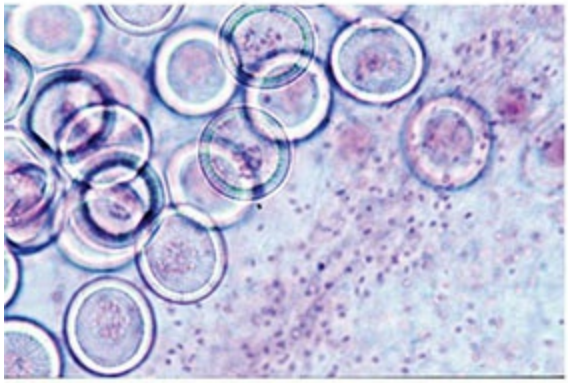


G

H

IP-158

I. Photograph of the tiny scolex *(arrow)* and the broad and narrow proglottids of *Diphyllobothrium latum*, the giant adult tapeworm. This tapeworm is the longest that infests humans, reaching between 3 and 10 m in length, often including more than 3,000 proglottids.

J. Close-up view of the spatula-shaped scolex of *D. latum*, demonstrating the characteristic central, longitudinal groove (bothrium), flanked on either side by a distinctive fold (phyllo "leaf"), the structures from which the genus name, *Diphyllobothrium*, is derived.

K. Close-up view of a proglottid of *Diphyllobothrium*, demonstrating the characteristic width-greater than-length dimensions, from which the genus name "*latum*" is derived. Note the nondescript centrally and linearly placed branching uterus, appearing as a compact rosette.

L. Photomicrograph of an egg of *Diphyllobothrium latum*. These eggs are relatively large, measuring from 60 to 75 µm long by 40 to 50 µm wide. They are oval in shape and have a thin, smooth shell, and the cleavage extends to the inner shell membrane. Characteristic is the presence of an inconspicuous, nonshouldered operculum, which is used to distinguish it from the egg of *Paragonimus westermani*, the operculum of which is distinctly shouldered (Image Plate 22-7P).

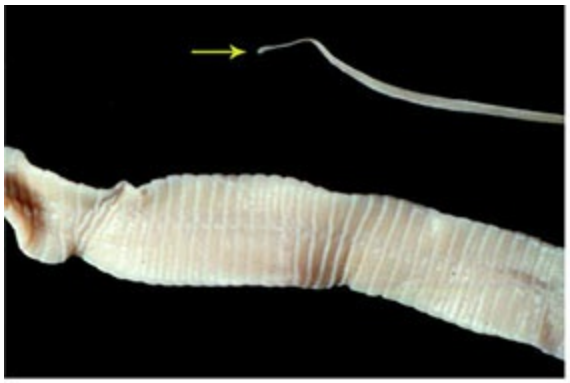
M. Photomicrograph of a *Hymenolepis nana* egg, comprised of a double membrane —an outer thin, smooth shell, and an inner membrane enclosing an oncosphere. Three pairs of hooklets are commonly contained (hexacanth), as illustrated in this photograph. These eggs range between 40 and 60 µm in diameter and are usually oval to subspherical in outline. The eggs of *H. nana* are also distinctive for the presence of polar thickenings on either side of the inner hexacanth membrane, from which four to eight slender filaments are derived, extending into the intermembrane space.

N. Photograph of an egg of *Hymenolepis diminuta*. These eggs are larger than those of *Hymenolepis nana*, with an average diameter of 60 to 80 µm, and usually are distinctively spherical in outline. The absence of polar thickenings and intermembrane filaments also distinguish this egg from those of *H. nana*. The six hooklets are clearly illustrated *(arrow)*.

O. Photomicrograph of an egg packet of *D. caninum*, demonstrating an aggregate of spherical eggs held in a matrix. The eggs shown in the photograph are immature, as the presence of internal hooklets indicate maturity and infectivity.

P. Photograph of a *Dipylidium caninum* proglottid, demonstrating a genital pore on either side, into which flow the delicate vitelline ducts. This double-pore arrangement is unique to *D. caninum* (from which the genus name is derived, pyle "gate or orifice"), in that the proglottids of other tapeworms possess only one lateral genital pore that alternates from one side to the other on adjacent segments. The proglottids are vase-form in shape, being longer than wide.

IP-159



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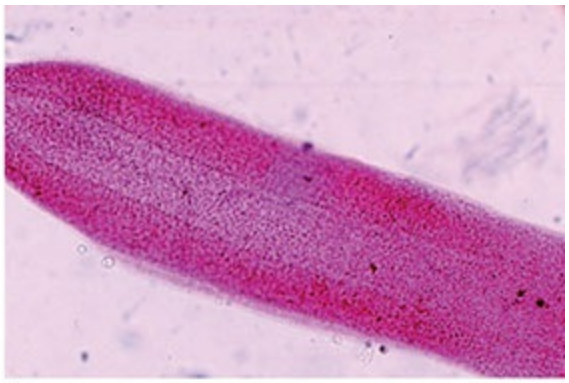

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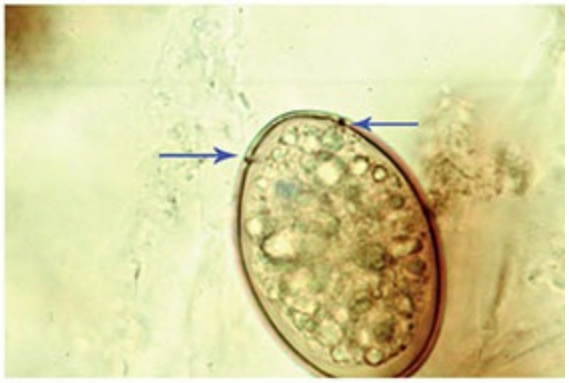

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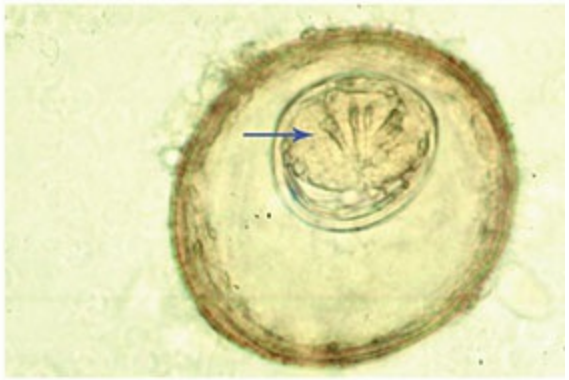

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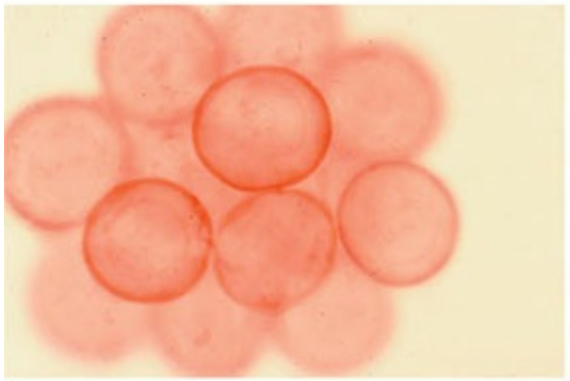

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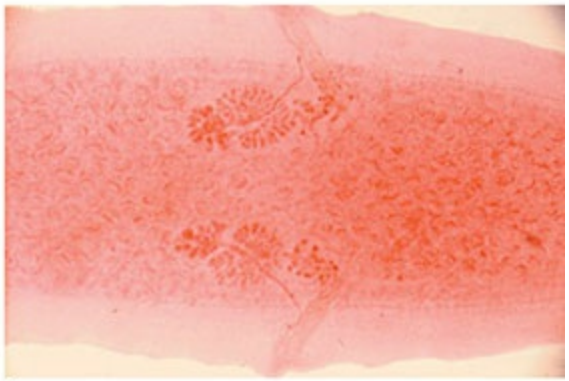

P

IMAGE PLATE 22-7

TREMATODES

A. Photograph of adult male and female *Schistosoma* adults. The male in this photograph is lighter staining and possesses a gynecophoric canal from which the copulating female, darker in staining, has become detached. Adult males average 20 to 30 mm in length, and females 14 to 17 mm. These flukes are threadlike in diameter, allowing them to occupy the lumina of venules without causing blockage of the blood flow, thus resulting in minimal edema.

B. Photomicrograph of an egg of *Schistosoma mansoni*, with its characteristic smooth, thin wall and oval outline with a prominent lateral spine. These eggs are relatively quite large, ranging from about 115 to 180 µm long by 45 to 70 µm wide. On maturity, the larval miracidium hatches intact, precluding a period of development in the external environment.

C. Photomicrograph of an egg of *Schistosoma haematobium*, also having a thin, smooth shell and an oval outline, but differing from the egg of *S. mansoni* by possessing a terminal, rather than a lateral spine. These eggs also are relatively large, ranging from 110 to 170 µm long, and 40 to 70 µm wide.

D. Illustrated here is an egg of *Schistosoma japonicum*, which is smaller (80 to 100 µm in diameter), more oval to spherical in outline, and has an internal cleavage that extends to the inner shell. Although a spine is not observed, a small lateral knob (not seen in this photograph), is distinctive.

E. Photomicrograph of a forked-tail cercaria, the infective stage in the *Schistosoma* life cycle. These free-swimming cercaria are released from the rediae of infected snails and have the capacity, through enzymatic activity, to directly penetrate the skin of an unsuspecting human, who may be wading or swimming in infested freshwater estuaries.

F. An adult fluke of *Fasciola hepatica*. These flukes are hermaphroditic and possess both female and male reproductive organs. The anterior portion of *F. hepatica* projects into a cone-shaped cephalic end, just posterior to which is the anterior sucker. Immediately posterior to the sucker, and darkly staining in this photograph, is the branching convoluted uterus. Immediately posterior to the uterus is the ventral sucker. The bulk of the posterior portion of the fluke includes the testes, appearing as a pink-staining meshwork in this photograph. These flukes measure

about 3 × 1 cm (about the size of a glass microscope slide). (From "A Pictorial Presentation of Parasites" by H. Zaiman.)

G. An adult fluke of *Fasciolopsis buski*, the giant intestinal fluke, which is also hermaphroditic and has structures similar to those described for *F. hepatica*. In contrast, the cephalic end is rounded and devoid of the conical protrusion. These flukes range from about 2 to 7.5 cm long, by 0.8 to 2 cm wide.

H. Photomicrograph of an egg of *F. hepatica* (which cannot be distinguished from those of *F. buski*), demonstrating a thin, smooth wall and an internal cleavage that extends to the inner shell membrane. Notice the nick on either side of the shell toward the narrow end *(arrow)*, representing the operculum, a lid-like structure that opens at the time the larva hatches. These eggs are large, averaging approximately 150 × 80 m in greatest dimensions.

IP-161

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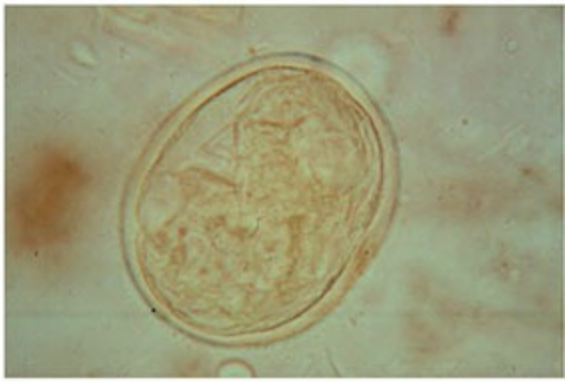

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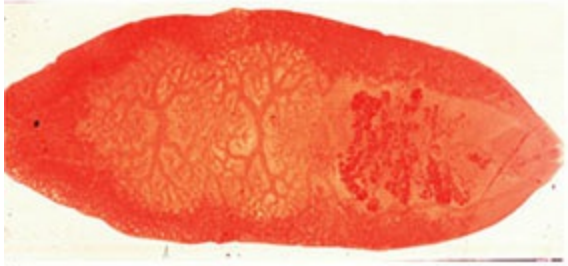

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IP-162

I. Photograph of an adult fluke of *Clonorchis sinensis*, demonstrating the long, bottle-shaped cephalic end with a prominent terminal sucker, dark brown-staining uterus, and the extensively branched, lighter pink-staining testes posteriorly. These internal structures, moving from mouth to tail, are illustrated in greater detail in Image Plates 22-7J-M.

J. Illustrated in this photograph is the anterior mouth or oral sucker, which leads to a blind digestive tract, called the ceca, a branch of which runs along the lateral margins of the worm.

K. Posterior to the oral sucker is the ventral sucker, which is immediately in front of the dark staining uterus, flanked on either side by the branches of the ceca. The delicate-staining vitelline glands are seen at the upper margin of the worm between the ceca and the outer integument. These glands produce the yolk for the eggs produced in the ovaries.

L. This field of view is just posterior to the uterus illustrating the ovary. Note the delicate vitelline duct entering the ovary on the right side.

M. This field of view is the posterior part of the worm, highlighting the extensively branching tests, from which the genus name (*clon* = divided; *orchis* = testes) is derived. Note that the ceca end in blind alleys. The excretory bladder is the ill-defined white space at the immediate posterior margin.

N. Hematoxylin-and-eosin-stained section through a dilated bile duct within which resides three *Clonorchis sinensis* adult flukes cut in cross section. Although a mild chronic inflammatory infiltrate is noted in the surrounding submucosa, extensive fibrosis and lumen occlusion is not evident.

O. Photomicrograph of an egg of *Clonorchis sinensis*, illustrating a thin, smooth wall. Characteristic is the prominent, shouldered operculum at the broader end. A tiny knob, not visible in this photograph, may be seen at the narrower, antiopercular end of the egg. These eggs are relatively small, ranging from 25 to 35 µm long by 14 to 17 µm wide.

P. Photomicrograph of an egg of *Paragonimus westermani*. The shell is smooth, relatively thin, and interrupted at one end by a prominent shouldered operculum. This egg appears morphologically similar to that of *Clonorchis sinensis*; however, these comparative photographs are deceiving, as *P. westermani* eggs are two to three times the size, ranging from 65 to 120 µm long by 40 to 70 µm wide. With this larger size, *P. westermani* eggs appear similar to those of *Diphyllobothrium*

latum, which, however, can be distinguished by its flat rather than raised, shouldered operculum.

IP-163

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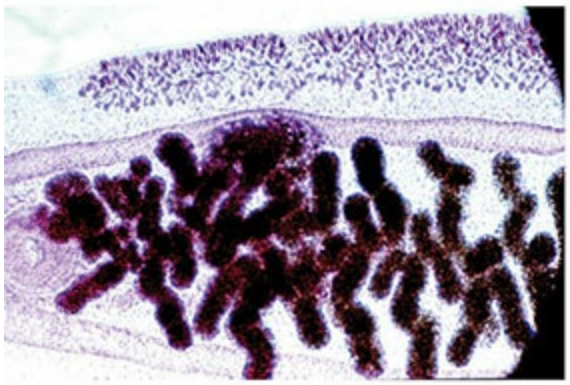

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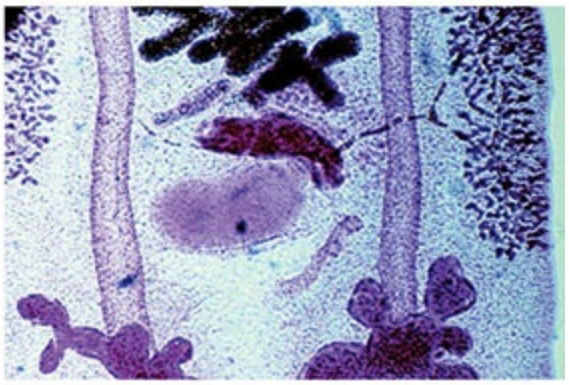

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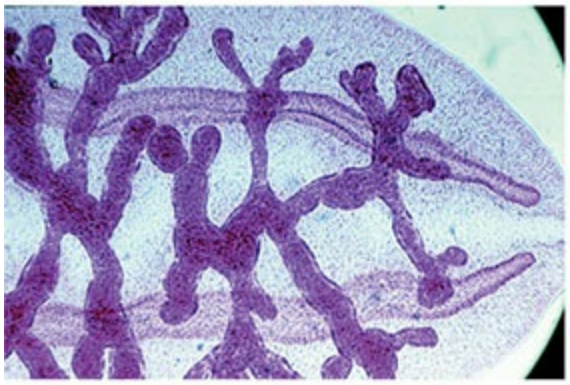

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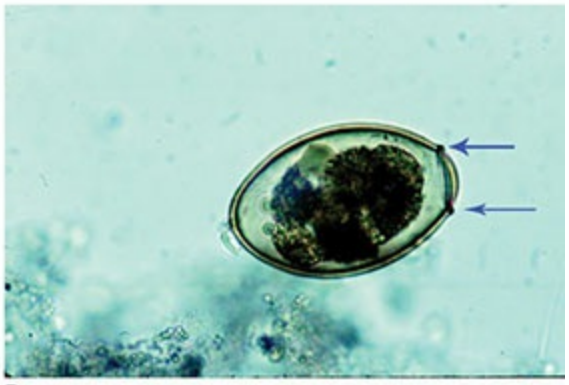

P

IP-164

IMAGE PLATE 22-8

PLASMODIUM

A. Photomicrograph of a Wright-Giemsa-stained peripheral blood smear illustrating several erythrocytes that are infected with ring forms of *Plasmodium* species. The infected erythrocytes are no larger in size than the surrounding cells, and Schüffner's dots are not present. The small size of the rings without development into advanced trophozoite forms and the high parasitemia are indicators of an infection with *Plasmodium falciparum*.

B. The infected erythrocytes in this photomicrograph of a stained peripheral smear also support a diagnosis of *Plasmodium falciparum*, both for the high parasitemia, and also the peripheral placement of tiny rings along the inner cell membranes, a so-called accolade effect or appliqué forms. Indeed, this smear was obtained from a patient with fulminant falciparum malaria.

C. Falciparum malaria can be definitively identified in the peripheral blood smear if the large, sickle or crescent-shaped gametocyte, as illustrated in the lower part of this photograph *(blue arrow)* is observed. Also note the small *Plasmodium* ring in the cell near the top of the field of view *(red arrow)*, again consistent with *Plasmodium falciparum*.

D. The infected erythrocyte illustrated in this stained peripheral smear is distinctly larger and paler than the surrounding cells and also has a distinctly stippled cytoplasm from the presence of Schüffner's dots. Note also that the ringed trophozoite is relatively large. These features are diagnostic for *Plasmodium vivax* infection.

E. *Plasmodium vivax* can be further suspected if a multicelled schizont, as illustrated by the enlarged erythrocyte in the center of this photomicrograph, is observed in a stained peripheral blood smear. These schizonts typically have more than 13 merozoites per schizont.

F. Illustrated in this stained peripheral smear is an enlarged, pale erythrocyte containing a uninucleated gametocyte of *Plasmodium vivax*.

G. Illustrated in this photomicrograph of a Wright-Giemsa-stained peripheral blood smear is an erythrocyte infected with a trophozoite of *Plasmodium malariae*. Note that the cytoplasm is condensed, does not develop into a typical ameboid form, but

rather bridges in a band form from one side of the cell membrane to the other. The red blood cell is not enlarged and Schüffner's dots are absent.

H. *Plasmodium malariae* can also be suspected if one observes a multinucleated schizont with less than 13 merozoites. Characteristically, the merozoites are arranged in a circular rosette pattern as seen in this photograph, often surrounding a coarse mass of malarial pigment that is absent in this particular infected cell.

IP-165

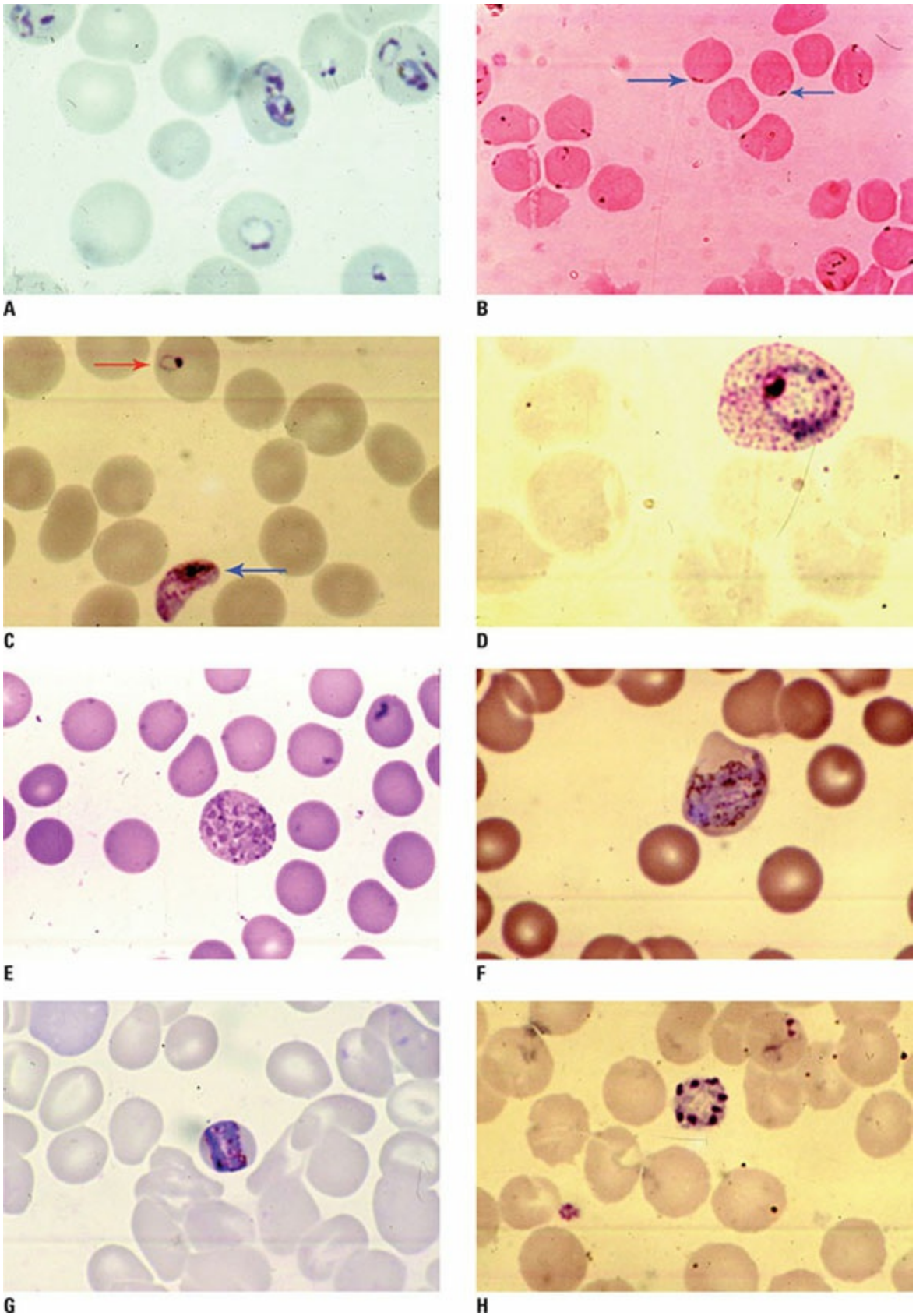
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IMAGE PLATE 22-9

BABESIOSIS/LEISHMANIASIS/TRYPANOSOMIASIS

A. Illustrated in this photomicrograph of a Wright-Giemsa-stained peripheral blood smear are several erythrocytes that are infected with the tiny pleomorphic ring-like forms of *Babesia* species. Tiny rings may be observed, suggestive of *Plasmodium falciparum*, as seen in one of the erythrocytes in the lower center field of view. The infected erythrocytes are not enlarged, are not pale, and never accumulate malarial pigment. Noteworthy is the arrangement of these forms into doublets resembling rabbit ears; or, more characteristically, into tetrads resembling a Maltese cross.

B. Photograph of the lower legs of a patient infected with *Leishmania tropica*, a species that typically causes cutaneous leishmaniasis, also called Chiclero ulcers. The ulcerating lesions as seen in this photograph result from the granulomatous inflammation caused by the infiltrating amastigotes.

C. Hematoxylin-and-eosin-stained section from subcutaneous tissue taken from the margin of an ulcer similar to that shown in Image Plate 22-9B. Note the tiny yeast-like forms seen in the cytoplasm of many of the infiltrating histiocytes and macrophages. These represent the amastigotes, the infective forms seen both in visceral and cutaneous leishmaniasis. These forms are difficult to differentiate from the intracytoplasmic yeast cells of *Histoplasma capsulatum*, but they often can be differentiated in stained smears of aspirates by demonstrating the rodlike kinetoplasts, as illustrated in Image Plate 22-9D and E.

D. Wright-Giemsa-stained impression smear illustrating a few background macrophages and numerous tiny 2 to 3 µm amastigotes of *Leishmania*. As mentioned before, these forms are often difficult to differentiate from the yeast cells of *Histoplasma capsulatum*. Careful observation, however, will reveal that many of these forms have a small, rod-shaped kinetoplast *(arrow)*, characteristic of *Leishmania* amastigotes.

E. Close-up view of a stained aspirate illustrating amastigotes of *Leishmania* species, identified by the distinctive kinetoplasts included within many of these forms.

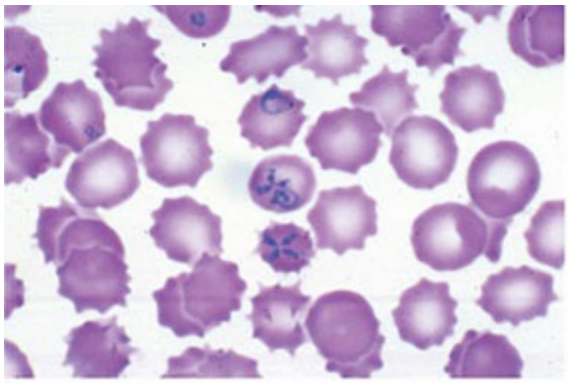
F. Photomicrograph of a Wright-stained peripheral blood smear in which several extracellular trypanosomes are observed. This smear was obtained from a patient with African trypanosomiasis. Each individual organism is long, slender, spindle-

shaped, and ranges from 15 to 30 µm long by 1.5 to 4 µm wide. The darker-staining, dot-like structure seen posteriorly in each trypanosome is the kinetoplast, from which a single flagellum originates. Each flagellum follows the outer contour of an undulating membrane that runs the length of the body, and then extends for some distance from the anterior end.

G. Photomicrograph of a Wright-stained peripheral smear obtained from a patient with American trypanosomiasis, also known as Chagas' disease. Note the characteristic "C" form of the trypanosomes. Each trypanosome has a deep-staining, dot-like structure posteriorly, the kinetoplast, from which a single flagellum originates. he kinetoplast of *T. cruzi* is larger and appears "bulging" compared with that of *T. brucei*.

H. Photograph of a triatomid (reduviid) bug, the vector for transmission of Chagas' disease. Meta cyclic trypanosomes develop in the hindgut of this bug, following ingestion of trypanosomes through the bite of an infected patient. The trypanosomes reenter the subcutaneous tissue after the bite of the second human host, not from the bite itself, but when contaminated fecal material discharged from the bug is scratched by the fingernails into the painful bite wound.

IP-167



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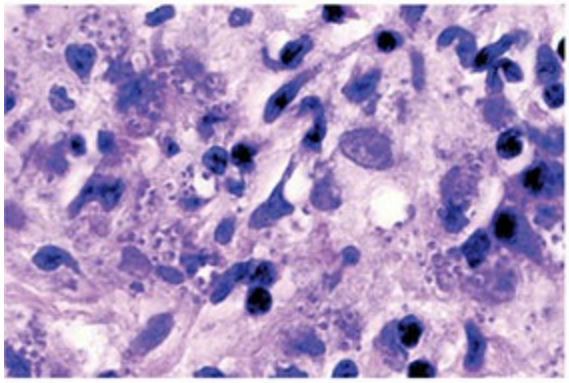

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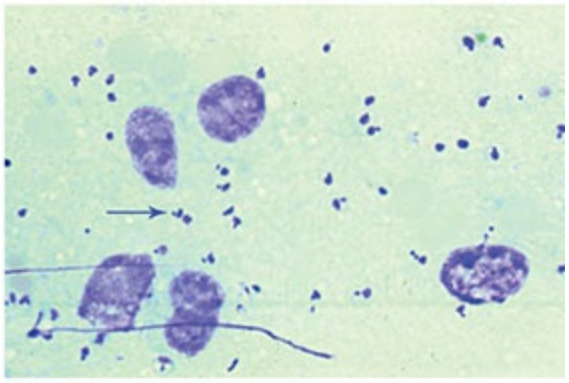

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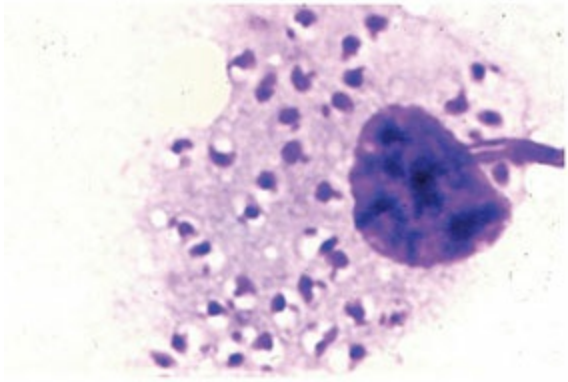

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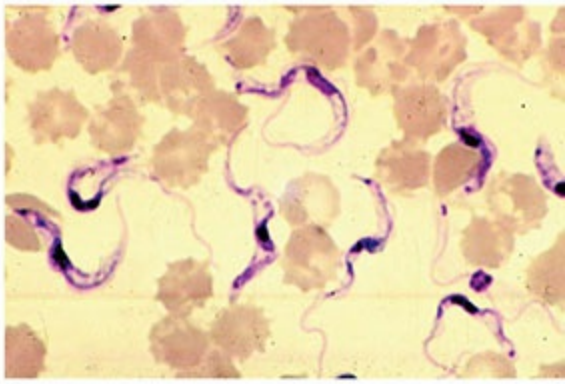

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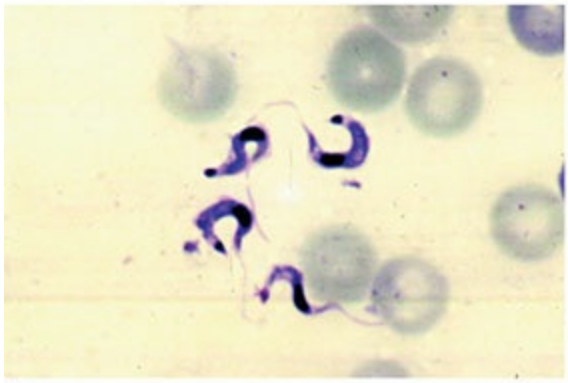

G

H

IP-168

IMAGE PLATE 22-10

FILARIA

A. Photomicrograph of a peripheral blood smear demonstrating a sheathed microfilaria. Microfilaria are extracellular parasites that measure about 245 to 295 µm in length and between 7 and 10 µm wide. They are released into the circulation from gravid female worms that reside within lymphatic channels in infected parts of the body. The microfilaria released by species pathogenic for humans have a sheath, as illustrated in this photograph *(arrow)*. As the column of nuclei seen in the parasite illustrated in this photograph does not appear to extend to the tip of the tail, the identification is *Wuchereria bancrofti*.

B. Photomicrograph of a peripheral blood smear illustrating the tail section of a microfilaria, high lighting the nuclear arrangement. Note that two detached nuclei extend into the tail, a feature characteristic of *Brugia malayi*.

C. A column of nuclei that extends into the tail section of a microfilaria, as illustrated in this photomicrograph, is characteristic of *Loa loa*.

D. Photomicrograph of a hematoxylin-and-eosin-stained section of a subcutaneous nodule, to include the cross section through the body of a female filarial worm, *Onchocerca volvulus*. Within the cavity of this organism are numerous microfilariae, which ultimately are released into the surrounding tissue, where they cause an inflammatory reaction ending in fibrosis. The microfilaria of *O. volvulus* rarely circulate in the peripheral blood, or they circulate in such small quantities that they virtually are never detected in stained smears.

E. Photograph of a dog heart heavily infested with the adult worms of *Dirofilaria immitis*. Canines serve as the definitive host for this tissue nematode. In humans, who may serve as inadvertent hosts, the larval forms never fully develop in the heart, but rather are swept into the pulmonary vasculature, where they produce infarcts and multifocal inflammatory granulomata.

F. Photomicrograph of a Giemsa-stained impression smear demonstrating numerous blue-staining, bow-shaped tachyzoites of *Toxoplasma gondii*. Each tachyzoite measures approximately 3 × 6 µm, is crescent or bow-shaped, and has a nucleus and various internal organelles. The tachyzoites are the infective form of the parasite, which enter the circulation and are carried to various organs, particularly the central nervous system, where they cause microfocal inflammatory lesions.

Tachyzoites can also transmigrate the placenta of pregnant women, enter the circulation of the developing fetus, and cause congenital infections in newborns.

G. Photomicrograph of a Giemsa-stained histologic section of a brain biopsy obtained from a patient with latent cerebral toxoplasmosis. Demonstrated are three cysts, ranging between 15 and 30 µm in diameter, within which are packed myriad tiny blue-staining bradyzoites. These are bradyzoite cysts, which will remain latent, unless the patient becomes immunocompromised.

H. A close-up view of a bradyzoite cyst. While the disease is latent in this cyst form, on suppression of immunity in a previously infected person, these cysts release active tachyzoites.

IP-169

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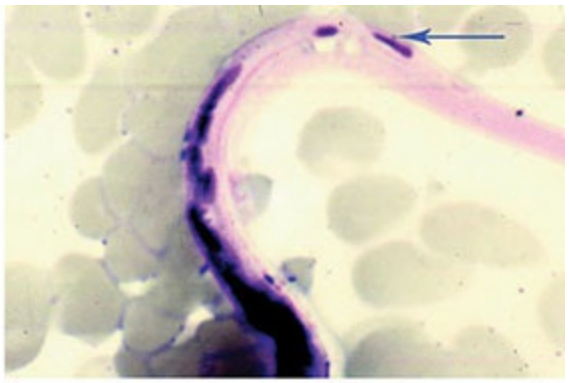

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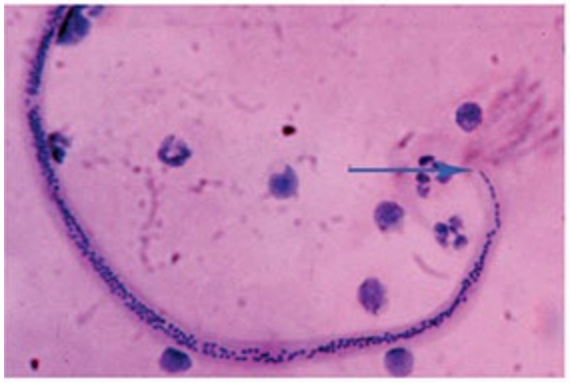

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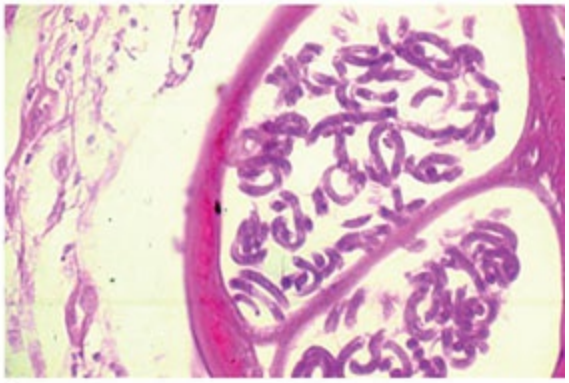

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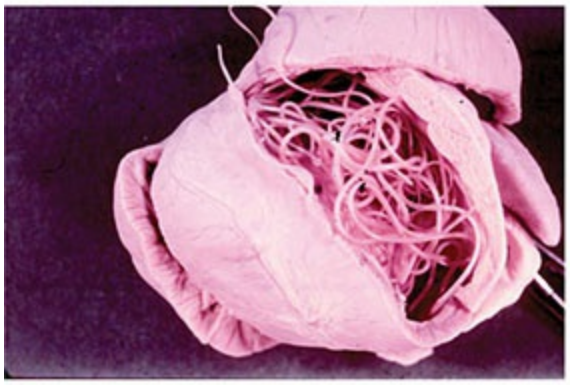

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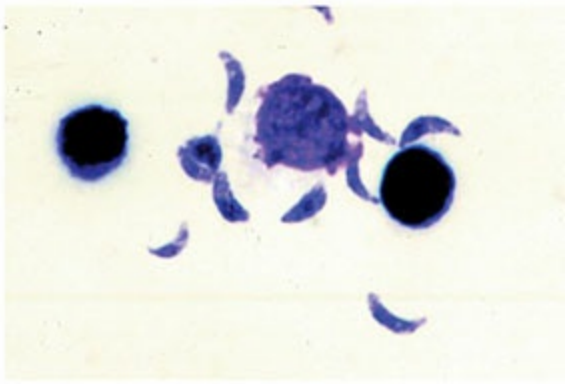

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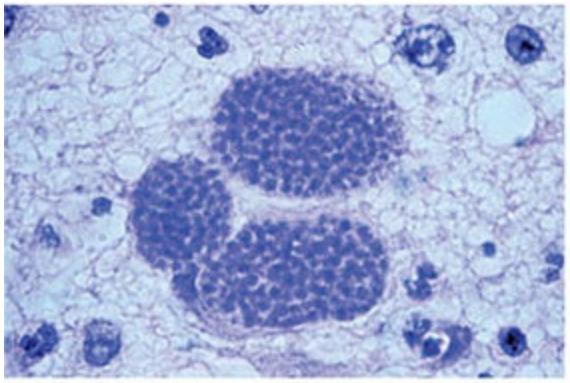

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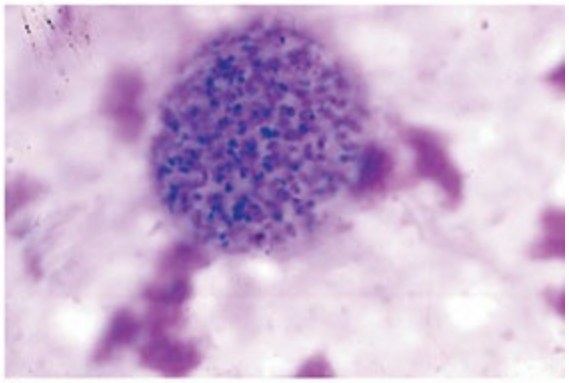


H

IP-170

IMAGE PLATE 22-11

Tissue Parasites

A. Hematoxylin-and-eosin-stained section of heart muscle, including a swollen fiber from top to bottom along the center of the field of view that is inhabited by dense collections of the amastigotic forms of *Trypanosoma cruzi*. Cardiac involvement in patients with Chagas' disease commonly results in heart failure and death.

B. Photograph of a liver cyst from which multiple small, variable-in-size, hydatid cysts were contained.

C. Close-up view of two hydatid cysts removed from the mesentery of a person with echinococcal disease, illustrating a thin, smooth outer membrane to the outer surface of which aggregates of fat are attached.

D. Hematoxylin-and-eosin-stained section taken through an echinococcal cyst. The cyst wall is composed of a layer of dense fibrous tissue derived from the host reaction. The inner parasite membrane is germinal, from which three daughter embryos (brood capsules), each with an inverted scolex, have been produced. On breaking free from the germinal membrane, these daughter cysts, along with released protoscolices, form free-floating hydatid sand.

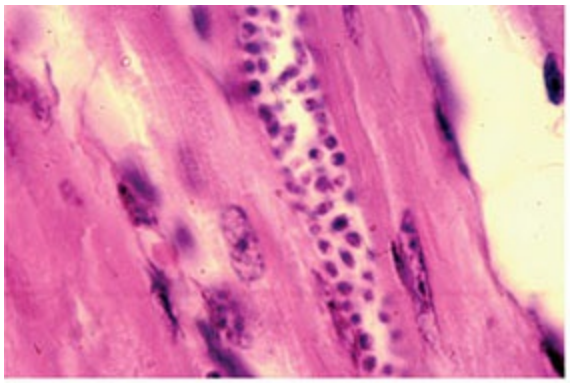
E. Illustrated are single protoscolices (fragments of "hydatid sand"), each containing an inverted rostellum with a prominent row of hooklets, representing the anlage of the protruding armed rostellum of the adult worm.

F. Photomicrograph of an adult *Echinococcus granulosus* tape worm. These adult worms, which reside in the intestinal tract of dogs and other canines, are very small, measuring no more than 6 mm long, and are composed of only three segments—an armed scolex, a neck, and a single proglottid, from which a relatively small number of eggs are released. However, as several hundred such worms may occupy the dog intestine, and the times of survival for each worm can reach 20 months, therefore, the total number of eggs released over a long period of time can be enormous, thereby guaranteeing completion of the life cycle.

G. Hematoxylin-and-eosin-stained section of skeletal muscle, including an encysted, coiled larval form of *Trichinella spiralis*. The dense infiltration of inflammatory cells into the tissue surrounding the cyst indicates relatively recent infection, most likely associated with local and possibly constitutional symptoms.

H. A second view of a muscle biopsy specimen including a coiled *Trichinella spiralis* larva. The absence of an inflammatory reaction and the laying down of dense fibrous tissue around the cyst indicate a chronic infection. In time, this inactive cyst may become calcified, representing the last stage of infection.

IP-171



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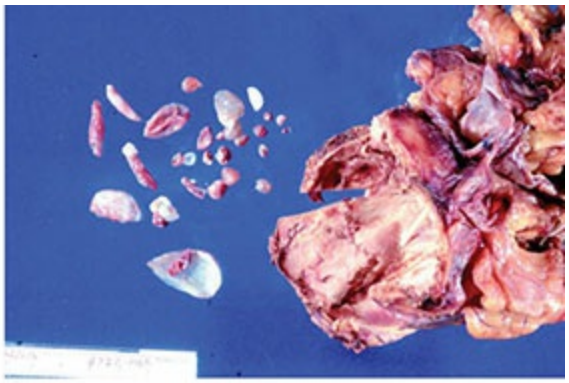

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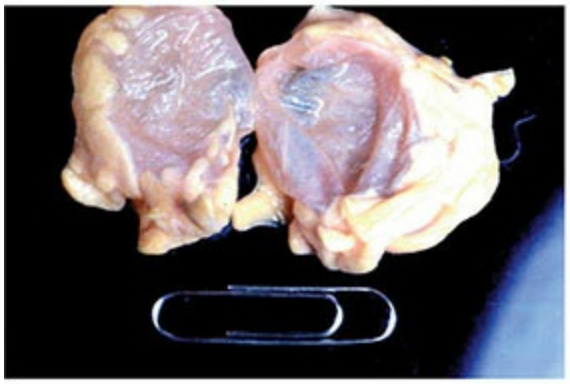

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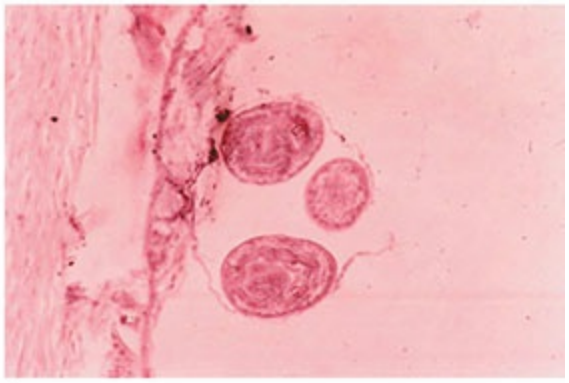

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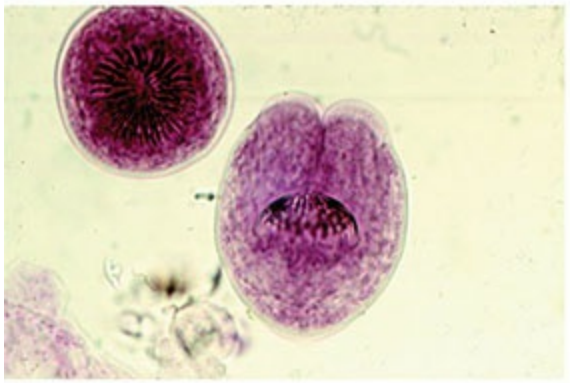

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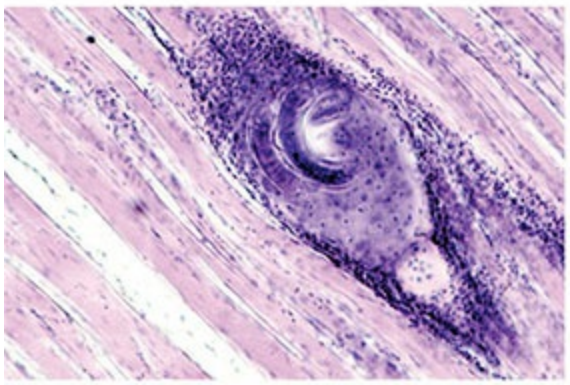

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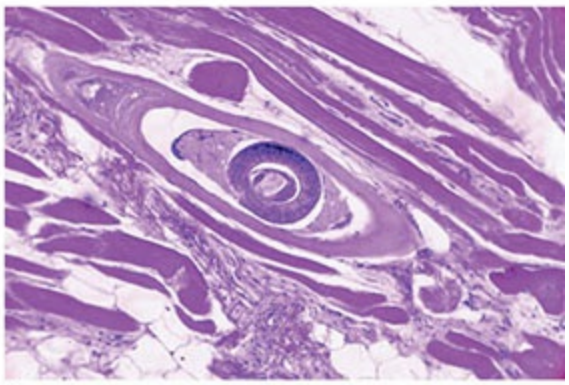

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IP-172

IMAGE PLATE 23-1

Viral Inclusions

A. Varicella-zoster virus hepatitis. The nuclei of several hepatocytes contain typical herpes virus inclusions. The mass of the inclusion is eosinophilic and colored red, and the residual nuclear chromatin, which is basophilic and colored blue, is crowded around the rim of the nuclear membrane. The inclusion stands out because of an artifactual clear halo between the inclusion and the peripheralized nuclear chromatin. This artifact is caused by formalin fixation. The inclusions of herpes simplex virus and varicella-zoster virus cannot be distinguished (Hematoxylin and eosin stain).

B. Herpes simplex cervicitis. The Papanicolaou stain demonstrates numerous multinucleated giant cells in a cervical scraping. The nuclei are molded against each other, and inclusions are present. The inclusions appear homogeneous, and nuclear chromatin is peripheralized to the rim of the nuclear membrane. The cytologic preparation was fixed promptly in ethanol, and the formalin halo artifact is not present. A mature squamous epithelial cell stains pink.

C. Herpes simplex vesicular lesion—Tzanck test. These cells were scraped from the base of a herpetic vesicle. The multinucleated cell is characteristic of herpes infection, but one cannot differentiate the effects of herpes simplex virus from those of varicella-zoster virus. The multiple nuclei are molded and homogeneous, but inclusions are difficult to see in air-dried smears that have been stained with a Giemsa or similar stain. The direct scrapings can also be stained by the Papanicolaou method or with hematoxylin and eosin.

D. Cytomegalovirus sialadenitis. The epithelial cells of a salivary gland contain the inclusions of cytomegalovirus. The large basophilic (blue or purple) inclusions fill much of the nucleus. In some cells, there are artifactual clear halos between the inclusion and the nuclear membrane that are produced by formalin fixation. Granular cytoplasmic inclusions are also visible in some cells. The architecture of the salivary gland is distorted by an intense lymphocytic infiltrate. Enlargement of the infected cells and nuclei inspired the name of the virus. Salivary gland virus was the original name for the virus, and salivary gland inclusion disease was the original name for the infection, because of the prominence of infection of this organ in neonatal disease (Hematoxylin and eosin stain).

E. Adenovirus pneumonia. In this biopsy from a patient with an adenovirus infection, there is a large amount of proteinaceous exudate, which is eosinophilic, in the air spaces, but there are few inflammatory cells. The nuclei of the infected respiratory epithelial cells are completely replaced by viral DNA, which produces a dense basophilic inclusion. Early inclusions may resemble those of herpes simplex virus. The mature inclusions, as seen here, fill the nucleus and obliterate the outline of the nuclear membrane. They are referred to as smudge cells (Hematoxylin and eosin stain).

F. Measles virus pneumonia. Several large multinucleated giant cells are in the airways of this patient with measles pneumonia. The multiple nuclei contain eosinophilic nuclear inclusions that resemble those of herpes simplex virus. The nuclear chromatin is pushed to the rim of the nuclear membrane, but the formalin clear halo artifact is not present in this section. In addition, in this biopsy there are also eosinophilic cytoplasmic inclusions, some of which are coalescent and/or surrounded by a clear space (Hematoxylin and eosin stain).

G. Respiratory syncytial virus infection. This is a monolayer of HEp-2 cells infected with respiratory syncytial virus. Two large syncytial giant cells have formed in the monolayer. There are no inclusions in the nuclei of the cells, but several brightly eosinophilic intracytoplasmic inclusions can be seen. (Hematoxylin and eosin stain.)

H. Rabies encephalitis. Two neurons each have an eosinophilic intracytoplasmic inclusion, which is known as a Negri body. Some vacuoles are evident in the inclusion. The irregular appearance comes from an admixture of normal cytoplasmic structures with masses of viral ribonucleic acid (Hematoxylin and eosin stain). (Courtesy of Daniel Perl, MD.)

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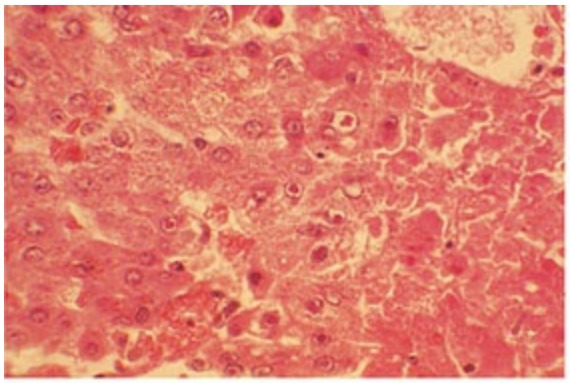

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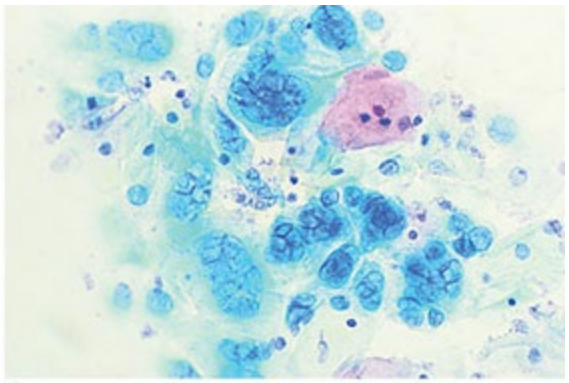

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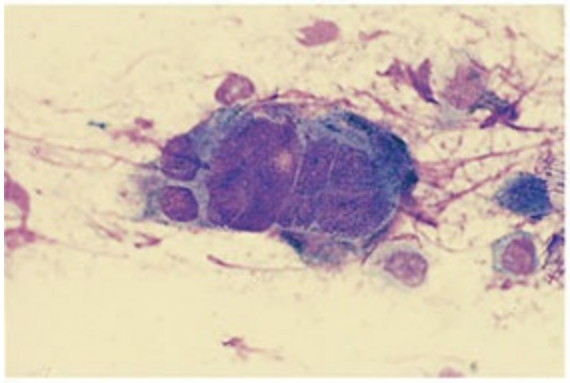

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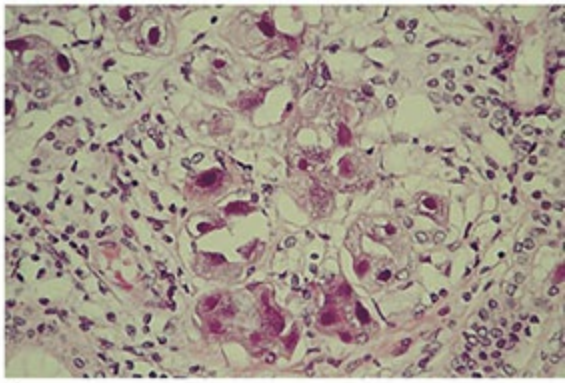

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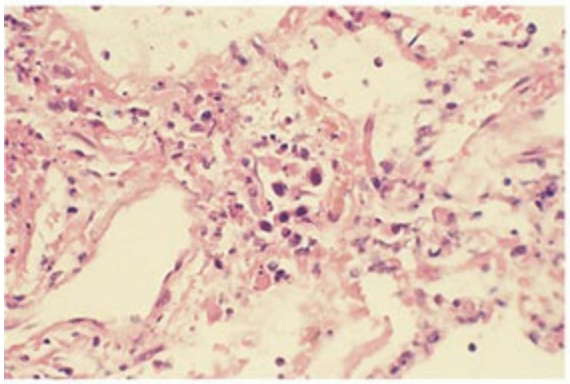

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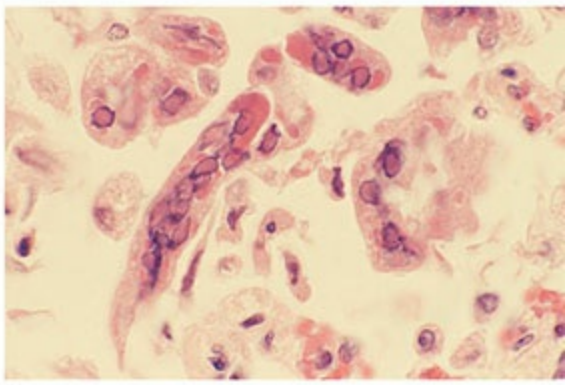

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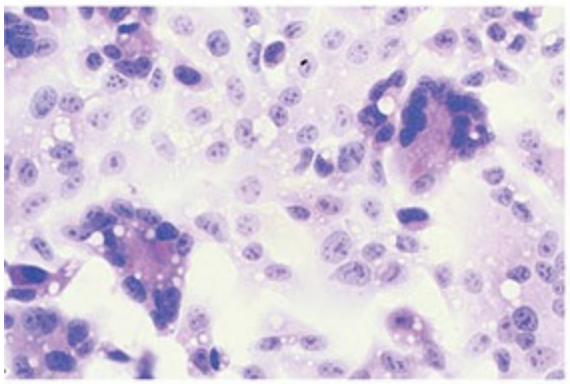

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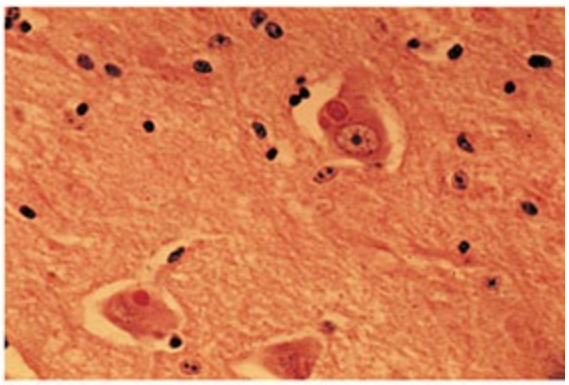


H

IMAGE PLATE 23-2

DIAGNOSIS OF INFECTIONS CAUSED BY VIRUSES, *CHLAMYDIA*, AND *EHRLICHIA*

A. *Chlamydia trachomatis* inclusion conjunctivitis. An epithelial cell that contains a chlamydial inclusion body is depicted. The cell has degenerated, leaving only the nucleus and the large inclusion. The large inclusion is molded around or capped over the underlying nucleus. Although these findings are not 100% specific, if present these should prompt diagnostic testing. Smear stained by the Giemsa method. (Photograph reproduced with permission of Julius Schachter and PSG Publishing Company, Inc., Littleton, MA.)

B. *C. trachomatis* cervicitis. Innumerable elementary bodies are present in this very heavy infection. The Evans blue counterstain causes the cells to fluoresce red, and the inclusions appear yellow in the photomicrograph. In areas with less background material or without a counterstain, the fluoresceinated antibody causes the elementary bodies to fluoresce apple green. A very heavily infected cell is present in the center of the photograph. Such specimens are of little challenge, but detecting positive specimens in which only a few elementary bodies are present is considerably more difficult.

C. This McCoy cell monolayer is infected with *C. trachomatis*. The specimen was centrifuged onto this monolayer of cycloheximide-treated McCoy cells, which were incubated for 48 hours. The cells have been stained with a fluorescein-conjugated monoclonal antibody to a major outer-membrane protein (MOMP) of *C. trachomatis*. Multiple intracytoplasmic inclusions, which correspond to the inclusion demonstrated in Image Plate 23-2A, stain bright green.

D. These HEp-2 cells are infected with herpes simplex virus, type 1. When a cytopathic effect became evident, the cells were scraped from the monolayer and placed on a slide. The cells have been stained with a fluorescein-conjugated monoclonal antibody to herpes simplex virus, type 1. There is bright, specific fluorescence, which is both nuclear and cytoplasmic.

E. This nasal aspirate contains cells that are infected with respiratory syncytial virus. Two cells in the smear show bright green cytoplasmic fluorescence. In addition to diffuse fluorescence, the discrete, punctate fluorescence represents viral inclusions, which are characteristic of this virus and help to confirm the specificity of the fluorescence. (Fluorescein isothiocyanate-conjugated monoclonal antibody to

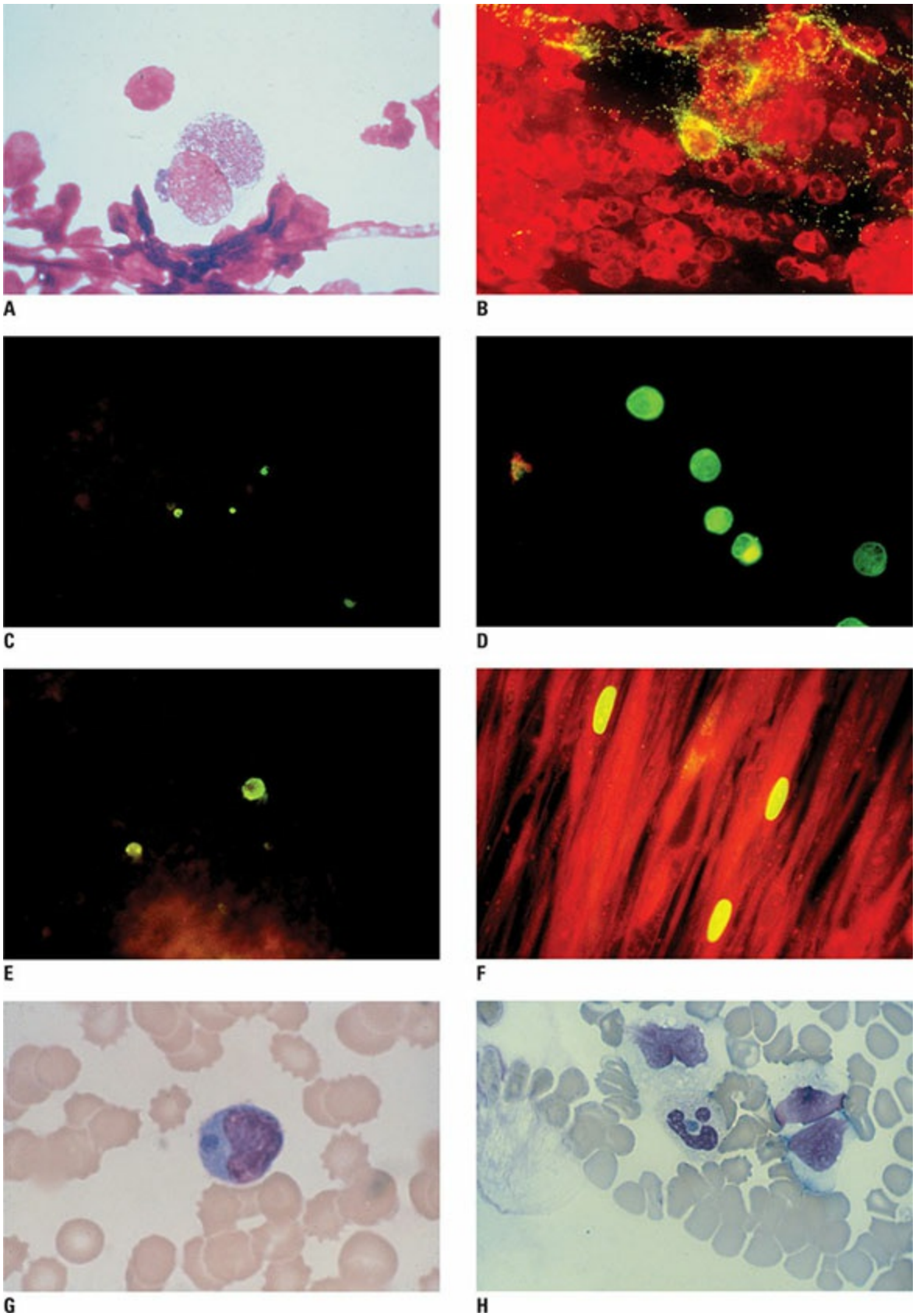
respiratory syncytial virus.)

F. This is a cytomegalovirus-infected human diploid fibroblast monolayer in a shell vial. The elongated fibroblast cells are colored by the counterstain. The infected cells are distinguished by the bright green fluorescence of the oval nuclei. (Fluorescein isothiocyanate-conjugated antibody to cytomegalovirus early-intermediate antigen.)

G. Human monocytic ehrlichiosis caused by *Ehrlichia chaffeensis*. A circulating mononuclear cell contains a distinct cytoplasmic inclusion (morula) that is made up of individual bacterial cells. Inclusions are rarely seen in cases of monocytic ehrlichiosis. (Wright stain. Slide contributed by J. Stephen Dumler, MD.)

H. Human granulocytic anaplasmosis is caused by *Anaplasma phagocytophilum*. A distinct bluish inclusion is present in the cytoplasm of a circulating neutrophil. (Wright stain. Slide contributed by J. Stephen Dumler, M.D.)

IP-175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IP-176

IMAGE PLATE A-1

IDENTIFICATION OF TICKS

A. Larval ticks, in this case *Ixodes scapularis*, are distinguished by the presence of six legs, three on each side. Nymphs and adults have a total of eight legs.

B. Instars (life stages) of *Ixodes scapularis* on a dime for comparison of size. From left to right: adult female (F), adult male (M), nymph (N), larva (L).

C. Ventral aspect adult female *Ixodes scapularis*, demonstrating relationship of mouthparts to basis capituli (B). Palp (P), hypostome (H), genital pore (G), anus (A), anal groove (U), spiracular plate (S). Inset shows detail of mouthparts. Note the teethlike denticles protruding from the lateral edges of the hypostome.

D. Coxal spurs of adult *Ixodes scapularis*. The spur on coxa I is long, pointed, and oriented internally. The spurs on coxae II through IV are short, blunt, and oriented externally.

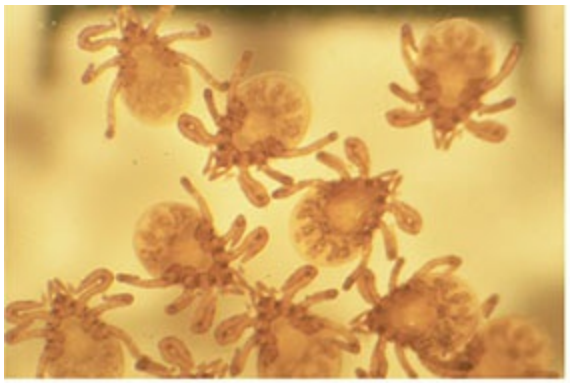
E. *Ixodes cookei* female dorsum. The scutum (S) is angular. The length of the palps (P) is approximately the same as the basis capituli (B). In this specimen, the hypostome (H) is broken.

F. *I. marxi* female dorsum. The scutum (S) is angular and resembles that of *I. cookei* (compare Image Plate A-1F), but the scutum of *I. marxi* is longer than it is wide in comparison to *I. cookei*.

G. *Dermacentor variabilis* adult females. The scutum demonstrates ornamentation (O). Festoons (F) are present at the posterior margin. One of the specimens is considerably engorged (E), changing the color of the dorsum from brown to cream.

H. *Dermacentor variabilis* adult female. The scutum is intensely ornamented (O). The basis capituli (B) is wider than it is tall. Note the "eyes" at the lateral margin bilaterally *(arrows)*.

IP-177



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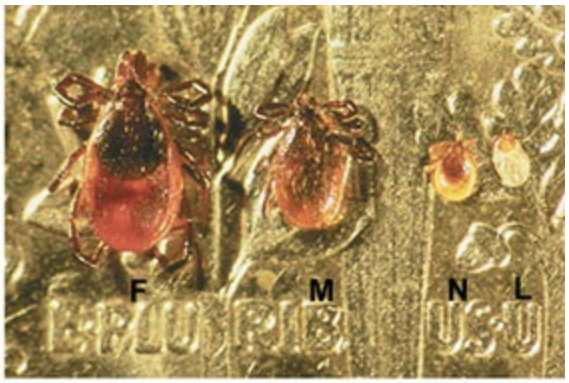

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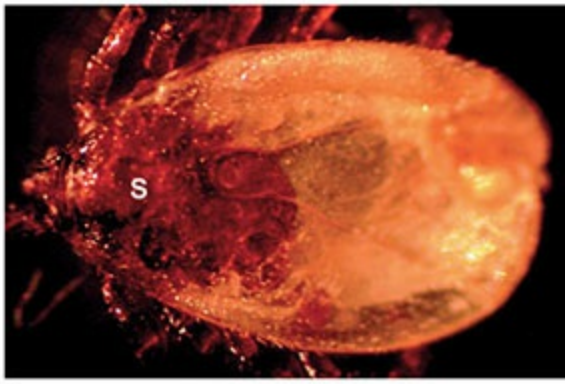

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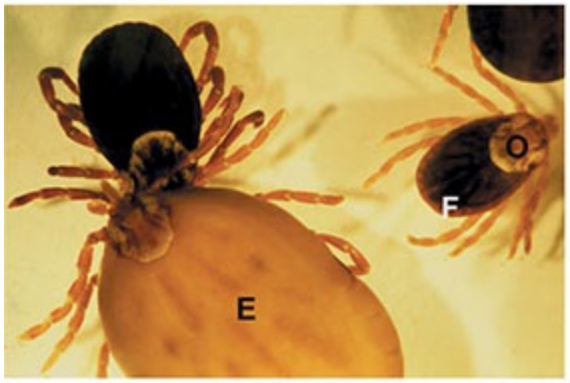

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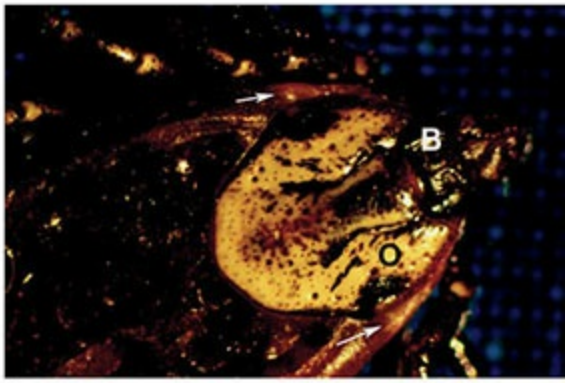

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IP-178

IMAGE PLATE A-2

IDENTIFICATION OF TICKS AND OTHER ARTHROPODS

A. *Amblyomma americanum* female dorsum. The ornamentation on the scutum (S) takes the form of a patch at the posterior edge—the lone star *(arrow)*. Festoons (F) are present around the posterior edge of the tick. "Eyes" are present, but are difficult to see in this partially engorged specimen.

B. *Amblyomma americanum* male dorsum. A very prominent "eye" *(arrow)* is present at the lateral edge of the scutum, which covers the entire dorsum. The mouthparts are missing.

C. *Amblyomma americanum* male dorsum. The prominent festoons (F) are evident at the posterior margin. The arrows delineate two horseshoe-shaped markings just lateral to the midline bilaterally.

D. *Amblyomma americanum* male ventral surface. A prominent internal spur is evident on coxa IV *(arrows)*. A detail of the spur is shown in the inset (I). The anus (A) is surrounded by a posterior facing groove (GR), in contrast to the anterior facing groove of *Ixodes* spp. The genital pore (G) is prominent. All of the mouthparts and the basis capituli are missing from this specimen.

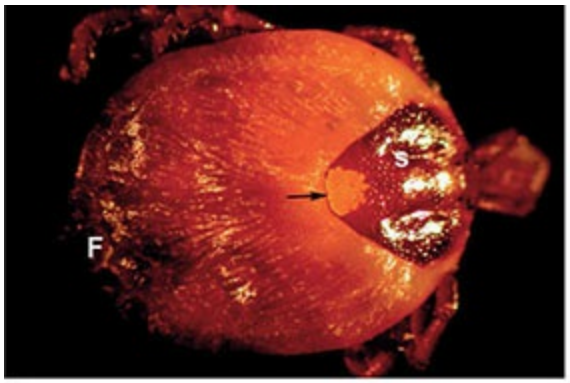
E. Dorsal view of *Ornithodoros* spp. This soft tick lacks a scutum, and the mouthparts are not visible from above.

F. *Sarcoptes scabiei* in its burrow. The mite has deposited eggs and defecated in its newfound home, adding to the discomfort of the victim.

G. *Loxosceles reclusa*, the brown recluse spider. The brown color and characteristic head marking distinguish this venomous spider. (Photograph courtesy of Robert Suter, Ph.D.)

H. Black widow spider. The deep black color and characteristic red, hourglass-shaped marking on the ventral side are clearly visible. (Photograph courtesy of Robert Suter, Ph.D.)

IP-179



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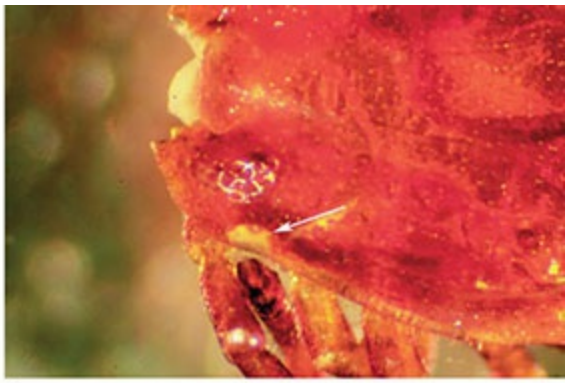

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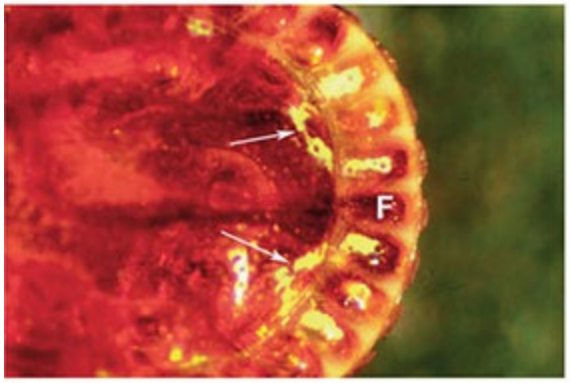

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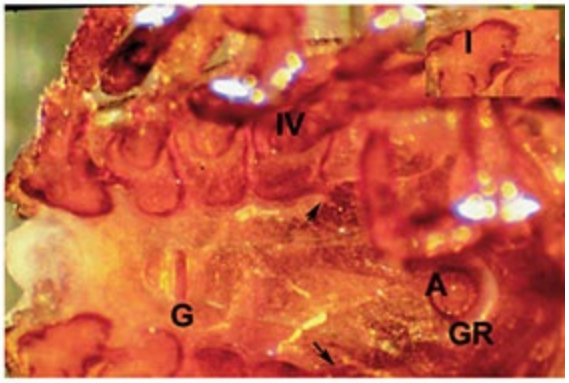

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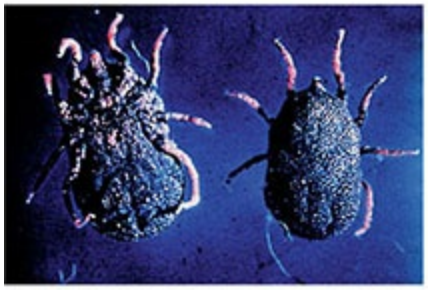

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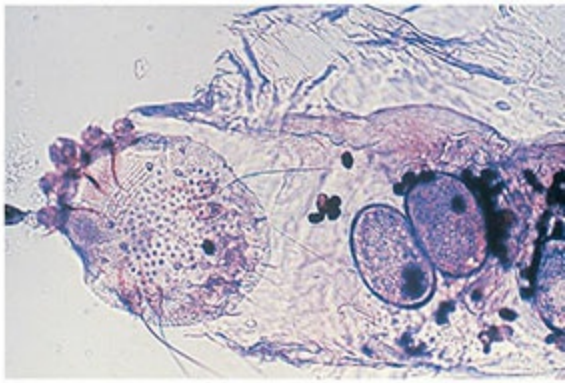

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IP-180

IMAGE PLATE A-3

IDENTIFICATION OF MISCELLANEOUS ARTHROPODS

A. *Dermatobia hominis*. The human botfly has been extracted from a pustular lesion. The flask shape, tapered neck, and encircling spines of the larva are evident. The neck was damaged at the time of surgical extraction.

B. Ventral view of *Cimex* spp., the bedbug. The orange-brown color and characteristic shape provide identifying features.

C. Dorsal view of *Triatoma* spp., a reduviid or kissing bug. The elongated body and long, thin head are characteristic. A pair of wings is evident.

D. *Tunga penetrans*, the jigger flea. Most fleas bite their host quickly and depart for completion of their life cycle, but the jigger flea remains embedded in the skin while producing eggs.

E. Female *Ctenocephalides* spp., the dog or cat flea. The long, muscular legs hang from the body and make the flea a prodigious jumper. The "hunched" appearance of the body is evident.

F. Female *Pediculosis humanus*, the human body louse. The long slender body supports three pairs of legs, each of which has clawlike hooks at the terminus.

G. Nit of a head louse. The egg is firmly attached to a hair shaft.

H. Female *Phthirus pubis*, the crab louse. The squat body and well-developed pincers on three pairs of legs make the derivation of the popular name clear. The louse hangs onto a hair shaft, which also supports a nit.

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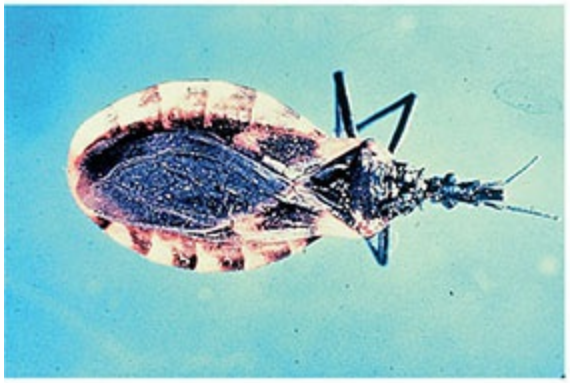

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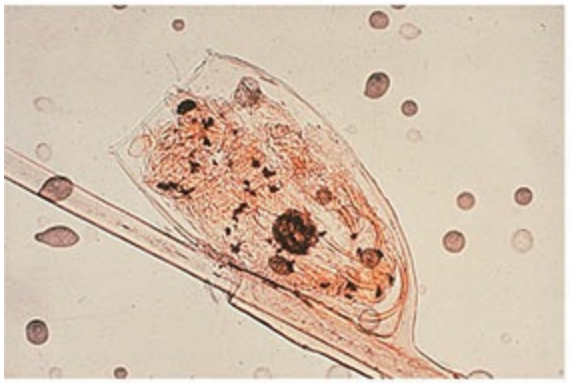

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Charts

CHART 1-1

Catalase

I. Principle

Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water and oxygen. Chemically, catalase is a hemoprotein, similar in structure to hemoglobin, except that the four iron atoms in the molecule are in the oxidized (Fe^{3+}), rather than the reduced (Fe^{2+}), state. Excluding the streptococci, enterococci, and a few other genera, most aerobic and facultative bacteria possess catalase activity.

Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. If allowed to accumulate, it is lethal to bacterial cells. Catalase converts hydrogen peroxide into oxygen and water as shown by the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (gas bubbles)}$$

The catalase test is most commonly used to differentiate members of the *Micrococcaceae*, which are positive, from members of the *Streptococcaceae*.

II. Reagents

A. Hydrogen peroxide 3% stored in a brown bottle under refrigeration

B. An 18- to 24-hour culture of the organism to be tested

III. Quality Control

The hydrogen peroxide reagent must be tested with positive and negative control organisms each day or immediately before unknown bacteria are tested.

A. Positive control: *Staphylococcus aureus*

B. Negative control: *Streptococcus* species

IV. Procedure

1. With an inoculating needle or a wooden applicator stick, transfer growth from the center of a colony to the surface of a glass slide.
2. Add one drop of 3% hydrogen peroxide and observe for bubble formation.

V. Results

A. Interpretation

The rapid and sustained appearance of bubbles or effervescence constitutes a positive test. Because some bacteria possess enzymes other than catalase that can decompose hydrogen peroxide, a few tiny bubbles forming after 20–30 seconds is not considered a positive test. In addition, catalase is present in red blood cells; so care must be taken to avoid carryover of red blood cells with the colony material.

CHART 1-2

Bile-Solubility Test

I. Principle

Bile salts, specifically sodium deoxycholate and sodium taurocholate, have the capability to lyse *Streptococcus pneumoniae* selectively when added to actively growing bacteria in agar or broth media. *S. pneumoniae* produces autolytic enzymes (autolysins) that account for the central depression or umbilication characteristic of older pneumococcal colonies on agar media. The addition of bile salts activates the autolysins and accelerates the natural lytic reaction observed with cultures or pneumococci.

C-2

The bile-solubility test can be performed either with a broth culture of the organism or with colonies growing on agar media. The turbidity of a broth suspension visibly clears on addition of bile salts if the organism is soluble. On agar medium, bile-soluble colonies dissolve when drops of the reagent are placed on them. Because sodium deoxycholate may precipitate at a pH of 6.5 or less, the broth culture medium used must be adjusted to pH 7.0 to prevent false-negative reactions.

II. Media and Reagents

1. A pure culture of the test organism grown at 35°C for 18–24 hours in Todd–Hewitt broth (or equivalent)
2. Sheep blood agar plate
3. Sodium deoxycholate (10% for tube test, 2% for plate test)
4. Phenol red solution (1% aqueous)
5. Sodium hydroxide (NaOH) solution, 0.10 N

III. Quality Control

A. Positive (bile-soluble) control: *Streptococcus pneumoniae*

B. Negative (bile-insoluble) control: Any of the viridans group streptococci

IV. Procedure

A. Broth Test

1. Transfer approximately 0.5 mL of an 18- to 24-hour broth culture to two clean test tubes. Alternatively, a suspension of the organism may be prepared from growth on agar media in phosphate-buffered saline, pH 7.0. If the latter is done, the pH need not be readjusted.
2. Add one drop of the phenol red indicator to each tube.
3. Add 0.10 N NaOH to adjust the pH to 7.0 (indicator a light pink color).
4. Add 0.5 mL of 10% sodium deoxycholate to one of the tubes (labeled "test").
5. Add 0.5 mL sterile normal saline to the other tube (labeled "control").
6. Gently agitate both test tubes and place them in an incubator or a water bath at 35°C for 3 hours, checking hourly.

B. Plate Test

1. To a few well-isolated colonies on the test organism growing on sheep blood agar, place a drop of 2% sodium deoxycholate.
2. Without inverting the plate, place it in a 35°C incubator for 30 minutes.

V. Results

A. Interpretation

1. Broth Test

   Bile-soluble (positive reaction): there is visible clearing of the suspension in the tube containing the sodium deoxycholate, with no change in the saline control suspension.

   Bile-insoluble (negative reaction): there is no change in the turbidity of the sodium deoxycholate–containing tube relative to the control saline suspension.

2. Plate Test

Bile-soluble (positive reaction): colonies on which the reagent was placed dissolve, leaving a partially hemolyzed area where the colony had been.

Bile-insoluble (negative reaction): colonies where the reagent was placed remain intact and visible.

VI. Limitations of Test

Only 86% of pneumococcal strains will lyse completely. Optochin susceptibility or other studies are useful for the identification of incompletely lysed strains.

CHART 1-3

The Slide Coagulase Test

I. Principle

Staphylococcal coagulase is a protein that has a prothrombin-like activity, which can convert fibrinogen into fibrin. A visible clot will result. Coagulase is believed to function in vivo by producing a fibrin barrier at the site of staphylococcal infection. This enzyme probably plays a role in localizing the abscesses (e.g., carbuncles and furuncles). In the laboratory, the coagulase test is used to identify *Staphylococcus aureus* and differentiate it from most other species of staphylococci.

Coagulase is present in two forms, bound and free, each having different properties that require the use of separate testing procedures.

C-3

Bound coagulase (slide test): Bound coagulase, also known as clumping factor, is attached to the bacterial cell wall and is not present in culture filtrates. Fibrin strands are formed between the bacterial cells when suspended in plasma (fibrinogen), causing them to clump into visible aggregates.

Free coagulase (tube test): Free coagulase is a thrombin-like substance present in culture filtrates. When a suspension of coagulase-producing organisms is prepared in plasma in a test tube, a visible clot forms as the result of coagulase reacting with a serum substance (coagulase-reacting

factor) to form a complex that, in turn, reacts with fibrinogen to produce the fibrin clot.

II. Media and Reagents

Rabbit plasma with EDTA (commercially available in lyophilized form). Reconstituted plasma should be refrigerated.

III. Quality Control

The coagulation of properties of plasma may be tested, for quality control purposes, by adding one drop of 5% calcium chloride to 0.5 mL of the reconstituted plasma. A clot should form within 10–15 seconds. A known *Staphylococcus aureus* strain and a *Staphylococcus epidermidis* strain serve as positive and negative controls, respectively. Each reconstituted vial of rabbit plasma with EDTA should be tested with 18- to 24-hour cultures of the control strains.

IV. Procedure

1. *Slide test* (bound coagulase): Place two drops of sterile water or saline in two circles drawn on a glass slide with a wax pencil. Gently emulsify colony material from the organism to be identified in liquid in each of the circles. Place a drop of coagulase plasma in the suspension in one of the circles and mix with a wooden applicator stick. Place another drop of water or saline in the other circle as a control. Rock the slide back and forth, observing for agglutination of the test suspension.
2. *Tube test* (free coagulase): Emulsify a small amount of the colony growth of the organism in a tube containing 0.5 mL of coagulase plasma. Incubate the tube at 35°C for 4 hours and observe for clot formation by gently tilting the tube. If no clot is observed at that time, reincubate the tube at room temperature and read again after 18 hours.

V. Results

A. Interpretation

1. *Slide test:* A positive reaction will be detected within 10–15 seconds of mixing the plasma with the suspension by the formation of a white precipitate and agglutination of the organisms in the suspension. The test is considered negative if no agglutination is observed after 2 minutes. The saline control should remain smooth and milky. If the control suspension agglutinates as well, then the test is uninterpretable. All strains that are coagulase-positive can be reported as coagulase-positive *Staphylococcus* or, if the colonial morphology is appropriate as, *Staphylococcus aureus*. All strains producing negative slide tests must be tested with the tube coagulase test.
2. *Tube test:* The tube coagulase test is considered positive if any degree of clotting is noted. The tube should be gently tilted and not

agitated, because this may disrupt partially formed clotted material. Fibrinolysins produced by the organism may also dissolve the clot soon after formation. Tube tests that are negative after 4 hours should be incubated at room temperature overnight and read after 18 hours.

CHART 1-4

Indole Test

I. Principle

Indole, a benzyl pyrrole, is one of the metabolic degradation products of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and deaminating tryptophan with the production of indole, pyruvic acid, and ammonia. Indole production is an important characteristic in the identification of many species of microorganisms, being particularly useful in separating *Escherichia coli* (positive) from members of the *Klebsiella- Enterobacter- Hafnia- Serratia* group (mostly negative).

The indole test is based on the formation of a red complex when indole reacts with the aldehyde group of *p*-dimethylaminobenzaldehyde. This is the active chemical in Kovac's and Ehrlich's reagents. A medium rich in tryptophan must be used. In practice, combination media such as sulfide indole motility (SIM), motility indole ornithine (MIO), or indole nitrate are used. Rapid spot tests, using filter paper strips impregnated with *p*-dimethylaminocinnamaldehyde reagent, are useful in screening for bacteria that are prompt indole producers.

II. Media and Reagents

A. Tryptophan Broth (1% tryptophan)

Peptone or pancreatic digest of casein (trypticase)	2 g
Sodium chloride	0.5 g
Distilled water	100 mL

B. Kovac's Reagent

Pure amyl or isoamyl alcohol	150 mL
p-Dimethylaminobenzaldehyde	10 g
Concentrated HCl	50 mL

C. Ehrlich's Reagent

p-Dimethylaminobenzaldehyde	2 g
Absolute ethyl alcohol	190 mL
Concentrated HCl	40 mL

III. Quality Control

Each new batch of medium or reagent should be tested for positive and negative indole reactions. The following organisms serve well as controls:

A. Positive control: *Escherichia coli*

B. Negative control: *Klebsiella pneumoniae*

IV. Procedure

Inoculate tryptophan broth (or other suitable indole media) with the test organism and incubate at 35°C for 18–24 hours. At the end of this time, add 15 drops of reagent down the inner wall of the tube. If Ehrlich's reagent is used, this step should be preceded by the addition of 1 mL xylene. This is not necessary with Kovac's reagent.

V. Results

A. Interpretation

The development of a bright fuchsia red color at the interface of the reagent and the broth (or the xylene layer) within seconds after adding the reagent is indicative of the presence of indole and is a positive test (Color Plates 6-4C and 7-2C).

VI. Bibliography

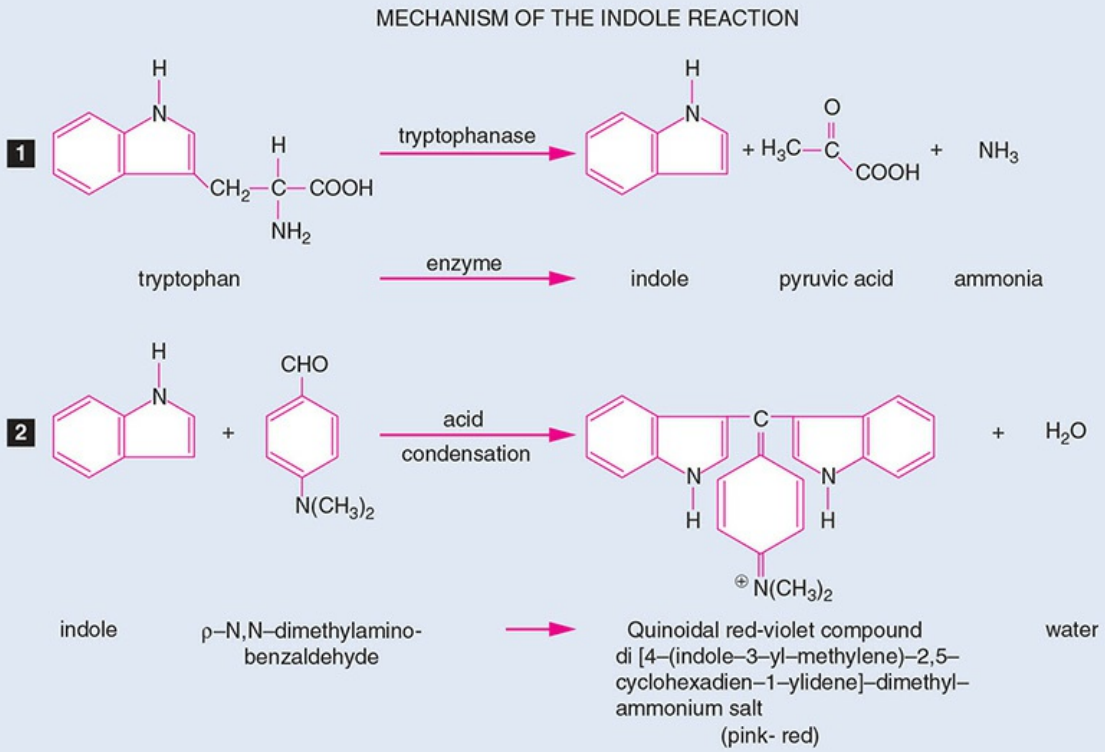
Blazevic DJ, Ederer GM. Principles of Biochemical Tests in Diagnostic Microbiology. New York, NY: Wiley, 1975:63–67.

Isenberg HD, Sundheim LH. Indole reactions in bacteria. J Bacteriol 1958;75:682–690.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:173–183.

Miller JM, Wright JW. Spot indole test: evaluation of four reagents. J Clin Microbiol 1982;15:589–592.

Vracko R, Sherris JC. Indole-spot test in bacteriology. Am J Clin Pathol 1963;39:429–432.



C-5

CHART 1-5

Cytochrome Oxidase Test

I. Principle

The cytochromes are iron-containing hemoproteins that act as the last link in the chain of aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water. The cytochrome system is found in aerobic, microaerophilic, and facultatively anaerobic organisms, and so the oxidase test is important in identifying organisms that either lack the enzyme or are obligate anaerobes. The test is most helpful in screening colonies suspected of being one of the *Enterobacteriaceae* (all negative) and in identifying colonies suspected of belonging to other genera such as *Aeromonas*, *Pseudomonas*, *Neisseria*, *Campylobacter*, and *Pasteurella* (positive).

The cytochrome oxidase test uses certain reagent dyes, such as *p*-phenylenediamine dihydrochloride, that substitute for oxygen as artificial electron acceptors. In the reduced state, the dye is colorless; however, in the presence of cytochrome oxidase and atmospheric oxygen, *p*-phenylenediamine is oxidized, forming indophenol blue.

II. Media and Reagents

A. Tetramethyl- *p*-phenylenediamine dihydrochloride, 1% (Kovac's reagent)

B. Dimethyl- *p*-phenylenediamine dihydrochloride, 1% (Gordon and McLeod's reagent)

III. Quality Control

Bacterial species showing positive and negative reactions should be used as controls at appropriate intervals. The following are suggested:

A. Positive control: *Pseudomonas aeruginosa*

B. Negative control: *Escherichia coli*

IV. Procedure

The test is commonly performed by one of two methods: (1) the direct plate technique, in which two to three drops of reagent are added directly to isolated bacterial colonies growing on plate medium; and (2) the indirect paper strip procedure, in which either a few drops of the reagent are added to a filter paper strip or commercial disks or strips impregnated with dried reagent are used. The tetramethyl derivative of *p*-phenylenediamine is recommended because the reagent is more stable in storage and is more sensitive to the detection of cytochrome oxidase and is less toxic than the dimethyl derivative. In either method, a loopful of suspected colony is smeared into the reagent zone of the filter paper.

V. Results

A. Interpretation

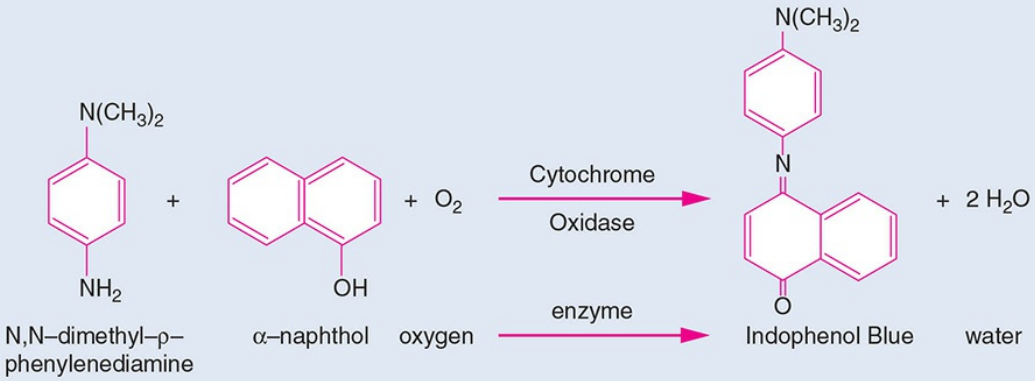
Bacterial colonies having cytochrome oxidase activity develop a deep blue color at the inoculation site within 10 seconds (Color Plate 6-1G). Any organism producing a blue color in the 10- to 60-second period is considered negative, and it can be concluded that it does not belong to the *Enterobacteriaceae*. *Pasteurella multocida* is unique in that it is tetramethyl oxidase-positive, but often dimethyl oxidase-negative; a feature to remember for isolates suspected to possibly represent this organism. Stainless steel or Nichrome inoculating loops or wires should not be used for this test because surface oxidation products formed when flame-sterilizing may result in false-positive reactions.

VI. Bibliography

Gordon J, McLeod JW. The practical application of the direct oxidase reaction in bacteriology. J Pathol Bacteriol 1928;31:185–190.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:249–260.
Steel KJ. The oxidase reaction as a taxonomic tool. J Gen Microbiol 1961;25:297–306.
Weaver DK, Lee EKH, Leahy MS. Comparison of reagent impregnated paper strips and conventional methods for identification of *Enterobacteriaceae*. Am J Clin Pathol 1968;49:494–499.

MECHANISM OF THE CYTOCHROME OXIDASE REACTION



C-6

CHART 1-6

PYR Test

I. Principle

The PYR test was first described by Facklam and coauthors in 1982; since that time, it has been widely used and is now a standard assay for the presumptive identification of both group A β-hemolytic streptococci and enterococci. Whereas the original test was described as a 16- to 20-hour agar test, subsequent PYR test formats included a 4-hour broth assay and now several rapid (minutes) tests in which the PYR reagent is impregnated in filter paper disks or strips that are inoculated with the organism to be tested. The PYR test also appears on several of the kit systems for identification of streptococci and *Streptococcus*-like bacteria. This chart describes the 4-hour

broth PYR test. Disks impregnated with the PYR substrate can also be used for an even more rapid test.

The substrate used for the PYR test is L-naphthylamide-β-naphthylamide. This compound is hydrolyzed by a specific bacterial aminopeptidase enzyme. Hydrolysis of the substrate by this enzyme releases free β-naphthylamide, which is detected by the addition of *N, N*-dimethylaminocinnamaldehyde. This detection reagent couples with the naphthylamide to form a red Schiff base.

II. Media and Reagents

1. PYR broth (Todd–Hewitt broth with 0.01% L-pyrrolidonyl-β-naphthylamide) dispensed into sterile tubes in 0.20-mL volumes
2. PYR reagent (0.01% *p*-dimethylaminocinnamaldehyde)

III. Quality Control

A. Positive control: *Enterococcus faecalis* or *Streptococcus pyogenes*

B. Negative control: *Streptococcus agalactiae*

IV. Procedure

1. With a sterile bacteriologic loop, pick up the growth of two to three morphologically similar colonies and emulsify them in the small volume of PYR broth.
2. Incubate the tube at 35°C for 4 hours.
3. Add one drop of the PYR reagent and observe for color change. The reaction should be read and recorded 1 minute after the addition of reagent.

V. Results

A. Interpretation

1. Positive: the development of a deep cherry red color within a minute of addition of the reagent
2. Negative: a yellow or orange color

VI. Procedure Notes

Other organisms (e.g., some aerococci, staphylococci, nutritionally variant streptococci, *Arcanobacterium haemolyticum*) may also be PYR-positive. Most laboratories now use commercially available products that work within minutes after exposure of the bacterium on filter paper to the reagents; PYR-positive organisms produce a purple color, whereas PYR-negative organisms produce no color change.

CHART 3-1

Complement Fixation (CF) Test

I. Principle

The complement fixation test is one of the major traditional tests for demonstration of antigens from infectious agents and the corresponding antibodies. It requires many reagents and numerous preparatory steps, which is why laboratories favor enzyme immunoassays and other methods to this one. There are almost as many versions as there have been users; the microtiter version developed at the Centers for Disease Control and Prevention (LBCF test) includes rigorous controls and is commonly used. The CF test depends on the ability of antibodies to fix complement, and the sera must be free of anticomplementary activity.

The terminal components of the complement cascade, C789 (i.e., the attack complex), can damage cell membranes in the presence of specific antibody, which fixes complement to the cell surface. In the CF test, erythrocytes are used as the target cell, because complement-induced cell lysis can be visualized or measured colorimetrically as an increase in free hemoglobin. In the presence of specific antibodies to an infectious agent, any complement in the system is bound, leaving no residual complement for reaction with antibodies to the indicator erythrocytes. Thus, the presence of specific antibody is indicated by the absence of hemolysis.

II. Materials and Reagents

Sheep erythrocyte suspension
Hemolysin (rabbit anti-sheep red blood cell antibody)
Guinea pig complement, free of antibodies to the agent of interest

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Barbital-buffered diluent
Plastic microtiter plate
Centrifuge adapter for microtiter plates
Water bath for incubation of plates
Color standards for judging hemolysis (prepared by lysing various concentrations of red blood cells)

III. Quality Control

Known positive antibody or antigen
Known negative antibody or antigen
Serum control without antigen (to detect anticomplementary activity)
Antigen controls without serum (to detect anticomplementary activity)
Tissue control (the cells or tissue in which the antigen was prepared)

Buffer control without antigen or antibody
Back titration of complement to document the use of 5 CH_{50} units
Cell control to demonstrate absence of hemolysis
Reference hemolysis standards

IV. Procedure

1. Sensitization of Erythrocytes

Serial dilutions of hemolysin are incubated with a 2.8% solution of sheep red blood cells in barbital buffer. As the concentration of hemolysin increases, the percentage hemolysis will increase to a plateau level. The concentration of hemolysin that represents the second point on the plateau is used for the test.

2. Quantitation of Complement

Varying amounts of a 1:400 dilution of cold, reconstituted guinea pig complement are incubated with sensitized cells. The volume of complement that produces 50% lysis of the red blood cells (CH_{50} unit) is calculated from the graphed results of the hemolysis. The complement stock may be aliquoted and frozen at –70°C, but adequate reactivity must be demonstrated before use in the test.

3. The Complement Fixation Test

A block titration of serial dilutions of both antigen and antibody is performed in the presence of 5 CH_{50} units of complement at 4°C for 18 hours. Serum is heated at 56°C for 30 minutes to inactivate any endogenous complement. The characteristics of either the antigen or antibody will be known; the serial dilutions of the known reagent may be abbreviated to include the dilution on each side of the expected optimum. Subsequently, sheep red blood cells that have been sensitized with the optimal dilution of hemolysin are incubated at 37°C for 30 minutes. Plates are centrifuged at low speed to sediment the unlysed erythrocytes.

V. Results

A. Interpretation

The highest dilution of antigen or antibody that provides 3+ to 4+ fixation of complement (<30% hemolysis) is the end point. All reagents must be free of anticomplementary activity, the correct quantity of complement must have been added, and the control specimens must react as expected.

VI. Bibliography

Hsiung GD. Diagnostic Virology. 3rd Ed. New Haven, CT: Yale University Press, 1982.

Standardized diagnostic complement fixation method and adaptation to

microtest. Atlanta: Public Health Service, 1965. Publication no. 1228 (Public Health Monograph 74).

CHART 3-2

Hemagglutination Inhibition (HAI) Test

I. Principle

The nucleic acids of many viruses encode surface proteins that agglutinate the red blood cells of a variety of species. The phenomenon is most commonly used for diagnosis of infections produced by orthomyxoviruses, paramyxoviruses, and the arboviruses–togaviruses (including rubella), flaviviruses, and bunyaviruses. The presence of virus in infected cell cultures can be detected by hemagglutination; the identity of the virus or of antibodies in a patient's serum can be determined by specific inhibition of that hemagglutination. Reagents and conditions for the test vary by virus.

Reaction of viral hemagglutinins with red blood cells results in a lattice of agglutinated cells which settle irregularly in a tube or microtiter well. Unagglutinated cells settle in a compact button. As with the complement fixation test, the HAI assay has largely been replaced by enzyme immunoassays and other techniques. This Chart is retained, since not all laboratories have made these transitions.

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II. Materials and Reagents

1. Red blood cells from an appropriate species (chicken, goose, guinea pig, trypsinized human O) collected in Alsever's solution or heparin
2. Diluent at appropriate pH
3. Solutions to remove nonspecific hemagglutinins from serum
4. Infected culture fluid or standard antigen for serology

III. Quality Control

A. Known positive serum
B. Known negative serum
C. Serum and cells without antigen (to detect nonspecific agglutination)
D. Back titration of hemagglutination activity of the antigen (to ensure that 4 hemagglutinating units [HAU] were tested)

IV. Procedure

A. Determination of Hemagglutinating Titer

1. A diluted suspension of red blood cells is incubated at 4°C or room temperature with serial dilutions of antigen until red blood cells in tubes without virus settle in a compact button (1–4 hours). The highest dilution that produces partial or complete agglutination represents 1 HAU.

B. Treatment of Sera

1. Removal of nonspecific inhibitors in sera is necessary for most viruses. Removal may be accomplished by physical means, such as kaolin; by enzymes, such as neuraminidase or the receptor-destroying enzyme (RDE) of *Vibrio cholerae*; or by a combination. The choice varies with the virus to be tested.

C. Inhibition Test

1. A dilution of antigen that contains 4 HAU is mixed with appropriate red blood cells and serial two-fold dilutions of treated serum. The suspension is incubated as for the hemagglutination test.

V. Results

A. Interpretation

The end point is the last well at which partial or complete agglutination of the red blood cells occurs. A smooth or jagged shield of cells or an irregular button indicates agglutination. Observation of the movement of the button of red blood cells when the plate is tilted may help to clarify the end point.

VI. Bibliography

Hsiung GD. Diagnostic Virology. 3rd Ed. New Haven, CT: Yale University Press, 1982.

Lennette EH, Schmidt NJ, eds. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. 5th Ed. Washington, DC: American Public Health Association, 1979.

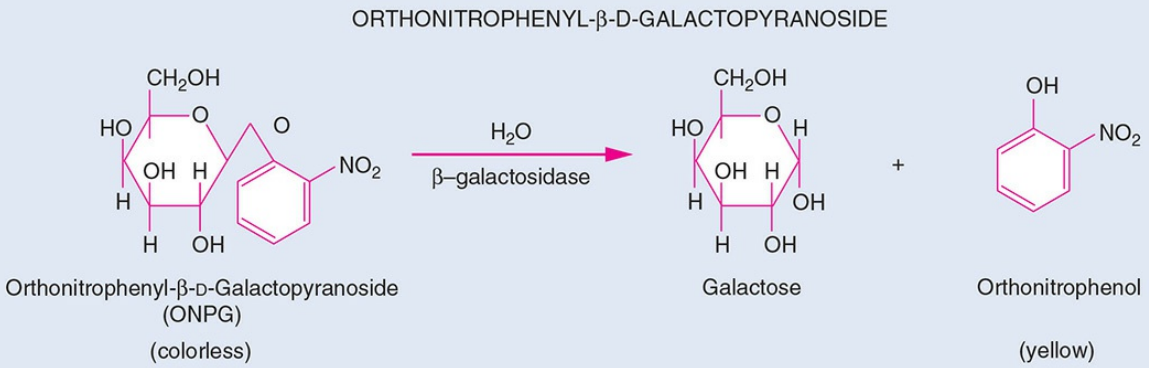
Smith TF. Viruses. In: Washington JA, ed. Laboratory Procedures in Clinical Microbiology. 2nd Ed. New York, NY: Springer-Verlag, 1985.

CHART 6-1

o -Nitrophenyl-β-D-Galactopyranoside

I. Principle

o-Nitrophenyl-β-D-galactopyranoside (ONPG) is structurally similar to lactose, except that orthonitrophenyl has been substituted for glucose, as shown in the following chemical reaction:



C-9

On hydrolysis, through the action of the enzyme β-galactosidase, ONPG cleaves into two residues, galactose and *o*-nitrophenol. ONPG is a colorless compound; *o*-nitrophenol is yellow, providing visual evidence of hydrolysis.

Lactose-fermenting bacteria possess both lactose permease and β-galactosidase, two enzymes required for the production of acid in the lactose fermentation test. The permease is required for the lactose molecule to penetrate the bacterial cell where the β-galactosidase can cleave the galactoside bond, producing glucose and galactose. Non–lactose-fermenting bacteria are devoid of both enzymes and are incapable of producing acid from lactose. Some bacterial species appear to be non–lactose-fermenters because they lack permease, but do possess β-galactosidase and give a positive ONPG test. So-called late lactose fermenters may be delayed in their production of acid from lactose because of weak permease activity. In these instances, a positive ONPG test may provide a rapid identification of delayed lactose fermentation.

II. Media and Reagents

1. Sodium phosphate buffer, 1 M, pH 7.0
2. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG), 0.75 M (Buffered ONPG tablets are commercially available.)
3. Physiologic saline
4. Toluene

III. Quality Control

A. Positive control: *Escherichia coli*

B. Negative control: *Proteus* species

IV. Procedure

Bacteria grown in medium containing lactose, such as Kligler's iron agar (KIA) or triple-sugar iron (TSI) agar, produce optimal results in the ONPG test. A loopful of bacterial growth is emulsified in 0.5 mL of physiologic saline to produce a heavy suspension. One drop of toluene is added to the suspension and vigorously mixed for a few seconds to release the enzyme for the bacterial cells. An equal quantity of buffered ONPG solution is added to the suspension, and the mixture is placed in a 37°C water bath.

When using ONPG tablets, a loopful of bacterial suspension is added directly to the ONPG substrate resulting from adding 1 mL of distilled water to a tablet in a test tube. This suspension is also placed in a 37°C water bath.

V. Results

A. Interpretation

The rate of hydrolysis of ONPG to *o*-nitrophenol may be rapid for some organisms, producing a visible yellow color reaction within 5–10 minutes (Color Plate 6-4A). Most tests are positive within 1 hour; however, reactions should not be interpreted as negative before 24 hours of incubation. The yellow color is usually distinct and indicates that the organism has produced *o*-nitrophenol from the ONPG substrate through the action of β-galactosidase.

VI. Bibliography

Belliveau RR, Grayson JW Jr, Butler TJ. A rapid, simple method of identifying *Enterobacteriaceae.* Am J Clin Pathol 1968;50:126–128.

Blazevic DJ, Ederer GM. Principles of Biochemical Tests in Diagnostic Microbiology. New York, NY: Wiley, 1975: 83–85.

Lederberg J. The beta-D-galactosidase of *Escherichia coli,* strain K-12. J Bacteriol 1950; 60:381–392.

Lowe GH. The rapid detection of lactose fermentation in paracolon organisms by the demonstration of β-galactosidase. J Med Lab Technol 1962;19:21–25.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:120–128.

CHART 6-2

Nitrate Reduction: General Applications

I. Principle

The capability of an organism to reduce nitrates to nitrites is an important characteristic used in the identification and species differentiation of many groups of microorganisms. All *Enterobacteriaceae* except certain biotypes of *Pantoa agglomerans* and certain species of *Serratia* and *Yersinia* demonstrate nitrate reduction. The test is also helpful in identifying members of the *Haemophilus, Neisseria,* and *Moraxella* genera.

Organisms demonstrating nitrate reduction have the capability of extracting oxygen from nitrates to form nitrites and other reduction products. The chemical equation is

$$\underset{\text{Nitrate}}{NO_3^-} + 2e^- + 2H \rightarrow \underset{\text{Nitrite}}{NO_2} + H_2O$$

The presence of nitrites in the test medium is detected by the addition of α-naphthylamine and sulfanilic acid, with the formation of a red diazonium dye, *p*-sulfobenzeneazo-α-naphthylamine. See Figure in Chart 19-12.

II. Media and Reagents

A. Nitrate Broth or Nitrate Agar (Slant)

Beef extract	3 g
Peptone	5 g
Potassium nitrate (KNO_3)	1 g
Agar (nitrite-free)	12 g
Distilled water	1 L

B. Reagent A

α-Naphthylamine	5 g
Acetic acid (5 N), 30%	1 L

C. Reagent B

Sulfanilic acid	8 g
Acetic acid (5 N), 30%	1 L

III. Quality Control

It is important to test each new batch of medium and each new formulation

of test reagents for positive and negative reactions. The following organisms are suggested:

A. Positive control: *Escherichia coli*

B. Negative control: *Acinetobacter baumannii*

IV. Procedure

1. Inoculate the nitrate medium with a loopful of the test organism isolated in pure culture on agar medium, and incubate at 35°C for 18–24 hours.
2. At the end of incubation, add 1 mL each of reagents A and B to the test medium, in that order.

V. Results

A. Interpretation

The development of a red color within 30 seconds after adding the test reagents indicates the presence of nitrites and represents a positive reaction for nitrate reduction (Color Plate 6-1H). If no color develops after adding the test reagents, this may indicate either that nitrates have not been reduced (a true negative reaction) or that they have been reduced to products other than nitrites, such as ammonia, molecular nitrogen (denitrification), nitric oxide (NO) or nitrous oxide (N_2O), and hydroxylamine. Because the test reagents detect only nitrites, the latter process would lead to a false-negative reading. Thus, it is necessary to add a small quantity of zinc dust to all negative reactions. Zinc ions reduce nitrates to nitrites, and the development of a red color after adding zinc dust indicates the presence of residual nitrates and confirms a true negative reaction.

VI. Bibliography

Finegold SM, Martin WJ, Scott EG. Bailey and Scott's Diagnostic Microbiology. 5th Ed. St Louis: Mosby, 1978:490.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:236–245.

Wallace GI, Neave SL. The nitrite test as applied to bacterial cultures. J Bacteriol 1927;14:377–384.

CHART 6-3

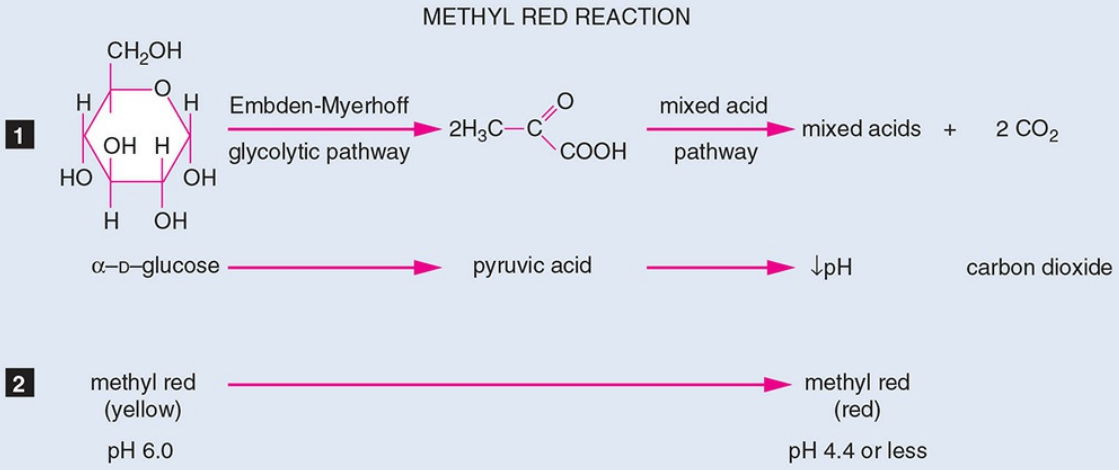
Methyl Red

I. Principle

Methyl red is a pH indicator, with a range between 6.0 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, to produce a color change, the test organism must produce large quantities of acid from the carbohydrate substrate being used.

C-11

The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic, acetic, formic) from glucose through the mixed acid fermentation pathway (Figure 6-2). Because many species of the *Enterobacteriaceae* may produce sufficient quantities of strong acids that can be detected by methyl red indicator during the initial phases of incubation, only organisms that can maintain this low pH after prolonged incubation (48–72 hours), overcoming the pH-buffering system of the medium, can be called methyl red–positive.



II. Media and Reagents

The medium most commonly used is methyl red–Voges-Proskauer (MR/VP) broth, as formulated by Clark and Lubs. This medium also serves for the performance of the Voges–Proskauer test.

A. MR/VP Broth

Polypeptone	7 g
Glucose	5 g
Dipotassium phosphate	5 g

Distilled water	1 L
Final pH	6.9

B. Methyl Red pH Indicator
Methyl red, 0.1 g, in 300 mL of 95% ethyl alcohol
Distilled water, 200 mL

III. Quality Control

Positive and negative controls should be run after preparation of each lot of medium and after making each batch of reagent. Suggested controls include the following:

A. Positive control: *Escherichia coli*

B. Negative control: *Enterobacter aerogenes*

IV. Procedure

1. Inoculate the MR/VP broth with a pure culture of the test organism. Incubate the broth at 35°C for 48–72 hours (no fewer than 48 hours).
2. At the end of this time, add 5 drops of the methyl red reagent directly to the broth.

V. Results

A. Interpretation

The development of a stable red color in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test (Color Plate 6-4C). Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate orange color between yellow and red may develop. This does not indicate a positive test.

VI. Bibliography

Barry AL, Bernsohn KL, Adams AP, et al. Improved 18-hour methyl red test. Appl Microbiol 1970; 20:866–870.

Blazevic DJ, Ederer GM. Principles of Biochemical Tests in Diagnostic Microbiology. New York, NY: Wiley, 1975:75–77.

Clark WM, Lubs HA. The differentiation of bacteria of the colon-aerogenes family by the use of indicators. J Infect Dis 1915;17:160.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980;209–214.

C-12

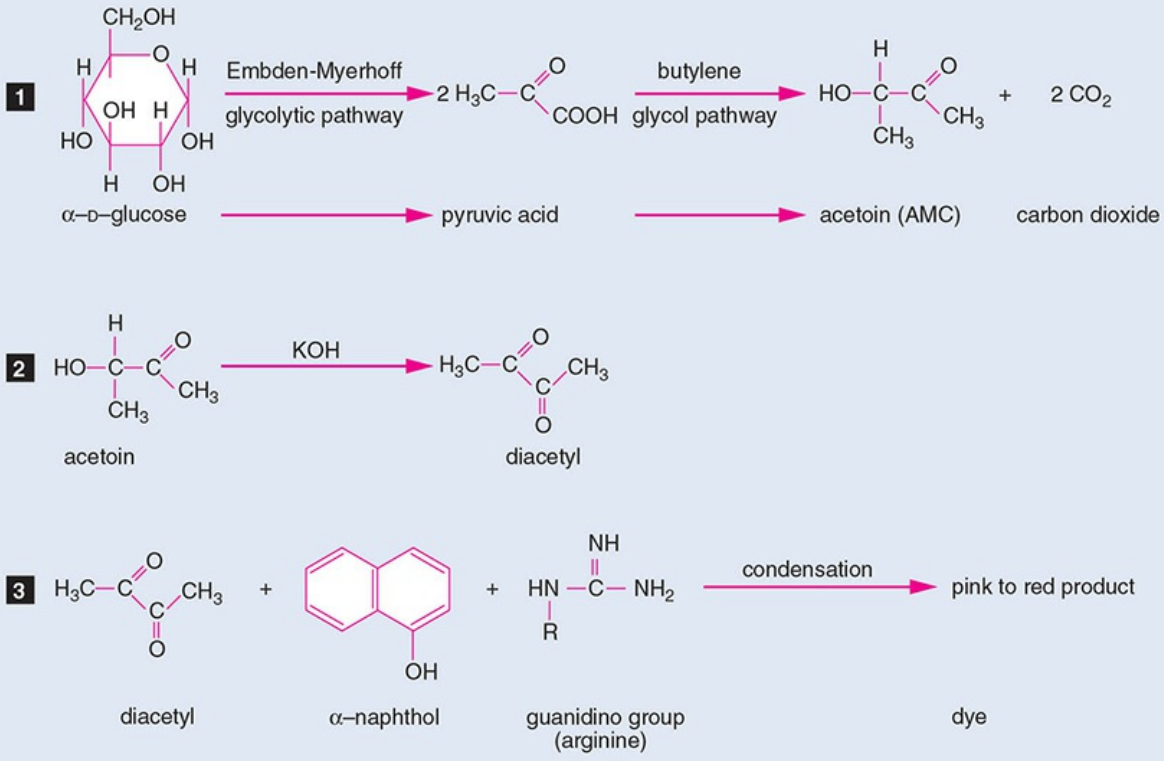
CHART 6-4

Voges-Proskauer Test

I. Principle

Voges-Proskauer is a double eponym, named after two microbiologists who worked at the beginning of the 20th century. They first observed the red color reaction produced by appropriate culture media after treatment with potassium hydroxide. It was later discovered that the active product in the medium formed by bacterial metabolism was acetyl methyl carbinol, a product of the butylene glycol pathway.

Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose, is further metabolized through various metabolic pathways, depending on the enzyme systems possessed by different bacteria. One such pathway results in the production of acetoin (acetyl methyl carbinol), a neutral-reacting end product. Organisms such as members of the *Klebsiella-Enterobacter-Hafnia-Serratia* group produce acetoin as the chief end product of glucose metabolism and form smaller quantities of mixed acids. In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and α-naphthol serves as a catalyst to bring out a red complex.



II. Media and Reagents

A. Medium

1. The medium is the MR/VP broth described in Chart 6-3.

B. Reagents

1. α-Naphthol, 5% color intensifier

α-Naphthol	5 g
Absolute ethyl alcohol	100 mL

2. Potassium hydroxide, 40%, oxidizing agent

Potassium hydroxide	40 g
Distilled water	100 mL

III. Quality Control

Positive and negative controls should be performed after preparation of each lot of medium and after making each batch of reagent. Suggested controls include the following:

A. Positive control: *Enterobacter aerogenes*

B. Negative control: *Escherichia coli*

IV. Procedure

Inoculate a tube of MR/VP broth with a pure culture of the test organism. Incubate for 24 hours at 35°C. At the end of this time, aliquot 1 mL of broth to a clean test tube. Add 0.6 mL of 5% α-naphthol,

followed by 0.2 mL of 40% KOH. It is essential that the reagents be added in this order. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10–15 minutes.

V. Results

A. Interpretation

A positive test is represented by the development of a red color 15 minutes or more after addition of the reagents, indicating the presence of diacetyl, the oxidation product of acetoin (Color Plate 6-4D). The test should not be read after standing for over 1 hour because negative Voges-Proskauer cultures may produce a copper-like color, potentially resulting in a false-positive interpretation.

VI. Bibliography

Barritt MM. The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. J Pathol Bacteriol 1936;42:441–454.

Barry AL, Feeney KL. Two quick methods for Voges-Proskauer test. Appl Microbiol 1967;15:1138–1141.

Blazevic DJ, Ederer GM. Principles of Biochemical Tests in Diagnostic

Microbiology. New York, NY: Wiley, 1975:105–107.
MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:308–320.
Voges O, Proskauer B. Beitrag zue Ernährungsphysiologie und zur Differential-diagnose der Bakterien der hämorrhagischen Septicamia. Z Hyg 1898;28:20–32.

CHART 6-5

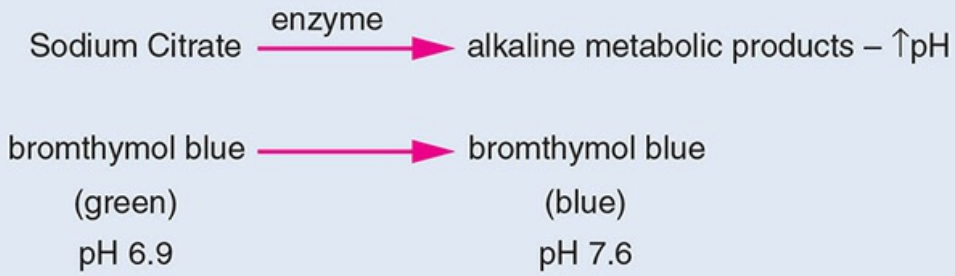
Citrate Utilization

I. Principle

Sodium citrate is a salt of citric acid, a simple organic compound found as one of the metabolites in the tricarboxylic acid cycle (Krebs cycle). Some bacteria can obtain energy in a manner other than by the fermentation of carbohydrates by using citrate as the sole source of carbon. The measurement of this characteristic is important in the identification of many members of the *Enterobacteriaceae.* Any medium used to detect citrate utilization by test bacteria must be devoid of protein and carbohydrates as sources of carbon.

The utilization of citrate by a test bacterium is detected in citrate medium by the production of alkaline by-products. The medium includes sodium citrate, an anion, as the sole source of carbon, and ammonium phosphate as the sole source of nitrogen. Bacteria that can use citrate can also extract nitrogen from the ammonium salts in the medium, producing sodium bicarbonate ($NaHCO_3$) and ammonia (NH_3), which results in an alkaline pH. Bromthymol blue—yellow below pH 6.0 and blue above pH 7.6—is the indicator.

THE CITRATE TEST



II. Media and Reagents

The citrate medium most commonly used is the formula of Simmons. The medium is poured into a tube on a slant. The formula of Simmons citrate medium is shown below:

Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium chloride	5 g
Sodium citrate	2 g
Magnesium sulfate	0.20 g
Agar	15 g
Bromthymol blue	0.08 g
Distilled water	1 L
Final pH	6.9

III. Quality Control

Each new batch of medium should be tested with a positive- and a negative-reacting organism. The following species are suggested controls:

A. Positive control—*Enterobacter aerogenes*

B. Negative control—*Escherichia coli*

IV. Procedure

1. A well-isolated colony is picked from the surface of a primarily isolation medium and inoculated as a single streak on the slant surface of the citrate agar tube. The tube is incubated at 35°C for 24–48 hours.

V. Results

A. Interpretation

A positive test is represented by the development of a deep blue color within 24–48 hours, indicating that the test organism has been able to utilize the citrate contained in the medium, with the production of alkaline products (Color Plate 6-4D). A positive test may also be read without a blue color if there is visible colony growth along the inoculation streak line. This is possible because, for growth to be visible, the organism must enter the log phase of growth, possible only if carbon and nitrogen have been assimilated. A positive interpretation from reading the streak line can be confirmed by incubating the tube for an additional 24 hours, when a blue color usually develops.

VI. Bibliography

BBL Manual of Products and Laboratory Procedures. Ed. 5. Cockeysville, MD: BioQuest, 1968:115, 138.

Blazevic DJ, Ederer GM. Principles of Biochemical Tests in Diagnostic Microbiology. New York, NY: Wiley, 1975:15–18.

Koser SA. Utilization of the salts of organic acids by the colon-aerogenes groups. J Bacteriol 1923;8:493–520.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:59–63.

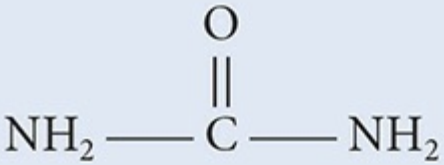
Simmons JS. A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolation of certain fungi. J Infect Dis 1926;39:209–214.

CHART 6-6

Urease: Conventional

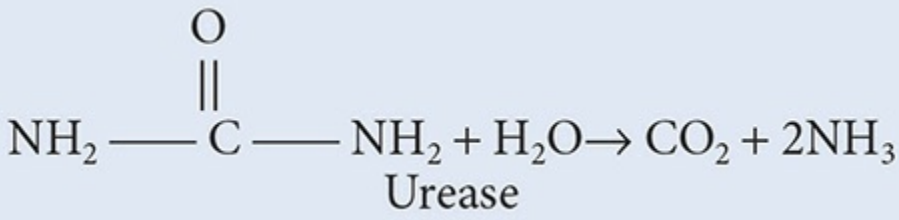
I. Principle

Urea is a diamide of carbonic acid with the formula



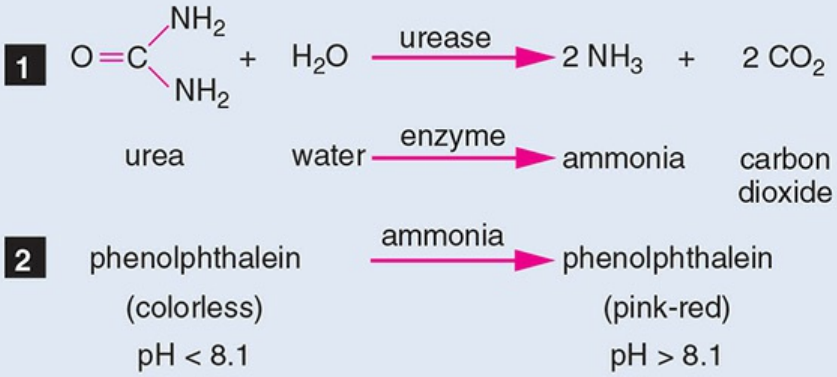
All amides are easily hydrolyzed with the release of ammonia and carbon dioxide.

Urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea following the chemical reaction



The ammonia reacts in solution to form ammonium carbonate ($(NH_4)_2CO_3$), resulting in alkalinization and an increase in the pH of the medium.

MECHANISM OF THE UREASE REACTION



II. Media and Reagents

Stuart's urea broth and Christensen's urea agar are the two media most commonly used in clinical laboratories for the detection of urease activity.

Stuart's Urea Broth		Christensen's Urea Agar	
Yeast extract	0.1 g	Peptone	1 g
Monopotassium phosphate	9.1 g	Glucose	1 g
Disodium phosphate	9.5 g	Sodium chloride	5 g
Urea	20 g	Monopotassium phosphate	2 g
Phenol red	0.01 g	Urea	20 g
Distilled water	1 L	Phenol red	0.012 g
Final pH	6.8	Agar	15 g
		Distilled water	1 L
		Final pH	6.8

III. Quality Control

Positively and negatively reacting control organisms should be run with each new batch of medium. The following organisms are suggested:

A. Positive control: *Proteus* species

B. Positive control (weak): *Klebsiella* species

C. Negative control: *Escherichia coli*

IV. Procedure

The broth medium is inoculated with a loopful of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism. Both media are incubated at 35°C for 18–24 hours.

V. Results

A. Interpretation

Organisms that hydrolyze urea rapidly may produce positive reactions within 1 or 2 hours; less active species may require 3 or more days. The reactions are as follows:

1. Stuart's broth

Red color throughout the medium indicates alkalinization and urea hydrolysis

2. Christensen's agar

Rapid urea hydrolyzers (*Proteus* species): red color throughout medium

Slow urea hydrolyzers (*Klebsiella* species): red color initially in slant only, gradually converting the entire tube

3. No urea hydrolysis: medium remains original yellow color (Color Plate 6-4E).

VI. Bibliography

BBL Manual of Products and Laboratory Procedures. 5th Ed. Cockeysville, MD: BioQuest, 1968:154.

Christensen WB. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. J Bacteriol 1946;52:461–466.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria.

2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:298–308.
Stuart CA, Van Stratum E, Rustigian R. Further studies on urease production by *Proteus* and related organisms. J Bacteriol 1945;49:437–444.

CHART 6-7

Decarboxylases

I. Principle

Decarboxylases are a group of substrate-specific enzymes that are capable of reacting with the carboxyl (COOH) portion of amino acids, forming alkaline-reacting amines. This reaction, known as decarboxylation, forms carbon dioxide as a second product. Each decarboxylase enzyme is specific for an amino acid. Lysine, ornithine, and arginine are the three amino acids routinely tested in the identification of the *Enterobacteriaceae.* The specific amine products are as follows:

Lysine	→	Cadaverine
Ornithine	→	Putrescine
Arginine	→	Citrulline

The conversion of arginine to citrulline is a dihydrolase, rather than a decarboxylase reaction, in which an NH_2 group is removed from arginine as a first step. Citrulline is next converted to ornithine, which then undergoes decarboxylation to form putrescine.

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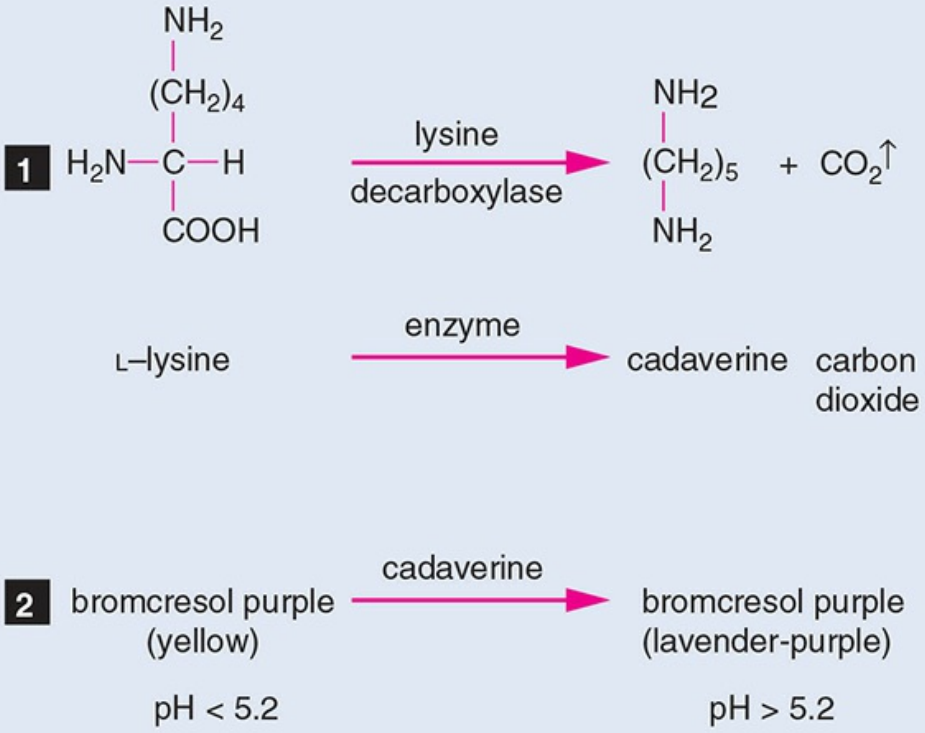
Møller decarboxylase medium is the base most commonly used for determining the decarboxylase capabilities of the *Enterobacteriaceae.* The amino acid to be tested is added to the decarboxylase base before inoculation with the test organism. A control tube, consisting of only the base without the amino acid, must also be set up in parallel. Both tubes are anaerobically incubated by overlaying with mineral oil. During the initial stages of incubation, both tubes turn yellow, owing to the fermentation of the small amount of glucose in the medium. If the amino acid is decarboxylated, alkaline amines are formed and the medium reverts to its original purple

color.

MECHANISM OF THE LYSINE DECARBOXYLASE REACTION



II. Media and Reagents

A. Møller Decarboxylase Broth Base

Peptone	5 g
Beef extract	5 g
Bromcresol purple	0.01 g
Cresol red	0.005 g
Glucose	0.5 g
Pyridoxal	0.005 g
Distilled water	1 L
Final pH	6.0

B. Amino Acid

Add 10 g (final concentration = 1%) of the L (*levo*) -form of the amino acid (lysine, ornithine, or arginine). Double this amount if the D (*dextro*) -form is to be used, because only the L-form is active.

III. Quality Control

The following organisms are suggested for positive and negative controls:

Amino Acid	Positive Control	Negative Control
Lysine	*Enterobacter aerogenes*	*Enterobacter cloacae*
Ornithine	*Enterobacter cloacae*	*Klebsiella pneumoniae*
Arginine	*Enterobacter cloacae*	*Enterobacter aerogenes*

IV. Procedure

From a well-isolated colony of the test organism previously recovered on primary isolation agar, inoculate two tubes of Møller decarboxylase medium, one containing the amino acid to be tested and the other to be used as a control tube devoid of amino acid. Overlay both tubes with sterile mineral oil to cover about 1 cm of the surface and incubate at 35°C for 18–24 hours.

V. Results

A. Interpretation

Conversion of the control tube to a yellow color indicates that the organism is viable and that the pH of the medium has been lowered sufficiently to activate the decarboxylase enzymes. Reversion of the tube containing the amino acid to a blue-purple color indicates a positive test owing to the formation of amines from the decarboxylation reaction (Color Plate 6-4F).

C-17

VI. Bibliography

Blazevic DJ, Ederer GM. Principles of Biochemical Tests in Diagnostic Microbiology. New York, NY: Wiley, 1975: 29–36.

Carlquist PR. A biochemical test for separating paracolon groups. J Bacteriol 1956;71:339–341.

Falkow S. Activity of lysine decarboxylase as an aid in the identification of salmonellae and shigellae. Am J Clin Pathol 1958;29:598–600.

Gale EF. The bacterial amino acid decarboxylases. In: Nord FF, ed. Advances in Enzymology and Related Subjects of Biochemistry. Vol. 6. New York, NY: Interscience Publishers, 1946.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980;78–93

Møller V. Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. Acta Pathol Microbiol Scand 1955;36:158–172.

CHART 6-8

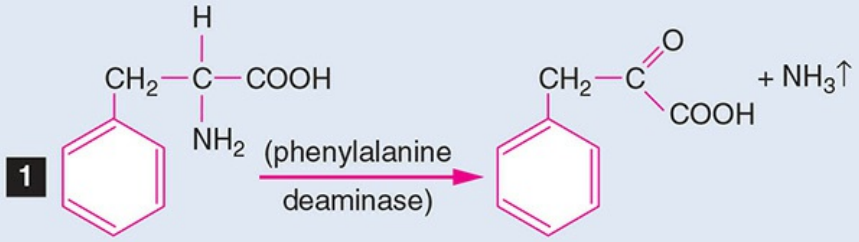
Phenylalanine Deaminase

I. Principle

Phenylalanine is an amino acid that on deamination forms a keto acid, phenylpyruvic acid. Of the *Enterobacteriaceae,* only members of the *Proteus, Morganella,* and *Providencia* genera possess the deaminase enzyme necessary for this conversion.

The phenylalanine test depends on the detection of phenylpyruvic acid in the test medium after growth of the test organism. The test is positive if a visible green color develops on addition of a solution of 10% ferric chloride.

MECHANISM OF THE PHENYLALANINE DEAMINASE REACTION



L–phenylalanine —(enzyme)→ phenylpyruvic acid + ammonia

2 phenylpyruvic acid + ferric ion (Fe^{+++}) —acid→ green complex

II. Media and Reagents

Phenylalanine agar is poured as a slant into a tube. Meat extracts or protein hydrolysates cannot be used because of their varying natural content of phenylalanine. Yeast extract serves as the carbon and nitrogen source. The formula is shown below:

Medium

DL-Phenylalanine	2 g
Yeast extract	3 g
Sodium chloride	5 g
Disodium phosphate	1 g
Agar	12 g
Distilled water	1 L
pH	7.3

Reagent	
Ferric chloride	12 g
Concentrated HCl	2.5 mL
Distilled water	100 mL

C-18

III. Quality Control

Each new batch of medium or reagent must be tested with positive- and negative-reacting organisms. The following are suggested:

A. Positive control: *Proteus* species

B. Negative control: *Escherichia coli*

IV. Procedure

The agar slant of the medium is inoculated with a single colony of the test organism isolated in pure culture of primary plating agar. After incubation at 35°C for 18–24 hours, 4 or 5 drops of the ferric chloride reagent are added directly to the surface of the agar. As the reagent is added, the tube is rotated to dislodge the surface colonies.

V. Results

A. Interpretation

The immediate appearance of an intense green color indicates the presence of phenylpyruvic acid and a positive test (Color Plate 6-4E).

VI. Bibliography

Blazevic DJ, Ederer GM. Principles of Biochemical Tests in Diagnostic Microbiology. New York, NY: Wiley, 1975: 23–28.

Hendriksen SD. A comparison of the phenylpyruvic acid reaction and unrease test in the differentiation of *Proteus* from other enteric organisms. J Bacteriol 1950; 60:225–231.

Hendriksen SD, Closs K. The production of phenylpyruvic acid by bacteria. Acta Pathol Microbiol Scand 1938;15:101–113.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria.

2nd Ed. Baltimore, MD: Williams & Wilkins, 1980;269–274.
Shaw C, Clarke PH. Biochemical classification of *Proteus* and *Providencia* cultures. J Gen Microbiol 1955;13:155–161.

CHART 7-1

Oxidative-Fermentative Test (Hugh and Leifson)

I. Principle

Saccharolytic microorganisms degrade glucose either fermentatively or oxidatively, as shown in Fig. 7-1. The end products of fermentation are relatively strong mixed acids that can be detected in a conventional fermentation test medium. However, the acids formed in oxidative degradation of glucose are extremely weak, and the more sensitive oxidation-fermentation medium of Hugh and Leifson (OF medium) is required for their detection.

The OF medium of Hugh and Leifson differs from carbohydrate fermentation media as follows:

The concentration of peptone is decreased from 1% to 0.2%.

The concentration of carbohydrate is increased from 0.5% to 1.0%.

The concentration of agar is decreased from 1.5% to 0.3%, making it semisolid.

The lower protein/carbohydrate ratio reduces the formation of alkaline amines that can neutralize the small quantities of weak acids that may form from oxidative metabolism. The relatively larger amount of carbohydrate serves to increase the amount of acid that can potentially be formed. The semisolid consistency of the agar permits acids that form on the surface of the agar to permeate throughout the medium, making interpretation of the pH shift of the indicator easier to visualize. Motility can also be observed in this medium.

II. Media and Reagents

For comparison, the formulas for a conventional carbohydrate fermentation medium and OF medium are as follows:

Carbohydrate Fermentation Medium

Peptone	10 g
Sodium chloride	5 g

D-glucose	5 g
Bromcresol purple	0.02 g
Agar[a]	15 g
Distilled water	1 L
pH	7.0

[a]Agar is omitted from broth medium.

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OF Medium of Hugh and Leifson

Peptone	2 g
Sodium chloride	5 g
D-glucose	10 g
Bromthymol blue	0.03 g
Agar	3 g
Dipotassium phosphate	030
Distilled water	1 L
pH	7.1

The OF sugar reactions listed in the Weaver CDC tables, described in Chapter 7, are based on tests using a "special" base with phenol red as the indicator:

Bacto casitone	2 g
Bacto agar	3 g
Phenol red, 1.5% aqueous	2 mL
Distilled water	1 L

The basal medium is adjusted to a pH of 7.3 and autoclaved. Carbohydrate solutions are passed through a Seitz filter and added to the basal medium to a final concentration of 1.0%. Studies at the CDC revealed that the correlation of test results between the Difco and the special CDC OF basal media is excellent and that either can be used when making interpretations from the Weaver tables.

OF medium should be poured without a slant into tubes with an inner diameter of 15–20 mm to increase surface area.

III. Quality Control

A. Glucose fermenter: *Escherichia coli*

B. Glucose oxidizer: *Pseudomonas aeruginosa*

C. Nonsaccharolytic: *Moraxella* species

IV. Procedure

Two tubes are required for the OF test, each inoculated with the unknown organism, using a straight needle, stabbing the medium three to four times halfway to the bottom of the tube. One tube of each pair is covered with a 1-cm layer of sterile mineral oil or melted paraffin, leaving the other tube open to the air. Incubate both tubes at 35°C and examine daily for several days.

V. Results

A. Interpretation

Acid production is detected in the medium by the appearance of a yellow color. In the case of oxidative organisms, color production may be first noted near the surface of the medium. Following are the reaction patterns:

Open Tube	Covered Tubes	Metabolism
Acid (yellow)	Alkaline (green)	Oxidative
Acid (yellow)	Acid (yellow)	Fermentative
Alkaline (green or blue)	Alkaline (green or blue)	Nonsaccharolytic

These color reactions are shown in Color Plate 7-1D. For slower-growing species, incubation for 3 days or longer may be required to detect positive reactions.

VI. Bibliography

BBL Manual of Products and Laboratory Procedures. 5th Ed. Cockeysville, MD: BioQuest, 1973:129–130.

Hugh R, Leifson E. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacilli. J Bacteriol 1953;66:24–26.

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:260–268.

C-20

CHART 7-2

Flagellar Stain

I. Principle

Most motile bacteria possess flagella, the shape, number, and position of which are important characteristics in the differentiation of genera and species identification, particularly when biochemical reactions are weak or equivocal. The Leifson staining technique (or modification) is most commonly used in clinical laboratories and is not difficult to perform, providing exact details that are followed in each step of the procedure.

Bacterial flagella can be stained by alcoholic solutions of rosaniline dyes that form a precipitate as the alcohol evaporates in the staining procedure. Basic fuchsin (pararosaniline acetate) serves as the primary stain with tannic acid added to the solution as a mordant. A counterstain, such as methylene blue, may be used to better visualize the bacteria in instances in which the primary stain is weak or does not react at all with the bacterial cell wall.

II. Media and Reagents

A. Leifson Method

1. Basic fuchsin, 1.2% in 95% alcohol

a. Commercial product must be certified for flagellar stain.

b. Pararosaniline acetate is preferred. If the dye solution is a mixture of pararosaniline acetate and pararosaniline hydrochloride, the hydrochloride compound must not compose more than two-thirds of the solution.

c. The stain must have the odor of acetic acid to be satisfactory.

d. When new stain is prepared, at least 1 day must be allowed for the dye to enter completely into solution.

2. Tannic acid, 3% in water

a. The suspension must have a light yellow color to be satisfactory.

b. Add phenol in a 1:200 concentration to discourage emergence of molds during storage.

3. Sodium chloride, 1.5% in water

4. Final stain

a. Combine the three stock solutions in equal volumes. The stain is ready for use immediately after preparation and should be stored in a tightly stoppered bottle.

b. A precipitate will form during storage. This should not be disturbed; rather, remove the staining solution from the top with a pipette.

c. Shelf life of the staining solution is 1 week at room temperature, 1 month in the refrigerator, and indefinite if frozen.

B. Ryu Method

1. Solution I

a. 5% phenol, 10.0 mL

b. Powdered tannic acid, 2.0 g

c. Saturated aluminum potassium sulfate 12-hydrate (crystals) (prepare 14 g potassium alum in 100 mL distilled H_2O), 10.0 mL

2. Solution II

a. Saturated solution of crystal violet in alcohol (prepare 12 g in 100 mL of 95% ethanol)

3. Final stain

a. Ten parts of solution I (mordant) are mixed with one part of solution II and stored in a plastic bottle at room temperature.

III. Quality Control

The following bacterial species may serve as controls:

A. Positive: peritrichous, *Escherichia coli*; polar, monotrichous, *Pseudomonas aeruginosa*; multitrichous, *Burkholderia cepacia*

B. Negative: *Acinetobacter baumannii*

IV. Procedure

A. Leifson Method

1. Using a young culture on an appropriate agar medium, prepare a light suspension of the bacteria in water.

2. Place 2 drops of the bacterial suspension toward one end of an acid-cleaned microscope slide. Allow to air-dry. With a wax pencil, draw a perpendicular line on the glass surface toward the opposite end from the dried suspension.

3. Place the slide on a tilted rack and overlay the dried bacterial suspension with a thin film of stain. The wax pencil mark prevents the stain from running off the end of the slide.

C-21

4. Stain for 5–15 minutes, allowing a precipitate to form as the alcohol evaporates. The staining time is decreased if the stain is fresh, if the room temperature is high, if there are air currents in the laboratory, or if the layer of stain is thin. The opposite effects increase the staining time.

5. When a precipitate forms, rinse the slide gently with distilled water, drain off excess water, and allow to air-dry.

B. Ryu Method

1. Prepare the smear by picking up some young growth from carbohydrate-free culture medium on an inoculating needle and lightly touching the center of each of 2 drops of distilled water on a new, precleaned slide. Let the drops air-dry at room temperature.

2. Flood the air-dried smears with the staining solution for 1–5 minutes (some trial and error may be needed to determine the time that best produces satisfactory staining of the flagella, but avoids excessive debris in the background). Wash the staining solution off in tap water.
3. After the smears have dried, examine them under the oil-immersion objective of the microscope. Cell bodies and flagella stain violet.

V. Results

A. Interpretation

Observe the stained slide under the oil-immersion (100×) objective of the microscope. Dark-staining red to blue-black flagella should be observed. Compare morphology with the photomicrographs provided in Leifson's *Atlas of Bacterial Flagellation,* cited in the bibliography, and see also Fig. 7-3.

VI. Bibliography

Clark WA. A simplified Leifson flagella stain. J Clin Microbiol 1976;3:632–634.

Leifson E. Atlas of Bacterial Flagellation. New York, NY: Academic Press, 1960.

Ryu E. A simple method of staining bacterial flagella. Kitasato Arch Exp Med 1937;14:218–219.

CHART 7-3

Fluorescence-Denitrification

I. Principle

The ability to produce fluorescein pigment and to reduce nitrate or nitrite completely to nitrogen gas are two important characteristics in the identification of the pseudomonads and other nonfermentative bacilli. Fluorescence-denitrification (FN) medium is formulated to detect these two characteristics. Fluorescence-lactose nitrate (FLN) medium is a modification in which lactose and phenol red indicator are added to permit detection of acid formed from utilization of lactose, which is helpful in identifying the strongly lactose-positive group of nonfermenters.

A. Fluorescence Test

Fluorescein is an organic luminescent pigment that on excitation with

ultraviolet light emits a green-yellow fluorescence. A few of the nonfermentative bacilli, notably *Pseudomonas aeruginosa,* are capable of producing fluorescein, detection of which is helpful in their identification. Fluorescence of colonies may not be detected on an ordinary isolation medium, such as blood agar or MacConkey agar; rather, media containing cationic salts, such as magnesium sulfate (included in FN medium), which act as activators or co-activators to intensify luminescence, often must be used.

B. Denitrification Test

The reduction of nitrate to nitrogen gas (N_2) or nitrous oxide (N_2O) is called denitrification and is shown by the following chemical equation:

$$2NO_3^- + 10e- + 12H^- \rightarrow N_2\uparrow + 6H_2O$$

In this reduction process, five electrons are accepted by the nitrate radical, resulting in formation of nitrogen gas and six molecules of water. The denitrification test is helpful in the separation of *Pseudomonas stutzeri* group (most strains are positive) from other nonfermentative bacilli (see Table 7-5).

II. Media and Reagents

A. The formula for FN medium is as follows:

Proteose peptone 3 (Difco Laboratories, Detroit MI)	1 g
Magnesium sulfate $7H_2O$	0.15 g
Dipotassium hydrogen phosphate	0.15 g
Potassium nitrate	0.2 g
Sodium nitrite	0.05 g
Agar	1.5 g
Distilled water	100 mL
pH	7.2

1. Suspend all the ingredients except the magnesium sulfate in distilled water. The magnesium sulfate is dissolved separately in a small amount of water before adding to the agar medium to avoid the formation of an insoluble precipitate.
2. If FLN medium is to be prepared, add 2 g of lactose and 0.002 g of phenol red indicator. Dispense 4 mL of medium into 13-mm

screw-cap tubes and let solidify to give a deep and a slant of approximately equal length.

3. Sellers' medium, available commercially, is also suitable for determination of fluorescence and denitrification by nonfermentative bacteria.

B. The formula for nitrate/nitrite reduction medium is as follows:

Heart infusion broth (Difco Laboratories, Detroit, MI)[a]	25 g
Potassium nitrate	2 g
Distilled water	1 L
pH	7.0

III. Quality Control

The following organisms are appropriate controls:

Positive fluorescence/positive denitrification: *Pseudomonas aeruginosa*
Negative fluorescence/positive denitrification: *Pseudomonas stutzeri*
Negative fluorescence/negative denitrification: *Escherichia coli*

IV. Procedure

Incubate the medium by stabbing the deep with a heavy suspension of the culture and then streaking the slant. Incubate at 35°C for 24–48 hours.

V. Results

A. Interpretation

Examine the tube for fluorescence with an ultraviolet light source (Wood's lamp). A bright yellow-green glow constitutes a positive test. The presence of gas bubbles in the deep of the medium indicates that nitrogen gas has been produced from denitrification, and a yellow slant with FLN medium indicates acid has been produced from the utilization of lactose by the microorganism. In broth medium, nitrogen gas formation is detected by noting the presence of a gas bubble in the upper portion of the inverted Durham tube (Color Plate 7-2B).

VI. Bibliography

Pickett MJ, Pedersen MM. Screening procedure for partial identification of nonfermentative bacilli associated with man. Appl Microbiol 1968;16:1631–1632.

[a] Agar is omitted from broth medium and inverted Durham tubes added to detect gas.

CHART 7-4

Esculin Hydrolysis Test

I. Principle

Esculin medium without bile is useful in differentiating several species on nonfermenting bacilli. Esculin is a substituted glucoside that can be hydrolyzed by certain bacteria to yield glucose and esculetin. Esculin is a fluorescent compound that fluoresces under long-wave UV light at 360 nm. When esculin is hydrolyzed, fluorescence is lost, and the medium turns black due to the reaction of esculetin with the ferric ions in the medium. Several species of *Chryseobacterium, Sphingobacterium,* as well as *C. luteola, B. vesicularis, S. paucimobilis, S. maltophilia, R. radiobacter,* and some species of *B. cepacia, B. pseudomallei,* and *O. anthropi* are esculin-positive.

II. Media and Reagents

The formula for esculin medium is as follows:

Esculin Agar Modified

Esculin	1 g
Ferric citrate	0.5 g
Heart infusion agar	40 g
Distilled water	1 L

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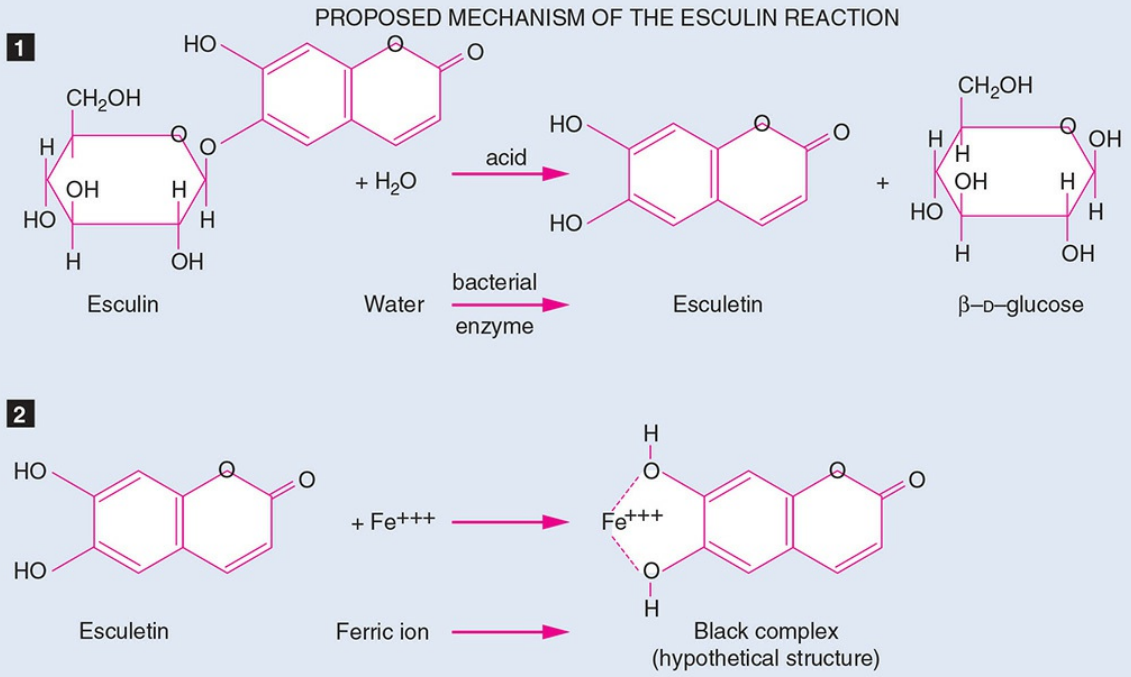
Esculin Broth

Heart muscle, infusion	10 g
Peptic digest of animal tissue	10 g
Sodium chloride	5 g
Esculin	1 g
Distilled water	1 L



III. Quality Control

A. Positive control: *Aeromonas hydrophila* ATCC 7965

B. Negative control: *P. aeruginosa* ATCC 27853

IV. Procedure

After touching the center of one well-isolated colony with a sterile inoculation loop or wooden stick, inoculate the organism onto the surface of the agar slant or into the broth. Incubate for 18–24 hours at 35°C.

V. Results

A. Interpretation

The development of a black color or the loss of fluorescence under UV light (360 nm) is interpreted as a positive test. Fluorescence or lack of a black color is interpreted as a negative test (Color Plate 7-2 E).

VI. Bibliography

MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 2nd Ed. Baltimore, MD: Williams & Wilkins, 1980:4–12.

Shigei J. Test methods used in the identification of commonly isolated aerobic gram-negative bacteria. In Isenberg HH, ed. Clinical Microbiology Procedures Handbook. Vol. 1. Washington, DC: American Society for Microbiology, 1992;11938–11940.

CHART 8-1

The CAMP Test

I. Principle

The presumptive identification of group B β-hemolytic streptococci can be done with the CAMP test, which is reliable and easy to perform. The hemolytic phenomenon was first described in 1944 by Christie, Atkins, and Munch–Petersen, and it is their names that provide the acronym (CAMP) for the test.

The hemolytic activity of the β-hemolysin produced by most strains of *Staphylococcus aureus* is enhanced by an extracellular protein produced by group B streptococci. Interaction of the β-hemolysin with this factor causes "synergistic hemolysis," which is easily observed on a blood agar plate. This phenomenon is seen with both hemolytic and nonhemolytic isolates of group B streptococci. Other bacteria, such as *Listeria monocytogenes*, *Rhodococcus equi,* and certain strains of *Vibrio cholera*, are also CAMP-positive.

II. Materials

1. β-hemolysin-producing strain of *Staphylococcus aureus*
2. Sheep blood agar plate

III. Quality Control

A. Positive control: group B *Streptococcus*

B. Negative control: group A *Streptococcus*

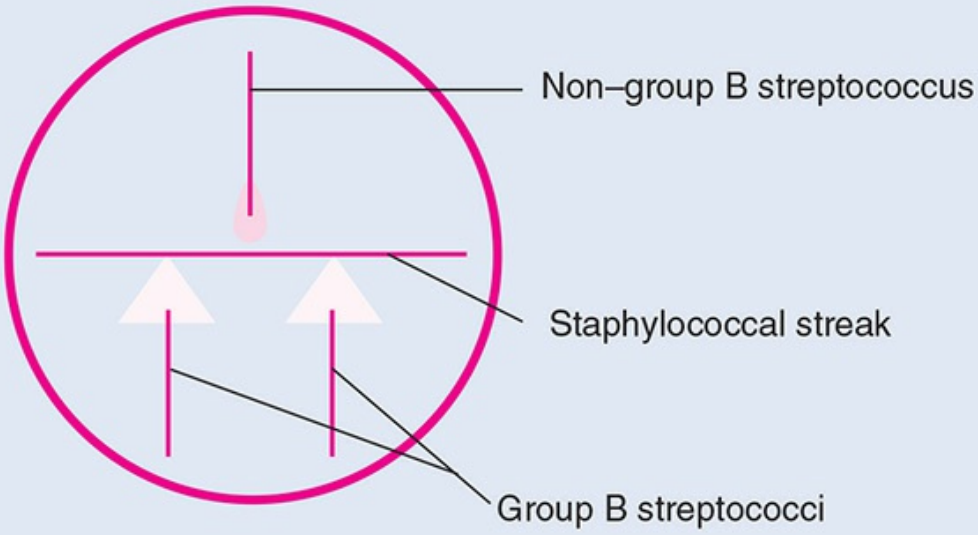
IV. Procedure

1. Down the center of a blood agar plate, and make a single straight line streak of β-hemolysin-producing *S. aureus.*
2. Taking care not to intersect the staphylococcal streak, inoculate a streak of the β-hemolytic streptococcus to be identified perpendicular to the staphylococcal streak. Make these streaks so that, after incubation, the growth of the two organisms will not be touching. The streptococcal streak should be 3–4 cm long. Known group A and B streptococcal strains should be similarly inoculated on the same plate as negative and positive controls, respectively.
3. Incubate the plate at 35°C in ambient air for 18–24 hours.

V. Results

A. Interpretation

As illustrated below, the area of increased hemolysis occurs where the β-hemolysin secreted by the *Staphylococcus* and the CAMP factor secreted by the group B *Streptococcus* intersect. Any bacitracin-resistant, trimethoprim-sulfamethoxazole–resistant, CAMP test-positive, β-hemolytic *Streptococcus* can be reported as "β-hemolytic *Streptococcus*, presumptive group B by CAMP test."



VI. Limitations

Some group A streptococci will be CAMP test-positive if the test plate is incubated in a candle jar, in a CO_2 atmosphere, or under anaerobic conditions. Therefore, ambient-air incubation should be used.

CHART 9-1

Test for X and V Factor Requirements

I. Principle

X factor (hemin, hematin) and V factor (NAD) are required either singly or in combination to support the growth of various species of *Haemophilus* on agar media. Filter paper strips or disks impregnated with X and V factors are

commercially available.

X and V factors, each being water-soluble, readily diffuse in agar culture media. Filter paper disks or strips impregnated with these factors are placed on the surface of a medium deficient in these factors, such as trypticase-soy or brain–heart infusion agar, which has been inoculated as a lawn with the test organism. Factor requirements of the organism are then determined, after overnight incubation, by observing the patterns of growth around the paper strips or disks (see following illustration).

II. Media and Reagents

1. Paper strips or disks impregnated with X and V factors
2. An agar medium deficient in X and V factors. Trypticase-soy or brain–heart infusion agars are suitable.
3. Brain–heart infusion broth

III. Quality Control

A. *Haemophilus parainfluenzae*: requires V factor only

B. *Haemophilus influenzae:* requires both X and V factors

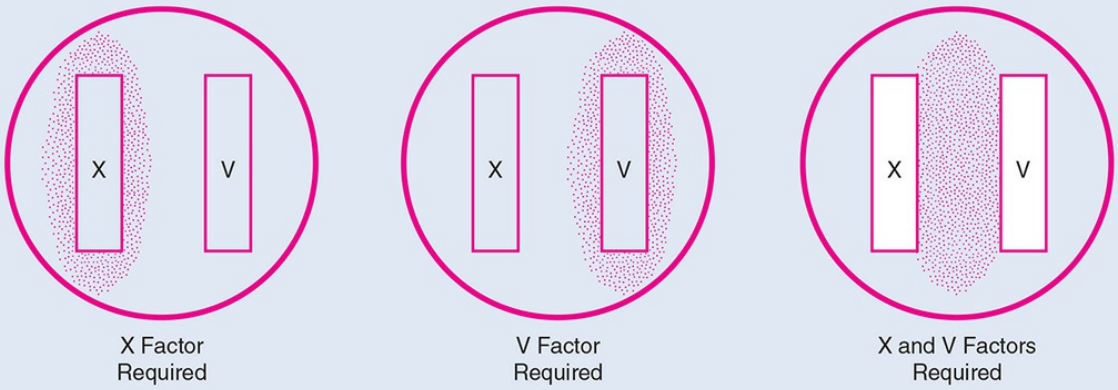
IV. Procedure

1. From a pure culture of the organism to be identified, prepare a light suspension of the growth in the brain–heart infusion broth. Be careful when transferring the growth not to pick up any of the hemin-containing medium from the culture plate. From this suspension, inoculate the surface of the factor-deficient agar medium.
2. Place an X and a V factor strip on the surface of the agar in the area of inoculation.
3. Position the strips about 1 cm apart.
4. Incubate the plate in 3%–5% CO_2 at 35°C for 18–24 hours.

V. Results

A. Interpretation

Visually inspect the agar surface, observing closely for visible growth between or around one or more of the strips or disks. The following patterns indicate the need for X factor only (*left*), V factor only (*center*), or both X and V factors (*right*):



VI. Bibliography

Parker RH, Hoeprich PD. Disk method for rapid identification of *Haemophilus* sp. Am J Clin Pathol 1963;37:319–327.

CHART 10-1

Protocol for Acid-Wash Decontamination of Sputum and Other Contaminated Specimens

1. Before performing the acid treatment of the specimen, prepare six smears for DFA testing, if this is performed, or obtain aliquots for PCR.
2. Inoculate one BCYE and one BMPA plate with three to five drops of untreated respiratory specimen and streak for isolation.
3. Add 0.5 mL of respiratory specimen to 4.5 mL of acid solution (0.2 M KCl–HCl) in a screw-cap tube.
   a. Tightly seal the tube, and thoroughly mix by vortexing under a safety hood.
   b. Allow the suspension to stand at room temperature for 5 minutes.
4. Inoculate a second set of BCYE and BMPA plates with three to five drops of the acid-specimen mixture prepared in step 3 and streak for isolation. Mark plates with an "A" to identify plates inoculated after acid treatment.
5. Place the four inoculated plates in CO_2-permeable plastic bags (two per bag), and incubate in CO_2 at 35°C for 2 weeks. Examine every 2–3 days for growth.

CHART 11-1

Rapid Carbohydrate Utilization Test for Identification of *Neisseria* Species

I. Principle

The rapid carbohydrate utilization test procedures have largely supplanted the use of cystine-tryptic digest semisolid agar with carbohydrates for the identification of *Neisseria* species. These, in turn, are largely being replaced by identification using mass spectrometry.

The rapid carbohydrate utilization test uses a balanced phosphate-buffered saline solution containing phenol red indicator. Small (0.10-mL) aliquots of the buffer are distributed in a series of tubes, one for each carbohydrate tested. Single drops of the individual carbohydrates (20% w/v) are added to individual tubes. A heavy suspension of the organism to be tested is prepared in the buffer without carbohydrates, and one drop of this suspension is added to each of the carbohydrate tubes. After 4 hours of incubation, the reactions are interpreted. Production of acid from carbohydrates is indicated by a change in the color of the phenol red indicator from red to yellow.

II. Media and Reagents

A. Phosphate Balanced Salt Solution (BBS) (a 10× stock solution having the following formula)

K_2HPO_4	0.40 g
KH_2PO_4	0.10 g
KCl	8.0 g
Distilled water	100 mL

Filter-sterilize and store at 4°C–8°C

B. Working Solution

Add 10 mL of the 10× BSS–90 mL of distilled water. After this, 0.5–0.8 mL of a 1% aqueous solution of phenol red is added to the solution, so that the final product is "cherry red." This working solution is then filter-sterilized. To ensure that the distilled water has the proper pH, the use of pharmacy-grade sterile distilled water is recommended.

C. Stock Carbohydrate Solutions

Weigh out 10 g each of glucose, maltose, sucrose, and lactose (and, if desired, fructose) individually. Each is dissolved in 50 mL of distilled water. The solutions are filter-sterilized, dispensed into sterile vials, and frozen at 20°C. It is important that "reagent-grade" carbohydrates be used, because the maltose from some bacteriologic media vendors may be contaminated with glucose. Carbohydrates may be obtained from Sigma Chemical Company (St. Louis, MO), Fisher Scientific Company (Fairlawn, NJ), or Mallinckrodt Chemical Works (St Louis, MO).

III. Quality Control

A. Positive glucose: *N. gonorrhoeae*
B. Positive maltose: *N. meningitidis*
C. Positive lactose: *N. lactamica*
D. Positive sucrose and fructose: *N. mucosa*

IV. Procedure

1. A series of nonsterile 12 × 75-mm tubes are labeled with the carbohydrate to be tested. Generally, glucose, maltose, sucrose, and lactose are tested. Some laboratories will also include fructose in the test battery. An additional tube, labeled with the isolate or specimen number, is also included for preparation of the inoculum.
2. For each of the carbohydrate tubes, 0.10 mL of working BSS is added. For the inoculum tube, 0.30–0.40 mL of BSS is placed in the tube.
3. A single drop of each of the carbohydrates is added to the appropriately labeled tube with a Pasteur pipette.
4. A heavy suspension of the organism to be tested is prepared in the inoculum tube using a sterile bacteriologic loop. The inoculum is prepared from an 18- to 24-hour, pure chocolate agar subculture of the organism. The inoculum suspension is carefully vortexed to obtain a uniform, heavy suspension.
5. Single drops of the inoculum suspension are added to each of the carbohydrate-containing tubes. The tubes are briefly agitated to ensure thorough mixing and are placed in an incubator or water bath at 35°C for 4 hours.

V. Results

A. Interpretation

The development of a yellow color in any of the carbohydrate-containing tubes indicates utilization of that carbohydrate by the organism. Negative carbohydrate-utilization tests remain red or red-orange.

VI. Bibliography

Janda WM. Identification of *Neisseria* species and *Branhamella catarrhalis*. In Identification of Aerobic Gram-Positive and Gram-

Negative Cocci. Washington, DC: American Society for Microbiology, 1986:115–131.
Kellogg DS, Holmes KK, Hill GA. Cumitech 4. In: Marcus S, Sherris JC, eds. Laboratory Diagnosis of Gonorrhea. Washington, DC: American Society for Microbiology, 1976.

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CHART 12-1

Furazolidone Disk Test

I. Principle

There are several methods available for differentiation of staphylococci from micrococci (Table 12-2). One of these is susceptibility to the compound furoxone (also called furazolidone). This test is performed as a disk susceptibility procedure: Staphylococci are susceptible to this compound, whereas micrococci are resistant.

II. Reagents

1. Furazolidone disks, 100 mg (FX 100, BD Microbiology Systems, Franklin Lakes, NJ)
2. Sheep blood agar plate

III. Quality Control

Known strains of *Staphylococcus* (either *S. aureus* or a coagulase-negative strain) and *Micrococcus* species should be tested with each new lot of disks, or on a weekly basis.

IV. Procedure

1. Prepare a suspension of the organism to be tested in sterile distilled water or broth. The suspension should be equivalent to a 0.5 McFarland turbidity standard.
2. With a swab, spread the organism suspension onto one-half of a blood agar plate.
3. Aseptically place an FX disk in the center of the inoculated area, and gently tap the disk so that it adheres to the agar surface.
4. Incubate the plate at 35°C in an ambient-air incubator for 18–24 hours.

V. Results

A. Interpretation

1. *Micrococcus* species are FX-resistant and will have zones of 6 mm (no zone)–9 mm.
2. *Staphylococcus* species are FX-susceptible and will have zones of inhibition of 15 mm or larger.

CHART 12-2

Novobiocin Disk Test

I. Principle

Coagulase-negative staphylococci can be divided into novobiocin-susceptible and novobiocin-resistant species. Among the novobiocin-resistant species, *S. saprophyticus* is the one commonly recovered from humans as a cause of urinary tract infections. Therefore, screening coagulase-negative staphylococci isolated from quantitative urine cultures for susceptibility to novobiocin provides a reliable presumptive identification of this species.

II. Reagents

1. Novobiocin disks, 5 mg (NB 5, BD Microbiology Systems, Franklin Lakes, NJ)
2. Sheep blood agar plate

III. Quality Control

A known *S. saprophyticus* strain and an *S. epidermidis* strain should be tested according to guidelines, possibly using the Individualized Quality Control Plan (IQCP) for this assay.

IV. Procedure

1. Prepare a suspension of the organism to be identified in sterile distilled water or broth.
2. The suspension should be equivalent in turbidity to a 0.5 McFarland standard.
3. With a sterile swab, spread some of the suspension over half of a blood agar plate.
4. Aseptically place a novobiocin disk on the inoculated area. Susceptibility to furazolidone (Chart 12-1) may be assessed on the same plate by placing the disks about 4 cm apart on the inoculated area. Gently tap the disk(s) with sterile forceps to assure contact with the agar surface.
5. Incubate the plate aerobically for 18–24 hours at 35°C.

V. Results

A. Interpretation

1. *S. saprophyticus* are novobiocin-resistant and will show zones of inhibition of 6 mm (no zone)–12 mm.
2. Other coagulase-negative staphylococci and *S. aureus* are novobiocin-susceptible and will show zones of 16 mm or larger.

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CHART 13-1

Bacitracin and SXT Susceptibility Tests

I. Principle

Susceptibility to low concentrations of the polypeptide antibiotic bacitracin and to the combination sulfonamide–trimethoprim–sulfamethoxazole (SXT) provides an easy and inexpensive method for the presumptive identification of both group A and group B β-hemolytic streptococci. Although still used in many laboratories, this test has largely been supplanted by reliable and relatively inexpensive serologic procedures, and more recently by mass spectrometry.

Group A streptococci are susceptible to relatively low concentrations of bacitracin and are resistant to SXT. Group B streptococci are resistant to both antibiotics. Other β-hemolytic streptococci show varying susceptibility to bacitracin, but these organisms are usually susceptible to SXT. Therefore, the performance of the SXT test along with the bacitracin test increases the sensitivity and predictive value of the bacitracin test.

II. Reagents

A. Sheep blood agar plate
B. Taxo "A" bacitracin differential disks (0.04 units/disk)
C. SXT disks (trimethoprim–sulfamethoxazole, 1.25 µg/23.75 µg)

III. Quality Control

A. Bacitracin S, SXT R: Group A *Streptococcus*
B. Bacitracin R, SXT R: Group B *Streptococcus*
C. Bacitracin S or R, SXT S: β-Hemolytic *Streptococcus*, groups C, F, or G

IV. Procedure

1. Pick three to four isolated colonies of the β-hemolytic *Streptococcus*, and streak the inoculum down the center of half of a blood agar plate.
2. Using a sterile swab or a bacteriologic loop, spread the inoculum as a lawn over the entire half of the plate.
3. Aseptically place a Taxo "A" bacitracin disk and an SXT disk on the inoculated area. Make sure that the disks are spaced evenly. Using flamed forceps, gently tap down the disks so that they adhere to the agar surface.
4. Incubate the plate in ambient air at 35°C.

V. Results

A. Interpretation

1. Susceptible (S): Any zone around either of the disks
2. Resistant (R): Growth up to the edge of the disk

Bacitracin	SXT	Identification
S	R	Presumptive group A
R	R	Presumptive group B
S/R	S	Not groups A or B

3. A reporting recommendation is: "β-hemolytic streptococci, presumptive group A, by bacitracin/SXT," or "β-hemolytic streptococci, presumptively not group A by bacitracin/SXT."
4. Because these tests are generally performed on throat isolates, for which group A streptococci are being sought, the presumptive group B is generally not reported.

VI. Limitations of Test

Only β-hemolytic streptococci should be tested, because many α-hemolytic streptococci (including pneumococci) are susceptible to low concentrations of bacitracin.

No data are available to indicate that zones of inhibition should be measured. Interpretation of SXT susceptibility may be difficult, because the organisms may grow slightly before total inhibition of growth occurs.

The lawn of bacterial inoculum should be confluent. Too light an inoculum will cause non–group A streptococci to appear susceptible to bacitracin.

CHART 13-2

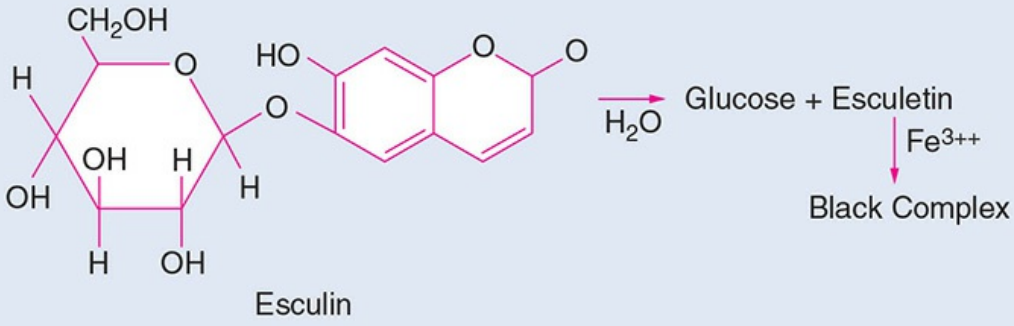
Bile Esculin Test

I. Principle

The bile esculin test is based on the ability of certain bacteria, notably the group D streptococci and *Enterococcus* species, to hydrolyze esculin in the presence of bile (4% bile salts or 40% bile). Esculin is a glycosidic coumarin derivative (6-β-glucoside-7-hydroxy-coumarin). The two moieties of the molecule (glucose and

7-hydroxycoumarin) are linked together by an ester bond through oxygen. For this test, esculin is incorporated into a medium containing 4% bile salts.

Bacteria that are bile esculin–positive are able to grow in the presence of bile salts. Subsequent hydrolysis of the esculin in the medium results in the formation of glucose and a compound called esculetin. Esculetin, in turn, reacts with ferric ions (supplied by the inorganic medium component ferric citrate) to form a black diffusible complex.



Esculin

II. Materials

A. Medium

Bile esculin agar medium is prepared as agar slants or plates. The medium has the following formula:

Peptone	5 g
Beef extract	3 g
Oxgall (bile)	40 g
Esculin	1 g
Ferric citrate	0.5 g

Agar	15 g
Distilled water	1 L
pH	7.0

III. Quality Controls

A. Positive control: *Enterococcus* species (e.g., *E. faecalis*)

B. Negative control: Viridans group streptococci, not group D

IV. Procedure

1. With an inoculating wire or loop, touch two or three morphologically similar streptococcal colonies and inoculate the slant of the bile esculin medium with an S-shaped motion, or streak the surface of a bile esculin plate for isolation.

2. Incubate the tube or plate at 35°C for 24–48 hours in an ambient air incubator.

V. Results

A. Interpretation

Diffuse blackening of more than half of the slant within 24–48 hours indicates esculin hydrolysis. On plates, black haloes will be observed around isolated colonies and any blackening is considered positive. All group D streptococci will be bile esculin–positive within 48 hours.

VI. Limitations of Test

Some viridans streptococci (approximately 3%) may also hydrolyze esculin in the presence of bile.

CHART 13-3

Optochin Susceptibility Test

I. Principle

Ethylhydrocupreine hydrochloride (optochin), a quinine derivative, selectively inhibits the growth of *Streptococcus pneumoniae* at very low concentrations (5 mg/mL or less). Optochin may also inhibit other viridans streptococci, but only at much higher concentrations. The test has a sensitivity of more than 95%, is simple to perform, and is inexpensive.

Optochin is water-soluble and diffuses readily into agar medium. Therefore, filter paper disks impregnated with optochin can be used in a disk diffusion test format to determine susceptibility of suspected pneumococci and, thereby, confirm their identity as such. *S. pneumoniae* cells surrounding

the disk are lysed owing to changes in the surface tension, and a zone of inhibition is produced.

II. Media and Reagents

1. Well-isolated colonies of the organism to be tested on sheep blood agar
2. Sheep blood agar plate

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3. Optochin disks (5 µg): Bacto Differentiation Disks, Optochin (Difco) Taxo "P" disks (BD Microbiology)
4. Disks should be stored at 4°C when not in use.

III. Quality Control

A. Positive control: *Streptococcus pneumoniae*

B. Negative control: Viridans streptococcus or *Enterococcus faecalis*

IV. Procedure

1. Using an inoculating loop or wire, select three to four well-isolated colonies of the organism to be tested and streak onto one-half to a one-third of a blood agar plate. The inoculated area should be about 3 cm^2.
2. Place an optochin disk in the upper third of the streaked area. Tap down the disk with flamed or otherwise sterilized forceps so that the disk adheres firmly to the agar surface.
3. Incubate the plate at 35°C for 18–24 hours in a candle jar or in 5%–7% CO_2.

V. Results

A. Interpretation

A viridans streptococcus can be presumptively identified as *S. pneumoniae* if it shows a zone of inhibition of 14 mm or more around a 6-mm (Taxo A) disk, or a zone of 16 mm or more around a 10-mm disk (Bacto). Organisms showing zones smaller than these should be tested for bile solubility.

VI. Limitations of Procedure

Occasionally, viridans streptococci will show small zones of inhibition around the optochin disk. These organisms, however, will not be bile-soluble.

CHART 13-4

Salt-Tolerance Test

I. Principle

Ability to grow in the presence of variable amounts of sodium chloride (NaCl) is a test that has been used to characterize several bacteria, including the viridans streptococci. It is particularly useful, however, for presumptive identification of the enterococcal group D organisms, which have the specific ability to grow in the presence of 6.5% NaCl incorporated into either a broth or an agar medium. This test, along with the bile esculin test, is used in many laboratories to distinguish *Enterococcus* species from the group D streptococci, *S. bovis* and *S. equinus*.

Heart infusion broth is a general-purpose nutritional broth that is used for the cultivation of many bacteria. It normally contains 0.5% NaCl. By increasing the salt concentration to 6.5%, the medium becomes semiselective for the growth of enterococci.

II. Medium

1. 6.5% NaCl broth has the following formula:

Heart infusion broth	25 g
Sodium chloride	60 g
Indicator (1.6 g of bromcresol purple in 100 mL of 95% ethanol)	1 mL
Glucose	1 g
Distilled water	1 L

2. The indicator may be omitted.

3. Dispense into tubes in 3- to 5 mL volumes and autoclave at 121°C for 15 minutes.

III. Quality Control

A. Positive growth: *Enterococcus faecalis*

B. Negative growth: *Streptococcus bovis*

IV. Procedure

1. Inoculate two or three colonies into broth.

2. Incubate overnight at 35°C in ambient air incubator.

V. Results

A. Interpretation

A positive test is the presence of obvious bacterial growth in the medium, with or without a color change in the indicator. If the organism is bile esculin–positive and grows in the 6.5% NaCl broth, the organism is an *Enterococcus* species. If the organism is bile esculin–positive and fails to grow in the 6.5% NaCl broth, the organism is a group D

Streptococcus.

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VI. Limitations of Procedure

1. If the medium is inoculated too heavily, inoculum may be interpreted as growth, resulting in a false-positive reaction.
2. To prevent interpretation of the test falsely as negative, agitate the tube gently before reading, as the growth may settle out during incubation.
3. Up to 80% of group B streptococci may be salt-tolerant, as will occasional isolates of group A streptococci. Aerococci may also be bile-esculin-positive and may grow in 6.5% NaCl.

CHART 13-5

Bile Solubility Test

I. Principle

Bile solubility reagent (10% sodium desoxycholate) is recommended for use in qualitative procedures for presumptive identification and differentiation of *Streptococcus pneumoniae* from other α-hemolytic streptococci. The bile solubility test is based on the observation that *S. pneumoniae* cells visibly lyse when 10% sodium desoxycholate is applied, while other α-hemolytic streptococci do not. *S. pneumoniae* produces an autolytic, intracellular enzyme that causes the organism to undergo rapid autolysis when cultivated on artificial medium. The actual mechanism for bile solubility is not fully understood, but it is speculated that bile salts lower surface tension at the medium–membrane interface and under these conditions, *S. pneumoniae* is particularly susceptible to disruption by enzyme action or by chemical agents.

II. Reagents

A. Sodium desoxycholate, 10% aqueous solution—store in its original container atom temperature until used. Some flocculation may occur if product is stored below 20°C. This will not reduce the effectiveness of the reagent. Protect product from light. This product should not be used if the color has changed, the expiration date has passed, or there are other signs of deterioration. Keep away from heat and flame. May cause

irritation to skin, eyes, and respiratory tract. Avoid breathing vapor and eye/skin contact.

B. Blood agar plate with growth of an α-hemolytic *Streptococcus*.

III. Quality Control

A. Bile (desoxycholate) soluble: *Streptococcus pneumoniae*

B. Bile (desoxycholate) insoluble: *Streptococcus sanguinis*

IV. Procedure

Perform preliminary tests (i.e., Gram's stain, catalase test) and note type of hemolysis to verify that the isolate is a viridans streptococcal species.

A. Tube Test

1. Prepare a saline suspension of the test isolate from an 18- to 24-hour, pure culture. Adjust turbidity to that of a 0.5 to 1.0 McFarland standard or equivalent.
2. Aliquot 0.5 mL of the suspension into each of two tubes. Label one tube as Test, the other as Control.
3. Add 0.5 mL of 10% bile solubility reagent to the tube marked Test, and 0.5 mL of saline (pH 7.0) to the tube marked Control. Gently agitate tubes to suspend bacteria.
4. Incubate tubes at 35°C to 37°C and examine periodically for up to 3 hours.
5. Observe for clearing in the Test suspension. The Control suspension should remain turbid.

B. Plate Spot Test

1. Place one drop of 10% bile solubility reagent near suspected 18- to 24-hour-old colonies growing on sheep blood agar. Gently roll the drop over several representative colonies by tilting the plate. Take care not to dislodge the colonies.
2. Incubate the plate aerobically in an upright position at 35°C to 37°C, and examine periodically for up to 30 minutes. Leave lid slightly ajar to enhance evaporation of the reagent.
3. Observe the colony for disintegration or solubility.

V. Results

A. Tube Test

1. **Positive test**—Clearing or loss of turbidity of the test suspension within 3 hours. The Control suspension remains turbid.
2. **Negative test**—Test and Control suspensions remain turbid after 3 hours.

B. Spot Test

1. **Positive test**—Disintegration of colonies and/or the appearance of an α-hemolytic zone on the plate where the colony was located within 30 minutes.

2. **Negative test**—Colonies on the plate remain intact with no change in colony integrity within 30 minutes.

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VI. Limitations

Test only α-hemolytic streptococci.

When performing the spot test, keep the plate level to prevent reagent from running, which may cause some nonpneumococcal colonies to be washed away and result in a false-negative test.

Colonies that are older than 24 hours may have lost their active enzyme, resulting in a false-negative test.

Optochin-susceptible and -resistant, bile-insoluble isolates of *S. pneumoniae,* and optochin-resistant, bile-soluble viridans streptococcal isolates have been reported.

CHART 14-1

Loeffler's Methylene Blue Stain

I. Principle

Methylene blue is a simple stain that has historically been useful in the identification of *Corynebacterium* species.

The metachromatic granules of *C. diphtheriae* readily take up methylene blue dye and appear deep blue. Although some authors have stated that the cytoplasmic granule formation characteristic of *C. diphtheriae* is rarely seen with saprophytic species of *Corynebacterium,* this criterion is unreliable and cannot be used for definitive identification of *C. diphtheriae* without further studies.

II. Media and Reagents

Methylene blue (80% dye content)	0.3 g
Ethyl alcohol (95%)	30 mL
Distilled water	100 mL

III. Procedure

1. Heat-fix the smear. Flood the surface of the smear with the methylene blue-staining solution for 1 minute. Wash the slide with water and blot dry.

2. In the past, it was necessary to add alkali to the above solution before use. However, methylene blue dyes prepared in recent years do not require this additional step because acid impurities found in older stains have been removed.

IV. Results

A. Interpretation

The corynebacteria are pleomorphic bacilli that range in size from 0.5 to 1.0 μm in width and from 2 to 6 μm in length and appear as straight, curved, or club-shaped rods. Characteristics for the microorganisms are the metachromatic granules that take up the methylene blue stain and appear dark blue. Although this finding is characteristic of the corynebacteria, species of *Propionibacterium,* some of the actinomycetes, pleomorphic strains of streptococci, and other bacteria may also morphologically resemble the corynebacteria and must be differentiated by other cultural and biochemical characteristics.

CHART 14-2

Loeffler's Serum Medium

I. Principle

Loeffler's serum medium is used primarily for the recovery of *C. diphtheriae* from clinical specimens. Because of this serum content, the medium may also be used more generally to determine the proteolytic activity of various microorganisms.

C. diphtheriae produces cells with characteristic morphologic features on Loeffler's medium. The medium is also helpful in the determination of pigment production by some bacteria.

II. Media and Reagents

A. Formula

Beef serum	70 g/L
Infusion dextrose broth (dry powder)	2.5 g/L
Egg (whole, dried)	7.5 g/L
Final pH	7.6

B. Preparation

1. To rehydrate the medium, dissolve 80 g of Loeffler's medium

(BBL) in 1 L of distilled water and warm to 42°C–45°C. The powder should be gradually added while the flask is gently rotated to minimize mixing air into the suspension.

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2. The medium should be dispensed in tubes and coagulated-sterilized in the autoclave as follows:
   a. When the suspension is uniform, dispense in tubes.
   b. Arrange the tubes in a slant position not more than four deep with several layers of newspaper or paper towels below and above the tubes to prevent rapid coagulation.
   c. Tightly close the autoclave, turn on the steam, and allow pressure to remain at 10 psi for 20 minutes.
   d. During this time, allow no air or steam to escape.
   e. Adjust the steam-inlet valve and open the air-escape valve such as to maintain a pressure of 10 psi. Abrupt changes in pressure may cause the medium to bubble.
   f. Close the outlet valve when all air has been replaced by steam and allow the pressure to reach 15 psi and hold there for 15 minutes.
   g. Allow the autoclave to cool slowly. When properly prepared, the slants are smooth and grayish white. Before inoculation, the slants should be incubated for 24 hours at 35°C as a sterility check.

III. Procedure

1. When *C. diphtheriae* is suspected, inoculate the Loeffler's medium as soon as possible after collection of the specimen.
2. Examine the slants for growth after 8–24 hours of incubation.
3. Prepare smears and stain with methylene blue.

IV. Results

A. Interpretation

See Chart 14-1 for interpretation of these stained smears.

V. Comment

Because Loeffler's medium is difficult to prepare, purchase of commercially prepared medium in sealed tubes is recommended.

VI. Bibliography

BBL. Manual of Products and Laboratory Procedures. 5th Ed. Cockeysville, MD: BBL, Division of Becton, Dickinson, and Co, 1973:118–119.

Buck T. A modified Loeffler's medium for cultivating *Corynebacterium*

diphtheriae. J Lab Clin Med 1949;34:582–583.

CHART 14-3

Tinsdale's Agar (as Modified by Moore and Parsons)

I. Principle

Tinsdale's medium supports the growth of all species of *Corynebacterium* while inhibiting the growth of normal inhabitants of the upper respiratory tract. Moore and Parsons modified the original Tinsdale's formula, simplifying the composition, but retaining the specificity for the recovery of *C. diphtheriae* and *C. ulcerans.*

The potassium tellurite is deposited within the colonies of *Corynebacterium,* turning them black. Tinsdale's medium is cystine-sodium thiosulfate tellurite, which is specifically helpful in the identification of *C. diphtheriae,* colonies of which are surrounded by a brown halo (Color Plate 14-3G).

II. Media and Reagents

A. Formula (Modified Tinsdale's Base)

Thiotone peptone	20 g
Sodium chloride	5 g
Agar	14 g
L-Cystine	0.24 g
Sodium thiosulfate	0.24 g
Distilled water	1 L

B. Preparation

1. Suspend 39 g of powder in 1 L of distilled water and heat with agitation. Boil for 1 minute. Autoclave for 15 minutes at 121°C.

2. Cool the modified base to 50°C and add:

Sterile bovine serum	100 mL
Tellurite solution, 1%	30 mL

3. Pour 15–20 mL of this medium into petri dishes and allow to harden.

4. The Tinsdale's agar base medium without serum and tellurite is stable indefinitely if stored in closed screw-capped tubes or bottles. However, the medium is ordinarily stable for only 2 or 3 days when

stored in the refrigerator following the addition of serum and tellurite.

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III. Procedure

Streak the plate so as to obtain well-isolated colonies. It is recommended that the agar be stabbed at intervals, because browning of the medium can be detected early in the stab areas.

IV. Results

A. Interpretation

A brown halo around the colony is considered presumptive evidence of *C. diphtheriae.* This can sometimes be seen after 10–12 hours of incubation, although 48 hours may be required for the appearance of typical dark-brown halos. The only related species other than *C. diphtheriae* that produces this halo is *C. ulcerans.* Other bacteria, such as coagulase-positive staphylococci, grow well on this medium, but do not have a brown halo. Bacteria, such as species of *Proteus* that produce a heavy, diffuse blackening of the medium, can be distinguished by their Gram stain reaction and biochemical characteristics.

V. Bibliography

Moore MS, Parsons EI. A study of modified Tinsdale's medium for the primary isolation of *Corynebacterium diphtheriae.* J Infect Dis 1958;102:88–93.

Tinsdale GFW. A new medium for the isolation and identification of *C. diphtheriae* based on production of hydrogen sulfide. J Pathol Bacteriol 1947;59:61–66.

CHART 15-1

Hydrolysis of Xanthine, Hypoxanthine, Tyrosine, and Casein

I. Principle

Aerobic actinomycetes may be characterized by their ability to hydrolyze xanthine, hypoxanthine, tyrosine, and casein. These compounds are incorporated into nutrient agar, and the organisms are seeded heavily on the agar surface. Results are interpreted by observing for clearing of the medium

under and around the bacterial growth, indicating hydrolysis.

II. Media and Reagents

A. Xanthine, Hypoxanthine, and Tyrosine Suspensions

1. Basal Medium

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1 L

2. Suspensions

Tyrosine	0.5 g
Xanthine	0.4 g
Hypoxanthine	0.4 g
Distilled water	100 mL

Heat the basal medium to bring the ingredients into solution and place 100-mL aliquots into 250-mL flasks. Sterilize by autoclaving at 15 psi for 15 minutes. Allow medium to cool almost to solidification. To each aliquot, add 10 mL of xanthine, hypoxanthine, or tyrosine suspensions. The suspensions are autoclaved before adding to the basal media. It is essential that the granules of xanthine, tyrosine, and hypoxanthine be kept in suspension until the medium solidifies; hence, the addition of the biochemical granules and the pouring of the medium into Petri dishes should be done when the basal medium has cooled just short of solidification. The media are poured into sterile 15 × 100-mm Petri dishes.

3. Casein Medium

a. Solution A

Skim milk (powdered)	10.0 g
Distilled water	100 mL

b. Solution B

Agar	2.0 g
Distilled water	100 mL

Autoclave solutions A and B separately, allow each to cool to about 50°C, and combine A with B and pour into 15 × 100-mm Petri dishes.

III. Quality Control

A. Negative control: *Nocardia asteroides* complex

B. Positive control: *Streptomyces* species

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IV. Procedure

Each xanthine, hypoxanthine, tyrosine, and casein plate is commonly divided into wedge-shaped thirds, providing an area to inoculate positive and negative controls along with the organism to be identified. The plates are incubated at 30°C and read at the following times:

A. Casein hydrolysis: Read after 2 weeks
B. Xanthine hydrolysis: Read after 3 weeks
C. Hypoxanthine hydrolysis: Read after 3 weeks
D. Tyrosine hydrolysis: Read after 3 weeks

V. Interpretation

Hydrolysis may be evident prior to the incubation end points provided above. Casein hydrolysis is read by observing for a complete clearing to transparency of the white, milky medium. The other three hydrolysis tests are read by observing the dissolution of the crystalline compounds around the colonies growing on the media.

VI. Bibliography

Gordon RE, Mihm JM. Identification of *Nocardia caviae* (Erickson) nov. comb. Ann NY Acad Sci 1962;98:628–636.

CHART 15-2

Lysozyme Test for the Identification of *Nocardia* Species

1. Prepare glycerol broth by adding 5 g of peptone and 70 mL of glycerol to 1,000 mL of distilled water (reduce amounts proportionately if smaller quantities are desired). Sterilize by autoclaving at 15 psi for 15 minutes.
2. Prepare lysozyme solution by dissolving 100 mg of lysozyme powder in 100 mL of 0.01 N HCl.
3. Combine 95 mL of glycerol broth with 5 mL of lysozyme solution and dispense in 5-mL aliquots into screw-capped tubes. Use 5-mL aliquots of glycerol broth without lysozyme for the growth control.
4. Prepare a light suspension of the unknown organism in sterile saline. Using a Pasteur pipette, inoculate 1 or 2 drops into the lysozyme and control tubes (keep the inoculum light).
5. Incubate at 25°C. Examine each tube for growth in 7–14 days. Growth will appear as a pellicle on the surface, as a sediment, or as both. The tubes can be gently shaken to observe bread crumb-like particles in the tubes showing

positive growth.

6. An organism resistant to lysozyme will grow in both the lysozyme tube and the control tube; an organism that is susceptible will grow only in the control tube.

CHART 16-1

Components of CDC Anaerobe Blood Agar

Ingredient	Amount
Trypticase-soy agar (BDMS)	15 g
Phytone (BDMS)	5 g
Sodium chloride	5 g
Agar	20 g
Yeast extract (Difco)	5 g
Hemin	5 mg
Vitamin K_1 (3-phytylmenadione)	10 mg
L-Cystine	400 mg
Demineralized water	1 L
Blood (sheep or rabbit), defibrinated	50 mL

CHART 16-2

Media and Characteristics Used for Identifying Anaerobes

Media	Characteristics
Blood agar	Relation to O_2[a], colony characteristics, hemolysis, pigment, fluorescence with UV light (Wood's lamp), pitting of agar, cellular morphology, Gram's stain reaction, spores, motility (wet mount); inhibition by penicillin, rifampin, and kanamycin
	Appearance of growth, rapidity of growth, gas production,

odor, cellular morphology

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Presumpto Plate 1	
LD agar	Indole, growth on LD medium, catalase[b]
LD esculin agar	Esculin hydrolysis, H_2S, catalase
LD egg yolk agar	Lipase, lecithinase, proteolysis
LD bile agar	Growth is in presence of 20% bile (2% oxgall), insoluble precipitate under and immediately surrounding growth
Presumpto Plate 2	
LD glucose agar	Glucose fermentation; stimulation of growth by fermentable carbohydrate
LD starch agar	Starch hydrolysis
LD milk agar	Casein hydrolysis
LD DNA agar	Deoxyribonuclease activity
Presumpto Plate 3	
LD Mannitol agar	Mannitol fermentation
LD Lactose agar	Lactose fermentation
LD Rhamnose agar	Rhamnose fermentation
LD Gelatin	Gelatin hydrolysis

[a] By comparing growth on anaerobe plate with blood agar (or chocolate agar) incubated in 5%–10% CO_2 incubator (or candle jar) or in room air.

[b] The catalase test can be performed by adding 3% hydrogen peroxide to the growth on LD agar, but the actions of catalase-positive cultures are more vigorous on LD esculin agar.

CHART 16-3

How to Inoculate Presumpto Quadrant Plates, Antibiotic Potency Disks, Nitrate Disks, and SPS Disks?

1. Prepare the inoculum from fresh growth of a pure culture of the anaerobic isolate. Use either a turbid cell suspension (McFarland 3) in LD broth prepared from isolated colonies or a 24- to 48-hour enriched thioglycolate medium subculture from an isolated colony. As an alternative, a 24- to 48-hour chopped-meat glucose broth subculture can be used.
2. Inoculate the quadrant plates as follows:
   a. Saturate one sterile swab for each quadrant plate to be inoculated in either the cell suspension or broth culture.
   b. Streak the middle portion of each quadrant with the swab containing bacteria.
3. Place a sterile, blank, 1/2-inch-diameter paper disk on the LD agar near the outer periphery of the quadrant. This disk is used in the test for indole after incubation of the plates.

   In addition to setting up the Presumpto plates, also:

   1. Inoculate the surface of an anaerobic blood agar plate evenly with a sterile swab that has been dipped in the cell suspension or broth culture.
   2. Place the antibiotic disks (penicillin, 2 U; rifampin, 15 µg; kanamycin, 1,000 µg) on the blood agar with sterile forceps. Space the disks evenly such that overlapping zones of inhibition will not be a problem.
   3. Place the sodium polyanetholsulfonate (SPS) and nitrate disks on a second anaerobic blood agar plate, if they are used in addition to the antibiotic disks. To prepare the SPS disk, pipette 20 µL of 5% SPS onto 1/4-inch (0.635-cm) sterile blank disks (Becton Dickinson [BD], Sparks, MD) and dry. SPS disks are stable at room temperature for 6 months. To prepare the nitrate disk, dissolve 30 g KNO_3 and 0.1 g sodium molybdate (Na_2MoO_4. $2H_20$) in 100 mL of distilled water and filter-sterilize the solution using a 0.45-µm membrane filter. Then add 20 µL of this reagent to 1/4-inch (0.635-cm) sterile blank disks and dry at room temperature for at least 72 hours.[519]

CHART 16-4

Interpretation of Presumpto Quadrant Plates (Color Plate 16-5)

I. Presumpto Quadrant Plate 1

LD Agar. Note and record the degree of growth on LD agar (light, moderate, heavy). Test for indole by adding two drops of para-dimethylaminocinnamaldehyde reagent to the paper disk on the medium. Observe for the development of a blue or bluish green color in the disk within 30 seconds, which is a positive

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reaction for indole. Development of another color (pink, red, violet) or no color is negative for indole. A lavender to violet color indicates a positive reaction for indole derivative(s).

LD Egg Yolk Agar. Formation of a zone of insoluble precipitate in the medium surrounding the bacterial colonies indicates a positive reaction for lecithinase production. This is best seen with transmitted light.

The presence of an iridescent sheen (a pearly layer on the surface of colonies and on the medium immediately surrounding the bacterial growth, best demonstrated with reflected light) indicates lipase production. If the reaction is questionable, add a few drops of water and look for a film that floats on top of water.

Clearing of the medium in the vicinity of the bacterial growth indicates proteolysis, as exhibited by certain proteolytic clostridia.

LD Esculin Agar. A positive test for esculin hydrolysis is indicated by the development of a reddish brown to dark brown color in the esculin agar surrounding the bacterial growth after exposure of the quadrant plate cultures to air for at least 15 minutes. Additional evidence for esculin hydrolysis can be obtained by examining the esculin agar quadrant under a Wood's lamp. Esculin agar exhibits a bright blue fluorescence under the ultraviolet light that is not present after the esculin is hydrolyzed.

Blackening of the bacterial colonies on the esculin agar indicates H_2S production. The blackening dissipates very rapidly after exposure to air. Therefore, the bacterial growth should be observed for blackening under anaerobic conditions (anaerobic glove box) or immediately after opening anaerobic jars to air. To test for hydrogen peroxide degradation as an indication of catalase, expose the plates to air for at least 30 minutes and then flood the esculin agar quadrant with a few drops of fresh 3% H_2O_2. Sustained bubbling after addition of the H_2O_2 is a positive reaction for catalase. In some cases, rapid bubbling may not be evident until after 30 seconds to a minute.

LD Bile Agar. Compare the degree of bacterial growth on the LD bile agar

with that on the plain LD agar and record as I (growth less than on the LD agar control) or E (growth equal to or greater than on the LD agar control). Using transmitted light, look for the presence or lack of an insoluble white precipitate underneath or immediately surrounding the bacterial growth. If in doubt, inspect under a stereomicroscope using transmitted light.

II. Presumpto Quadrant Plate 2 (Color Plate 16-5)

LD Glucose Agar. Fermentation of glucose is indicated by acid production or a yellow color in and around the growth in the medium. A blue color around the growth is a negative reaction. Some bacteria reduce the indicator; therefore, it is sometimes necessary to flood the quadrant with dilute bromthymol blue reagent to determine if acid has been produced.

Glucose stimulation can be observed by comparing the amount of bacterial growth on LD glucose agar with that on plain LD agar.

LD Starch Agar. To detect hydrolysis of starch, flood the quadrant with Gram's iodine solution; clearing around the growth indicates a positive reaction. A brownish color indicates unhydrolyzed starch and a negative reaction.

LD Milk Agar. A clear zone around the growth in the quadrant indicates hydrolysis of casein (i.e., digestion of milk proteins) and a positive reaction. If casein is unhydrolyzed, the medium remains cloudy (a negative reaction).

LD DNA Agar. A pink to reddish zone around the growth on the quadrant indicates a positive reaction for the degradation of DNA (i.e., deoxyribonuclease activity). If the medium remains blue around the growth, DNA was not degraded and the reaction is negative.

III. Presumpto Quadrant Plate 3

LD Gelatin Agar. To detect the hydrolysis of gelatin, flood the quadrant with acidified mercuric chloride reagent. This reagent binds to unhydrolyzed gelatin. A zone of complete clearing around the growth on the quadrant indicates that gelatin has been hydrolyzed and is recorded as a positive test.

LD Mannitol Agar, LD Lactose Agar, and LD Rhamnose Agar. Examine and interpret these quadrants as described for LD glucose agar.

CHART 16-5

Description of Presumpto Plates for Identification of Anaerobes

Three types of quadrant plates (Presumpto 1, 2, and 3) containing 12 differential

agar media were developed by Dowell, Lombard, and associates into a system that allows determination of 20 different characteristics (Chart 16-4) of anaerobic isolates at a minimal cost.[519] The information derived from using the three-quadrant plates, along with the other characteristics obtained from anaerobe blood agar and enriched thioglycolate medium plus metabolic product analysis using GLC, permits definitive identification of many clinically significant anaerobic bacteria that are encountered in the laboratory. In addition, the quadrant plates, especiall y the Presumpto 1 and 2

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plates, can be used to supplement several of the commercial packaged kit systems, as well as traditional broth identification systems, with important tests or characteristics these nonagar systems lack.

The basal medium in the Presumpto quadrant plate system is Lombard–Dowell (LD) medium. This is a specially designed medium that supports growth of a wide variety of anaerobes, including fastidious species. It is noteworthy that LD broth (the same medium without agar or with only a small amount) is the inoculum broth for some of the commercial packaged ID systems. The Presumpto 1 plate is a four-quadrant Petri dish containing the following media: LD agar, LD esculin agar, LD egg yolk agar, and LD bile agar, initially devised for presumptive identification of *Bacteroides* and *Fusobacterium* (without Presumpto plates 2 and 3) but since has been useful for identifying other anaerobic bacteria.

The Presumpto 2 plate contains LD glucose agar, LD DNA agar, LD milk agar, and LD starch agar. It is useful for characterization of clostridia, the anaerobic, non–spore-forming gram-negative and gram-positive bacilli and the anaerobic cocci. Like the Presumpto 1 plate, it can be used along with packaged micromethod kits that lack the tests it contains, or along with conventional tube tests.

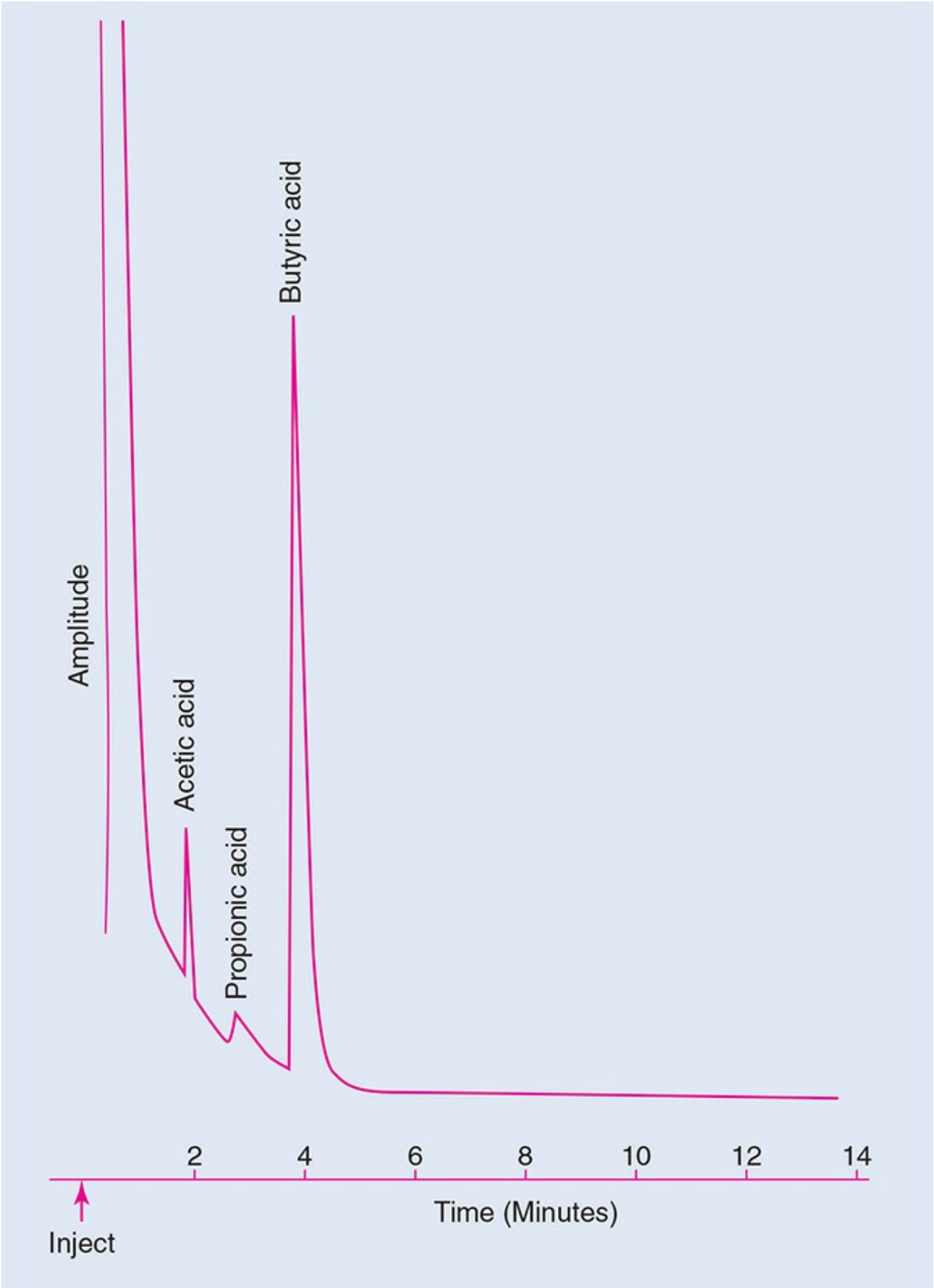
The Presumpto 3 plate contains LD gelatin agar, LD mannitol agar, LD lactose agar, and LD rhamnose agar. Use of LD gelatin agar and the use of the carbohydrates in this quadrant plate have been described.[123,519]

CHART 16-6

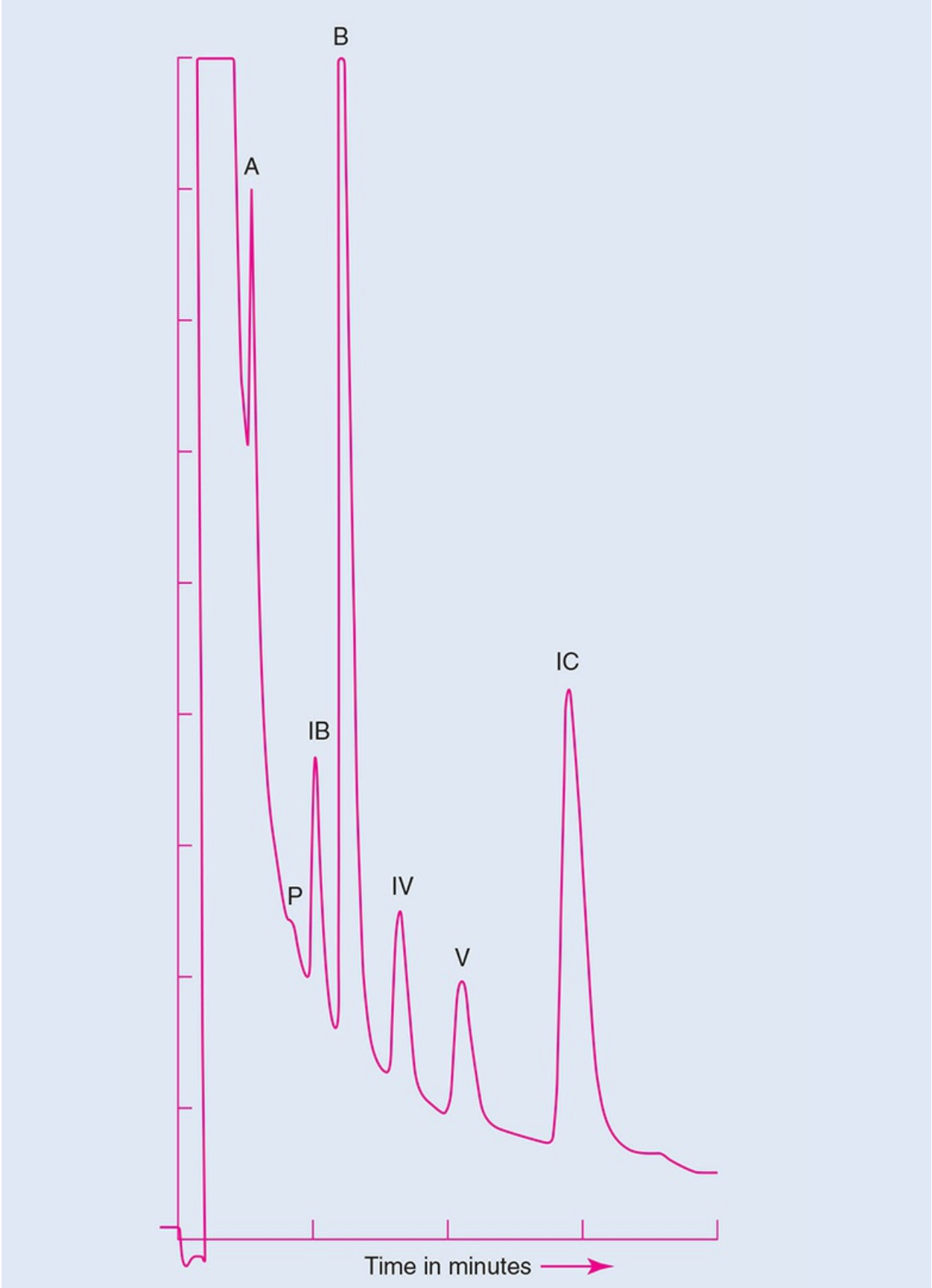
Identifying Volatile Fatty Acids

1. Inoculate tubes containing 7–8 mL of prereduced peptone-yeast extract-glucose

(PYG) broth with a few drops (0.05–0.1 mL) of an actively growing culture.


Amplitude
Acetic acid
Propionic acid
Butyric acid
2
4
6
8
10
12
14
Time (Minutes)
Inject

■ **FIGURE 16-6A** Volatile acid chromatogram of a 48-hour peptone–yeast extract–glucose broth culture of *Fusobacterium mortiferum.* The retention times of the products in the broth culture are compared with those of the standard tracing (Fig. 16-6A) to identify the unknown acids. The peaks indicate major amounts of butyric and acetic acids, but only a minor amount of propionate for this culture. The same instrument and operating conditions were used as for the tracing in Figure 16-10.


B
A
IC
IB
P
IV
V
Time in minutes

■ **FIGURE 16-6B** Example of a volatile acid chromatograph of a 48-hour peptone–yeast extract–glucose broth culture of *Clostridium difficile*. *A,* acetic acid; *P,* propionic acid; *IB,* isobutyric acid; *B,* butyric acid; *IV,* isovaleric acid; *V,* valeric acid; *IC,* isocaproic acid.

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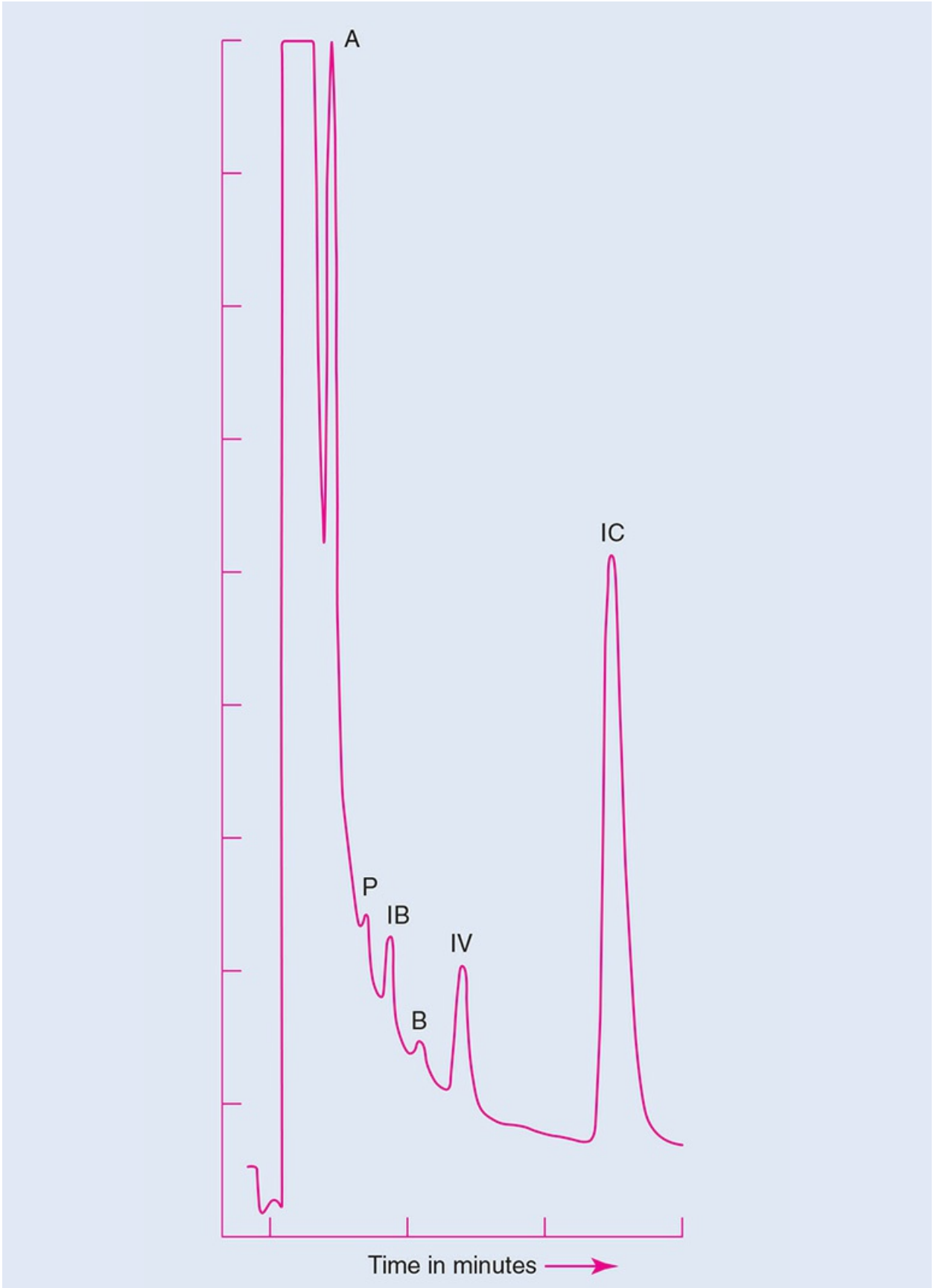
A
IC
P
IB
IV
B
Time in minutes

■ **FIGURE 16-6C** Example of a volatile acid chromatograph tracing of a 48-hour peptone–yeast extract–glucose broth culture of *Peptostreptococcus anaerobius*. *A,* acetic acid; *P,* propionic acid; *IB,* isobutyric acid; *B,* butyric acid; *IV,* isovaleric acid; *IC,* isocaproic acid.

2. Incubate under anaerobic conditions for 48 hours, or until adequate growth is obtained.
3. Transfer 2 mL of the culture to a clean, 13- × 100-mm screw-capped tube.
4. Acidify the culture to pH 2.0 or lower by adding 0.2 mL of 50% (v/v) aqueous H_2SO_4.
5. Add 1 mL of ethyl ether, tighten the cap, and mix by gently inverting the tube about 20 times.
6. Centrifuge briefly in a clinical centrifuge (1,500–2,000 rpm) to break the ether–culture emulsion.
7. Place the ether-culture mixture in a freezer at −20°C or lower or in an alcohol–dry ice bath until the aqueous portion (bottom) is frozen.
8. Rapidly pour off the ether layer into a clean screw-capped tube. If desired, add one or two anhydrous $CaCl_2$ pellets to the ether extract to allow removal of residual water.
9. Inject 14 µL of the extract into the column of a gas chromatograph packed with SP-1220.

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CHART 16-7

Identifying Nonvolatile Fatty Acids

1. Transfer 1 mL of the original PYG culture to a clean 13 × 100-mm screw-capped tube.
2. Add 0.4 mL of H_2SO_4 (v/v) and 2 mL of methanol.
3. Place the tube in a 55°C water bath for 1 hour or overnight.
4. Add 1 mL of distilled water and 0.5 mL of chloroform, and centrifuge briefly to break any emulsion in the chloroform layer. (Note: The chloroform layer will be in the bottom of the tube.)
5. Fill a syringe with the chloroform extract after placing the tip of the needle

beneath the aqueous layer.

6. Wipe off the outside of the needle with a clean tissue and inject 14 µL into a GLC column packed with SP-1000.

CHART 16-8

The Nagler Test and the CAMP Test for *C. perfringens*

Historically, the Nagler test has been used for the presumptive identification of *Clostridium perfringens*, even though the antitoxin used in the test is not specific. The popularity of the Nagler test has waned because the antitoxin has not been widely available. Additional details describing the Nagler test are given in previous editions of this textbook.

An alternative to the Nagler test, used in some laboratories, is the so-called reverse CAMP test.[62,519] To perform the test, inoculate an anaerobe blood agar plate with a single streak of an unknown organism that is thought to possibly represent *C. perfringens* isolate. Next, make a single streak at a 90-degree angle to within 1–2 mm of the first streak, using an inoculum of a group B β-hemolytic *Streptococcus*. An arrowhead of synergistic hemolysis, with the tip of the arrow pointing from the *Streptococcus* toward the *C. perfringens*, is a positive test. Both false-positive and false-negative results may occur. Additional data on the specificity of this test for clinical isolates of *C. perfringens* are needed; however, most laboratories experienced in anaerobic bacteriology may find the Nagler test and the reverse CAMP test, while perhaps "novel," to be unnecessary.

CHART 16-9

Assay for Toxin in Feces by Tissue Culture Cytotoxicity Procedure[a]

1. Centrifuge liquid stool or extract of formed stool (2,000 *g* for 20 minutes or 10,000 *g* for 10 minutes).
2. Filter through 0.45-µm membrane filter.

3. Add 0.1 mL of cell-free supernatant plus 0.1 mL buffered gelatin diluent (pH 7.0–7.2) or phosphate-buffered saline (PBS) to a tissue culture tube. Commercially available human diploid lung fibroblasts (WI-38 cells) are convenient to use.
4. Observe the tissue culture cells for cytotoxicity at 4, 24, and 48 hours. Most positives will be observed at 24 hours.
5. For the antitoxin neutralization test, add 0.1 mL of *C. difficile* antitoxin (TechLab, Blacksburg, VA) to 0.1 mL cell-free supernatant and carry out steps 1 and 4. Rounding of WI-38 cells (so-called actinomorphic change) or other cytopathic effects should not be seen if toxin that is present in the stool is neutralized.
6. The toxin titer is determined using serial twofold or tenfold dilutions of the filtered fecal sample in buffered gelatin diluent (pH 7.0–7.2) or PBS. Correlation between the toxin titers and severity of illness is very crude.

[a]Commercial kits for performing *C. difficile* cytotoxin neutralization assays are available from at least two manufacturers (Bartels Diagnostics; Bio-Whittaker/Wampole). The kits can be purchased complete with human foreskin fibroblasts in microbroth dilution trays, and they provide the necessary antitoxin and other reagents to accomplish the cytotoxin neutralization assay.

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CHART 16-10

Some Key Characteristics of Commonly Isolated Anaerobic, Gram-Negative Rods on CDC Differential Agar Media

		Presumpto 1 Plate								Presumpto 2 Plate				Presumpto 3 Plate			
Species	No. of Strains Examined	Indole	Indole Derivativer	Esculin Hydrolysis	H_2S	Catalase	Lecithinase	Lipase	Growth on Bile Agar	Glucose Fermentation	Starch Hydrolysis	Milk Proteolysis	DNase	Gelatin Hydrolysis	Mannitol Fermentation	Lactose Fermentation	Rhamnose Fermentation
B. distasionis	21	–	–	+	–	+/–	–	–	E	+	–	–/+	–	–	_	+	+
B. fragilis	135	–	–	+	–	+	–	–	E-ppt	+	–/+	–/+	–	–	–	+	–
B. ovatus	5	+/–	–	+	–	V	–	–	E-ppt	+	V	V	V	–	+/–	+	+
B. thetaiotaomicron	71	+	–	+	–	+	–	–	E	+	–/+	–/+	+/–	–	–	+	+
B. uniformis	5	+	–	+/–	–	–	–	–	E	+	–	V	V	–	–	+	–/+
B. vulgatus	23	–	–	–/+	–	–	–	–	E	+	–	–	–	–	–	+	+
B. splanchnicus	2	+	–	+	v	–	–	–	E	+	–	–	–	V	–	+	–
B. ureolyticus	1	–	–	–	–	–	–	–	V	–	–	–/+	–	–/+	–	–	–
Campylobacter gracilis	10	–	–	–	–	–	–	–	V	–	–	–	–/+	–	–	–	–
Porphyromonas asaccharolytica	6	+/–	–	–	–	–	–	–/+	NG	–	–	+/–	–/+	V	–	–	–
P. gingivalis	13	+	–	–	v	–	–	–	NG	–	–/+	+	–	+	–	–	–
Prevotella bivia	14	–	–	–	–	–	–	–	NG	+	–/+	+/–	+/–	V	–/+	+	–/+
P. disiens	3	–	–	–	–	–	–	–	V	+/–	–	+	V	+	–	–	–
P. denticola	5	–	–	–/+	–	–	–	–	NG	+	+	–	+/–	–/+	–	+	–
P. intermedia/ nigrescens	16	+	–	–	–	–	–	+	NG	+/–	+/–	+	+	+	–	–	–
P. melaninogenica	10	–	–	–	–	–	–	–	NG	+	+	–	+/–	V	–	+	–
P. oralis	2	–	–	+	–	–	–	–	NG	V	V	–	V	–	–	V	V
P. oris/buccae	9	–	–	+	–	–	–	–	NG	+	+	+/–	+	–	–	+	–/+
P. veroralis	2	–	–	v	–	–	–	–	NG	+	+	–	V	–	–	+	V
Fusobacterium gonidiaformans	6	+/–	–	–	–/+	–	–	–	NG	–/+	–	–	–	–	–	–	–
F. mortiferum	5	–/+	–	+	–	–	–	–	E	+	–	–	–	–	–	V	–
F. naviforme	6	+	–	–	–	–	–	–	NG	–/+	–	–	–	–	–	–	–
F. necrophorum	18	+	–	–	V	–	–	+	NG	V	–	+	–/+	–/+	–	–	–
F. nucleatum	31	+	–	–	V	–	–	–	NG	V	–	–	–	–	–	–	–
F. varium	6	v	–	–	V	–	–	–	E	–	–	–	–	–	–	–	–

+, positive reaction in <90% strains; , negative reaction in <90% strains; +/– , most strains positive, occasional strain negative; –/+, most strains negative, occasional strain positive; V, variable; E, growth equal to control without bile; E1, equal growth with an occasional strain inhibited; E-ppt, equal growth with a precipitate adjacent to or under growth; NG, no growth.

Adapted from references 509 and 519.

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CHART 16-11

Reactions of Gram-Positive, Non–Spore-Forming Bacilli on CDC Differential Agar Media

Species	No. of Strains Examined	Presumpto 1 Plate								Presumpto 2 Plate				Presumpto 3 Plate			
		Indole	Indole Derivativer	Esculin Hydrolysis	H_2S	Catalase	Lecithinase	Lipase	Growth on Bile Agar	Glucose Fermentation	Starch Hydrolysis	Milk Proteolysis	DNase	Gelatin Hydrolysis	Mannitol Fermentation	Lactose Fermentation	Rhamnose Fermentation
Actinomyces																	
A. israelii	12	–	–	+	–	–	–	–	V	+	–	–/+	–/+	–	V	–/+	–/+
A. meyeri	39	–	–	–	–	–	–	–	I^{NG}	+/–	–	–	–/+	–	–	V	–
A. naeslundii	31	–	–	+/–	–	–	–	–	V	+	–	–	–	–	–/+	+/–	–
A. odontolyticus	56	–	–	V	–	–	–	–	V	+	–	–	–	–	–	V	–/+
A. viscosus	25	–	–	V	–	+/–	–	–	NG^{I}	+	–	–	–	–	–	V	–
Arcanobacterium																	
A. pyogenes	12	–	–	–	–	–	–	–	I^{NG}	+	–	+	–/+	+/–	–	–/+	–
Bifidobacterium																	
B. adolescentis	8	–	–	V	–	–	–	–	E	+	–/+	–	–	–	–/+	+	–
B. bifidum	4	–	–	–	–	–	–	–	E	+	–	–	–	–	–	+	–
B. breve	4	–	–	+/–	–	–	–	–	E	+	–/+	–	–	–	V	+	–
B. dentium[a]	3	–	–	–	–/+	–	–	–	E	+	–	–	–	–	+	+/–	–
B. infantis/ longum	3	–	–	–	–	–	–	–	E	+	V	–	–	–	–	+	–
Collinsella																	
C. aerofaciens	3	–	–	–/+	–	–	–	–	E	+	–	+/–	–	–	–	+	–
Eggerthella																	
E. lenta	33	–	–	–	–/+	V	–	–	E^{I}	+	–	+/–	–	–	–	+	–
Eubacterium																	
E. brachy	2	–	–	–	–	–	–	–	NG	–	–	–	–	–	–	–	–
E. limosum	8	–	–	–	+	–	–	–	E	+	V	–	–	–	+	–	–
E. nodatum	3	–	–	–	–	–	–	–	NG	–	–	–	–	–	–	–	–
E. saburreum	2	v	–	V	–	–	–	–	NG	–	–	–	–	–	–	–	–
E. timidum	3	–	–	–	–	–	–	–	NG	–	–	–	–	–	–	–	–
Propionibacterium																	
P. acnes	144	+/–	–	–	–	+	–	–/+	V	+	–	+/–	–	V	–	–	–
P. avidum	5	–	–	+/–	–	+	–	+/–	E	+	–	V	–	+/–	–	–	–
P. granulosum	7	–	–	–	–	+	–	–/+	V	+	–	V	–/+	–/+	–/+	–/+	–
P. propionicum	10	–	–	–	–	–	–	–	I	V	–	_	–	–	V	V	–
Pseudoramibacter																	
P. alactolyticum	4	–	–	–	–	–	–	–	I	V	–	–	–	–	–	–	–

[a]Formerly called *B. eriksonii*.

+, positive reaction in >90% of strains; –, negative reaction in >90% of strains; +/–, most strains positive, occasional strain negative; –/+, most strains negative, occasional strain positive; I, growth inhibited; V, variable; E, growth equal to control without bile; E^{I}, equal growth with an occasional strain inhibited; I^{NG}, inhibited growth with occasional strains not growing.

Adapted from references 509 and 519.

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CHART 16-12

Reactions of *Clostridium* Species on CDC Differential Agar Media

		Presumpto 1 Plate								Presumpto 2 Plate				Presumpto 3 Plate			
Species	No. of Strains Examined	Indole	Indole Derivativer	Esculin Hydrolysis	H_2S	Catalase	Lecithinase	Lipase	Growth on Bile Agar	Glucose Fermentation	Starch Hydrolysis	Milk Proteolysis	DNase	Gelatin Hydrolysis	Mannitol Fermentation	Lactose Fermentation	Rhamnose Fermentation
C. baratii	8	–	–	+	–	–	+	–	E	+	V	V	–	–	–	+/–	–
C. bifermentans	16	+	–	+	–	–	+	–	E	+	–	+/–	–	+	–	–	–
C. butyricum	31	–	–	+	–	–	–	–	I	+	+/–	–	–	–	–	+	–
C. cadaveris	6	+/–	–/+	–	V	–	–	–	E^{NG}	+	+/–	–	+	–/+	–	–	–
C. clostridioforme	18	–/+	–	V	–	–	–	–	V	+/–	–	–	–/+	–	–	+/–	V
C. difficile	490	–	–	+	–	–	–	–	E	+	–	–	–	–	+	–	–
C. histolyticum	5	–	–	–	–	–	–	–	V	–	–	+	+	+/–	–	–	–
C. innocuum	18	–	–	+	–	–	–	–	E	+	–	–	–	–	+/–	–	–
C. limosum	2	–	–	–	–	–	–	–	NG	V	V	V	V	–	–	–	–
C. malenominatum	7	+	–	–	V	–	–	–	I^{E}	–	–	–	–/+	–	–	–	–
C. paraputrificum	13	–	–	+	–	–	–	–	E	+	–/+	–	–	–	–	+	–
C. perfringens	79	–	–	V	–/+	–	+	–	E	+	+/–	V	+	+	–	+	–
C. ramosum	16	–	–	+	–	–	–	–	E	+	–	–	–	–	V	+	V
C. septicum	56	–	–	+/–	–	–	–	–	E	+	–	–	+	+/–	–	+/–	–
C. sordellii	32	+	–	–	V	–	+/–	–	E	+	–	+	–	+	–	–	–
C. sphenoides	8	+/–	–	V	–	–	–	–	V	+	–	–	V	–	V	V	V
C. sporogenes	52	–	+	+	–	–	–/+	+	E	+	–	+	–/+	+	–	–	–
C. subterminale	7	–	–	–	V	–	–	–	V	–	–/+	V	–/+	+/–	–	–	–
C. symbosium	2	–	–	–	–	–	–	–	V	V	–	–	–	–	V	–	–
C. tertium	44	–	–	+	–	–	–	–	E	+	V	–	+/–	–	+	+	–
C. tetani	27	+/–	–	–	V	–	–	–/+	E^{I}	–	–	–	V	+	–	–	–

+, positive reaction in >90% of strains; –, negative reaction in >90% of strains; +/–, most strains positive, occasional strain negative; –/+, most strains negative, occasional strain positive; V, variable; E, growth equal to control without bile; E^{I}, equal growth with occasional strain inhibited; E^{NG}, equal growth with occasional strains not growing; NG, no growth; I^{E}, most strains inhibited with equal growth for occasional strains.

Adapted from references 509 and 519.

CHART 17-1

Disk Diffusion (Bauer–Kirby) Susceptibility Test for Nonfastidious Bacteria

I. Principle

Continued evaluation and revision of the disk diffusion procedure has been performed by a committee of the Clinical Laboratory Standards Institute (CLSI). Designating a national standard test for disk diffusion has not only permitted more exacting quality control, but has also allowed valid comparison of results among different laboratories using the procedure. The CLSI publishes periodic updates that incorporate new information and changes that have been suggested by users. It is important to maintain currency of the procedure. This chart summarizes the basic procedure; consult the referenced CLSI document for variation used to test fastidious bacteria.

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The principle of the disk diffusion procedure has been discussed in the text. The zone diameters that are generated by the test are meaningless without reference to the minimum inhibitory concentration (MIC) correlates and interpretive guidelines published by the CLSI.

II. Media and Reagents

- **A.** Bacterial isolate from an infectious process
- **B.** Nutrient broth (tryptic soy broth is recommended) for growth of the inoculum
- **C.** McFarland 0.5 standard for adjusting the turbidity of the inoculum
- **D.** Vortex mixer for suspension of the inoculum
- **E.** Viewbox for comparison of broth with standard. A photometer may be used if satisfactory results are documented.
- **F.** Mueller–Hinton agar plates (100 mm diameter for a maximum of five disks; 150 mm diameter for a maximum of 12 disks) from a lot that produces satisfactory quality control results. The pH must be 7.2–7.4, and the depth must be approximately 4 mm.
- **G.** Calipers, ruler, or template for measuring the diameters of inhibitory zones

III. Quality Control

Reference strains of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* should be tested each time a new batch of disks or agar is used. Additional controls should be performed each day the test is done or according to the laboratory's Individualized Quality Control Plan (IQCP) for this test (Table 17-14).

IV. Procedure

- **A.** Preparation of Inoculum (Growth Method)
  1. With a wire loop, touch the tops of four or five similar-appearing, well-isolated colonies on an agar plate culture. Transfer the growth

to a tube that contains 4–5 mL of a suitable broth medium.

2. Allow the culture to incubate at 35°C until it matches the turbidity of the standard prepared in the foregoing (usually 2–6 hours). Vigorously agitate the standard on a mechanical vortex mixer immediately before use. Use adequate light to read the tube against a white background with a contrasting black line. Add sterile saline or broth if necessary to obtain a turbidity that is visually comparable with that of the standard. For the *E. coli* quality control strain, approximately 1–2 $\times 10^8$ colony-forming units (CFU)/mL should result. Alternatively, a direct broth or saline suspension of colonies from an 18- to 24-hour agar plate may be adjusted to the standard. In this case, the bacteria must have been grown on a nutrient, nonselective medium, such as blood agar. Commercially available devices to standardize the inocula without adjustment of turbidity and without preincubation in broth media have also performed acceptably.
3. Within 15 minutes after adjusting the turbidity of the inoculum suspension to that of the standard, dip a sterile nontoxic cotton swab into the inoculum suspension and rotate the swab several times with firm pressure on the inside wall of the tube to remove excess fluid.
4. Inoculate the dried surface of a Mueller–Hinton agar plate that has been brought to room temperature by streaking the swab three times over the entire agar surface, rotating the plate approximately 60 degrees each time to ensure an even distribution of the inoculum. Finally, swab the rim of the agar. Replace the lid of the dish. Allow at least 3–5 minutes but no longer than 15 minutes for the surface of the agar to dry before adding the antibiotic disks.

B. Alternative Inoculum Preparation (Direct Colony Suspension; DCS)

1. Make a direct saline or broth suspension of colonies from a nutrient nonselective agar plate (e.g., blood agar) that has been incubated for 18–24 hours.
2. Immediately adjust the inoculum to the standard density, as for the growth method.
3. This method is recommended for *Haemophilus* species, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, and *Staphylococcus* species when testing susceptibility to oxacillin.

C. Testing of Antibiotics

1. Place the appropriate antimicrobial-impregnated disks on the surface of the agar, using either forceps or a multidisk dispenser.
2. Gently tap each disk down onto the agar to provide uniform

contact. Do not move a disk once it has contacted the agar, because some of the drug diffuses almost immediately. Disks must be evenly distributed on the agar so that they are no closer than 24 mm from center to center.

3. Invert the plates and place them in a 35°C air incubator for 16–18 hours (24 hours when testing staphylococci against methicillin or oxacillin or enterococci against vancomycin).

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V. Results

A. Interpretation

Measurement of zone diameters: Examine the plates after overnight incubation. With the use of sliding calipers, a ruler, or a template, the zones of complete growth inhibition around each of the disks are carefully measured to within the nearest millimeter; the diameter of the disk is included in this measurement.

All measurements are made with the unaided eye while viewing the back of the Petri dish with reflected light against a black, nonreflecting background. The plates should be viewed from a directly vertical line of sight to avoid any parallax that may result in misreadings. Susceptibility plates prepared with blood must be viewed from the agar surface and measurements made with the cover of the Petri dish removed, again using reflected light to illuminate the plate. Common problems in delineation of zone sizes are discussed in the text.

An interpretive correlate (susceptible, moderately susceptible, intermediate, or resistant) is provided by reference to published guidelines. Zones that fall into the intermediate range should be considered equivocal; if therapy with the drug is desired, a dilution susceptibility test should be performed to clarify the issue.

VI. Bibliography

Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests: approved Atandard- Twelfth Edition (M02-A12). Wayne, PA: Clinical Laboratory Standards Institute, 2015.

CHART 17-2

Performance of Microbroth Dilution Susceptibility Tests With Nonfastidious Bacteria

I. Principle

The microdilution test is an adaptation of the reference macroscopic test. Large numbers of isolates can be tested against multiple drugs easily, so the procedure is well adapted to routine use. This test generates more quantitative information, which may be useful or even essential in certain circumstances, than does the disk diffusion test. This chart summarizes the basic procedure; consult the referenced Clinical Laboratory Standards Institute (CLSI) document for the variation used to test fastidious bacteria.

The principle of the microdilution test is the same as that of the macroscopic test. Bacterial isolates are exposed to serial dilutions of each antimicrobial agent, after which the concentration of drug that inhibits growth is determined by visual inspection.

II. Media and Reagents

A. Nutrient broth (tryptic soy broth is recommended)

B. 0.5 McFarland standard for adjusting inoculum

C. White background with black lines for comparison of turbidity of inoculum with 0.5 McFarland standard

D. Frozen or lyophilized microtiter plates containing antibiotics in cation-supplemented Mueller–Hinton broth

E. Multiprong inoculators—either wire loops or disposable plastic pins

F. Viewing mirror and template for visualization of endpoints. A photometric or fluorometric analysis of endpoints may be done if demonstrated to be equivalent to visual inspection.

III. Quality Control

Quality control should be performed with each new lot of plates or according to the laboratory's Individualized Quality Control Plan (IQCP).

The organisms used for the dilution test are *Staphylococcus aureus* (American Type Culture Collection [ATCC] 29213), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27583), *Neisseria gonorrhoeae* (ATCC 49226), and *Haemophilus influenzae* (ATCC 49247). Note that the *S. aureus* strain differs from that used in the disk diffusion test. *E. coli* (ATCC 35218) may be used to control combinations that include clavulanic acid, a b-lactamase inhibitor (Table 17-22).

IV. Procedure

A. Preparation of Inoculum: Proceed as for the disk diffusion test (Chart 17-1).

B. Inoculation of Plates

Inoculate the bacterial suspension into each well of the microplate within 15 minutes of adjusting the density of the inoculum. One well should contain bacteria without antibiotic (growth control) and one should contain broth only (sterility control). If the volume of the inoculum exceeds 10% of the volume

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of the well, the dilution effect on the antimicrobial agent must be calculated. To prevent drying, seal the plates in a plastic bag, with plastic tape, or with a tight-fitting plastic cover. A portion of a nutrient agar plate, such as blood agar, should be inoculated to detect contamination.

C. Incubation

Incubate the plates at 35°C for 16–20 hours (24 hours when testing staphylococci against oxacillin, methicillin, or vancomycin or when testing enterococci against vancomycin) in a forced-air incubator. Do not place more than four plates in a stack, so that a uniform temperature of incubation is maintained.

V. Results

A. Interpretation

The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic that inhibits growth of the isolate. It may be determined by viewing the wells with the unaided eye, comparing the growth in the wells that contain antibiotics with that in the growth control well. A viewing mirror, with which the bottom of the microtubes may be visualized, facilitates observation. A template on which the contents of each well are detailed is useful. For a test to be valid, adequate growth must be present (diffuse turbidity or at least a 2-mm button). For trimethoprim-sulfamethoxazole, the endpoint is at least 80% inhibition of growth. A single skipped well may be ignored; if there are more skipped wells, the test should be repeated.

Refer to the guidelines for interpretation of MIC results as susceptible, dose-dependent susceptible, intermediate, or resistant.

Examine the purity plate and repeat the test if contamination is present.

VI. Bibliography

Clinical Laboratory Standards Institute. M07-A10 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard. 10th Ed. Wayne, PA: Clinical Laboratory Standards Institute, 2015.

Clinical Laboratory Standards Institute. M100-S25 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. Wayne, PA: Clinical Laboratory Standards Institute, 2015.

CHART 17-3

Gradient Diffusion Test (Etest) for Bacterial Susceptibility

I. Principle

The gradient diffusion test uses a nonporous plastic strip that has been calibrated with minimum inhibitory concentration (MIC) values that cover 15 twofold dilutions. A predefined antibiotic gradient is immobilized on the surface of the strip that is opposite the MIC scale. When transferred to agar, antibiotic diffuses from the strip, similar to the disk diffusion test. This chart summarizes the general procedure, which should be used only for combinations of antibiotic and organism that have been validated by comparison to reference methods.

II. Materials and Reagents

- **A.** Nutrient broth or 0.85% saline
- **B.** Sterile swabs (not too tightly spun)
- **C.** 0.5 and/or 1.0 McFarland standard
- **D.** Etest strips
- **E.** Etest applicator kit or forceps
- **F.** Mueller–Hinton agar plates (100 mm or 150 mm) with supplements if appropriate

III. Quality Control

is performed using strains appropriate for dilution tests (Chart 17-2).

IV. Procedure

- **A.** Allow the Etest strips to reach room temperature, approximately 30 minutes after removal from a –20°C freezer.
- **B.** Preparation of inoculum is performed as for the disk diffusion or microbroth dilution tests (Charts 17-1 and 17-2, respectively).
- **C.** Inoculate the agar plate as for the disk diffusion test (Chart 17-1).
- **D.** Allow the agar surface to dry 10–15 minutes at room temperature or in the incubator before proceeding.
- **E.** Open the Etest package and place the strip(s) in a dry Petri dish or applicator tray.

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F. Apply the strip(s) to the surface of the agar with forceps or an applicator, with the MIC scale facing up. If the strip is positioned upside down, the positioning can be corrected if the error is recognized immediately. If the strip is incubated in the inverted position, the test is invalid.

G. Incubate as appropriate for the organism being tested.

V. Results

A. After the appropriate incubation period, interpret the test only if there is adequate growth and the ellipse of inhibition is clearly visible.

B. Read the MIC where a clearly defined zone of inhibition intersects the strip. Factors to be considered when determining the MIC are discussed in the text.

C. As the antibiotic gradient is continuous (linear) and the interpretive criteria are discontinuous (twofold dilutions), round up the MIC value to the next twofold dilution when reporting the result.

VI. Bibliography

Etest Technical Guide 3B. Solna, Sweden: AB Biodisk.

CHART 18-1

Diene's Stain Procedure for Identification of Mycoplasmas

I. Principle

Mycoplasma colonies from genital cultures are easily identified by observing typical colonies on agar medium. Visualization of colony morphology is facilitated by application of Diene's stain directly to the agar surface.

Diene's stain is a nonspecific stain that imparts a contrasting appearance to *Mycoplasma* colonies on agar, allowing easier visualization of colony morphology and characteristics.

II. Reagents

A. Modified Diene's Stain (Stock Solution)

Methylene blue	2.5 g
Azure II	1.25 g
Maltose	10.0 g

Na_2CO_3	0.25 g
Distilled water	100 mL

Diene's stain working solution is prepared by diluting an aliquot of the stock solution 1:3 with distilled water.

III. Procedure

1. Flood an agar plate containing mycoplasmal growth with 1 mL of Diene's stain working solution.
2. Immediately rinse the agar surface with distilled water to remove the stain.
3. Decolorize the medium by adding 1 mL of 95% ethanol. Leave the ethanol in contact with the agar for 1 minute, and then remove. Repeat the wash step a second time.
4. Rinse the plate with distilled water and allow to dry.
5. Observe for colonies under the low power of a microscope (50× to 100×).

IV. Results

A. Interpretation

Mycoplasmas with the "fried-egg" colony morphology will stain with a dark blue center and a light blue periphery and will appear highly granular. The agar background will be clear or slightly violet.

Mycoplasmas other than *M. pneumoniae* will remain stained; *M. pneumoniae* reduces the methylene blue after a time and will become colorless. If the isolate is from the genital tract and produces typical colonies and staining, the isolate can be reported as *M. hominis*.

V. Bibliography

Velleca WM, Bird BR, Forrester FT. Laboratory Diagnosis of *Mycoplasma* Infections. Atlanta: Department of Health and Human Services, 1980.

CHART 18-2

Hemadsorption Test for Identification of *Mycoplasma pneumoniae*

I. Principle

Among the respiratory mycoplasmas, *M. pneumoniae* is the only species that will specifically absorb red blood cells. This property, therefore, provides a method for the presumptive identification of *M. pneumoniae*.

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When colony growth is noted on mycoplasma isolation media inoculated with respiratory tract specimens, a suspension of guinea pig erythrocytes is placed on the agar surface for a given time and then washed off. *M. pneumoniae* colonies will adsorb some of the red blood cells to the colony surface.

II. Reagents

A. *Mycoplasma* glucose agar with colonies thought to possibly represent *Mycoplasma* present.

B. Washed guinea pig erythrocytes (0.2%–0.4%) suspended in *Mycoplasma* broth medium.

III. Quality Control

A. Positive control: *M. pneumoniae*

B. Negative control: *Mycoplasma* species other than *M. pneumoniae*

IV. Procedure

1. Flood the surface of the agar plate with 2 mL of the red blood cell suspension.

2. Incubate the plate at 35°C for 30 minutes and periodically rotate the plate to prevent the red blood cells from settling out.

3. Wash the surface of the plate three times with 3 mL of *Mycoplasma* broth by gently rotating the plate. Remove wash fluid by aspiration with a pipette.

4. Examine the colonies at 50× to 100× magnification under a dissecting microscope.

V. Results

A. Interpretation

1. Positive test: Colonies with red blood cells adsorbed onto the surface = *M. pneumoniae*.

2. Negative: Colonies with no red blood cells adsorbed = *Mycoplasma* species, not *M. pneumoniae*.

VI. Bibliography

Manchee RJ, Taylor-Robinson D. Hemadsorption and hemagglutination by mycoplasmas. J Gen Microbiol 1968; 50:465.

CHART 18-3

Manganous Chloride–Urea Test for Identification of *Ureaplasma urealyticum*

I. Principle

Of the human mycoplasmas, *U. urealyticum* is the only species that is able to hydrolyze urea to ammonia. This property not only facilitates isolation of the organism, but also provides a rapid and convenient way to identify the species in culture. The manganous chloride ($MnCl_2$)–urea test provides a visual method for detecting ammonia formed by *U. urealyticum*.

Urease activity of *U. urealyticum* is detected by exposing the colonies to a solution of $MnCl_2$ in the presence of urea. The urease of the organism hydrolyzes urea to ammonia, and then the following reaction occurs:

$$2NH_3 + 2\ H_2O \rightarrow 2NH_4OH \rightarrow 2NH_4^+ + 2OH^-$$

$$MnCl_2 + 2OH^- \rightarrow MnO_2 + 2\ HCl$$

The manganese oxide formed in the reaction is insoluble and forms a dark brown precipitate around the colonies. This reaction is then detected under a dissecting microscope.

II. Reagents

A. $MnCl_2$-Urea Reagent

Urea (Sigma, St Louis, MO)	1.0 g
$MnCl_2$ (Fisher Scientific, Hampton, NH)	0.8 g
Distilled water	100 mL

B. Sterilize by filtration, dispense in 3-mL aliquots, and freeze at –20°C

C. Growth of suspect colonies on agar medium

III. Quality Control

A. Positive control: *U. urealyticum*

B. Negative control: *M. hominis*

IV. Procedure

1. Flood the plate with 2–3 mL of the $MnCl_2$–urea reagent.
2. After 5 minutes at room temperature, examine the colonies under 50× to 100× magnification with a dissecting microscope.

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V. Results

A. Interpretation

Colonies of *U. urealyticum* will stain dark brown. Other mycoplasmas (e.g., *M. hominis*) will remain unstained.

VI. Comments

This test works optimally on colonies that are not too close together on the agar medium. Colonies should not be older than 48 hours when the test is performed.

VII. Bibliography

Shepard MC, Howard DR. Identification of "T" mycoplasmas in primary agar cultures by means of a direct test for urease. Ann NY Acad Sci 1970;174:809.

CHART 18-4

Medium for Isolation of *Mycoplasma pneumoniae*

I. Base Reagents for Preparation of Complete Media

A. *Mycoplasma* Base Agar

This medium may be prepared using commercial PPLO agar as directed according to the package insert, or may be prepared from raw materials using the following formula:

Beef heart infusion	50 g
Peptone	10 g
NaCl	5 g
Purified agar	14 g
Distilled water	1 L

Mix together and melt the agar in a boiling water bath. Dispense in 70-mL aliquots and autoclave at 15 psi for 15 minutes. Store at 4°C.

B. Yeast Extract

1. Purchase commercial 25% yeast extract or prepare as follows: Weigh out 250 g of active bakers' yeast and place in 1 L of distilled water. Heat to boiling, cool, and filter to remove particulate matter. Adjust the pH to 8.0, and filter-sterilize.

2. Yeast extract (20 mL) is then mixed with uninactivated horse serum (10 mL) in a 2:1 ratio and frozen in 30-mL aliquots at –20°C.

3. Phenol red solution (0.4%): Phenol red (1 g) is dissolved in 3 mL of 1 N NaOH and then 247 mL of distilled water is added. Solution

is filter-sterilized and stored at 4°C to 8°C.

4. Methylene blue solution (1%): Methylene blue (1 g) is dissolved in 100 mL of distilled water and autoclaved for 15 minutes.

C. Thallium Acetate Solution (10%): Thallium acetate (10 g) is dissolved in 100 mL of distilled water and filter-sterilized.

D. Glucose Solution: Glucose (50 g) is dissolved in 100 mL of distilled water and filter-sterilized.

E. Penicillin Solution: Penicillin powder is dissolved in distilled water to achieve a concentration of 100,000 units/mL.

II. Methylene Blue-Glucose Diphasic Medium

1. Melt 70 mL of *Mycoplasma* agar in a boiling water bath. Cool to 50°C and add 30 mL prewarmed yeast extract-serum.
2. Add the following:

Methylene blue solution (1%)	0.1 mL
Phenol red solution (0.4%)	0.5 mL
Glucose solution	2.0 mL
Thallium acetate solution (10%)	0.25 mL
Penicillin solution	3.0 mL

3. Adjust the pH to 7.8 with sterile 1 N NaOH.
4. Dispense agar in 1-mL aliquots into sterile 13 × 100-mm screw-capped tubes.
5. Mix 140 mL of mycoplasma broth (A, above, without agar) with 60 mL of the yeast extract-serum. Add the following to this mixture:

Methylene blue solution (1%)	0.2 mL
Phenol red solution	1.0 mL
Glucose solution	4.0 mL
Thallium acetate solution (10%)	6.0 mL
Penicillin solution	0.5 mL

Adjust the pH to 7.8 with sterile 1 N NaOH. Dispense 2.0 mL into each of the tubes containing the solidified agar medium.

III. Mycoplasma Glucose Agar Medium

1. Melt 70 mL of mycoplasma agar in a boiling water bath. Cool to 50°C.
2. Prewarm 30 mL of yeast extract-serum and mix with the agar medium.
3. Aseptically add the following reagents:

Glucose solution	2.0 mL

Thallium acetate solution (10%)	0.25 mL
Penicillin solution	3.0 mL

4. Adjust the pH to 7.8 with sterile 1 N NaOH.
5. Dispense 6 mL of medium into 60 × 15-mm petri dishes. Allow to cool. Store media at 4°C.

IV. SP-4 Medium (also used for culture of M. pneumoniae, but mostly for culture of highly fastidious species)

A. Basal Medium

Mycoplasma Broth Base (BBL)	3.5 g/L
Bacto Tryptone (Difco)	10.0 g/L
Bacto Peptone (Difco)	5 g/L
Glucose	5.0 g/L
Deionized water	615 mL

Adjust to pH 7.5 to 7.6
Autoclave at 121°C, 15 minutes

B. Sterile Supplements

CMRL 1066 Tissue Culture Supplement with glutamine (Gibco)	50.0 mL
Fresh yeast extract (25% solution)	35.0 mL
Yeastolate (2% solution)	100.0 mL
Fetal bovine serum (heated to 56°C for 1 hour)	170.0 mL
Penicillin (100,000 U/mL stock)	10 mL
Phenol red solution (0.1%, aqueous)	20 mL

C. Final Formulation

Combine basal medium (615 mL) with supplements (385 mL) and dispense aseptically in 3-mL volumes into 4-mL, screw-capped vials.

V. Bibliography

Velleca WB, Bird BR, Forrester FT. Laboratory Diagnosis of Mycoplasma Infections. Atlanta, GA: Department of Health and Human Services, 1980.

CHART 18-5

Medium for Isolation of the Genital Mycoplasmas

A-8 Agar

This differential agar is used to differentiate *Ureaplasma* spp. from other genital *Mycoplasma* spp., including *M. hominis*. It is often used for primary plating in conjunction with a broth such as 10B broth or bromthymol blue broth.

Colonies of *U. urealyticum* will be brown and granular; colonies of *M. hominis* will have the typical larger and "fried-egg" appearance.

To prepare one liter, mix the following:

Ultrapurified (filtered) water	825 mL
$CaCl_2$ dihydride	0.15 g

(dissolve before adding the following ingredients)

TSA broth	25 g
Yeast extract	2 g
Putrescine dihydrochloride	1.7 g
DNA	0.2 g
Agar	10.5 g

Mix in flask; adjust pH to 5.5 with 2 N HCl; sterilize in autoclave, 121°C; cool in 56°C waterbath.

Supplements (filter-sterilize each separately (except serum) before adding:

Horse serum	200 mL
IsovitaleX	5 mL
10% urea	10 mL

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	1 mL
GHL tripeptide solution[a]	
2% L-cysteine (Freshly prepared)	5 mL
Penicillin	1000 IU/mL

[a] GHL tripeptide solution can be purchased from many chemical distributors, such as CalbioChem-Novabiochem.

Add supplements under sterile conditions. Adjust pH to 6.0. Pour into petri dishes; can be stored at 4°C for up to 3 months.

10B Broth

This broth is used for isolation of *M. hominis* and *Ureaplasma* spp. It is often used in conjunction with A8 Agar for primary plating. 10B broth is

also referred to as Shephard's broth when made without arginine and used specifically for isolation of *Ureaplasma* spp.

To prepare one liter, mix the following:

Ultrapurified water	825 mL
Mycoplasma broth w/o crystal violet) Base (purchase from BD or other)	14 g
Arginine	2 g
DNA	0.2 g
1% phenol red	1 mL

(Can store for 1 month)

Mix in flask; adjust pH to 5.5 with 2 N HCl; sterilize in autoclave, 121°C; cool in 56°C waterbath.

Supplements (filter-sterilize each separately [except serum] before adding):

Horse serum[a]	200 mL
25% Yeast extract	100 mL
IsovitaleX	5 mL
10% urea	4 mL
4% L-cysteine	2.5 mL

(Freshly prepared)

Adjust pH after addition of supplements to pH 5.9–6.1

Can store broth at 4°C for up to 3 months

[a]Can use fetal bovine serum in place of horse serum.

SP-4 Broth or Agar

This broth or agar medium can be used for isolation of *M. pneumoniae*, *M. hominis,* and *Ureaplasma spp*. The agar and broth can be used as a primary plating media. Supplements such as arginine, urea, and antibiotics can be added for coverage of all mycoplasmas and for inhibition of bacterial contamination. This is probably one of the most commonly used media for isolation of genital mycoplasmas.

Mix the following in a liter flask:

Ultrapurified (filtered) water	643 mL
Mycoplasma broth base w/o Crystal violet (BD)	3.5 g
Tryptone	10 g

Peptone	5.3 g
Arginine	2.0 g
1% phenol red (prepared fresh monthly)	2 mL
DNA	0.2 g
Agar (if making SP-4 agar)	15 g

Mix in flask; adjust pH to 5.5 with 2 N HCl; sterilize in autoclave, 121°C; cool in 56°C waterbath.

Supplements (filter-sterilize each separately [except serum] before adding):

Fetal bovine serum	170 mL
25% Yeast extract	35 mL
2% yeastolate (BD)[a]	100 mL
10X CMRL 1066 (Sigma)[b]	50 mL
50% glucose	10 mL
Penicillin	1,000 IU/mL

[a] Yeastolate products are animal-free and water-soluble portions of autolyzed yeast or *Saccharomyces cerevisiae*. Yeastolate is a mixture of peptides, amino acids, carbohydrates, simple and complex, as well as vitamins; distributed by BD Biosciences.

[b] CMRL-1066 is a chemically defined medium developed in the late 1950s by Connaught Medical Research Laboratories; distributed by SIGMA.

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Adjust pH after addition of supplements to pH 7.4–7.6, if media is to be used primarily for *M. pneumoniae* isolation.

Can be stored for up to 3 months at 4°C.

Bromthymol Blue Broth

This medium contains the indicator bromthymol blue instead of phenol red and is used for isolation and differentiation of *M. hominis* and *Ureaplasma* spp. The uninoculated broth is yellow. The presence of *M. hominis* is suggested if the color does not go beyond chartreuse, whereas *Ureaplasma* spp. is demonstrated by a full-green color. A blue-green color may indicate a mixture of *M. hominis* and *Ureaplasma* spp. Subculturing onto an agar media is usually needed for definitive identification. The advantage of this media is the lower concentration of urea, that reduces the likelihood of killing of the organisms in the culture, which may occur in media that afford the generation of higher urea concentrations. When the color change occurs,

the organisms are in their logarithmic phase, which is optimal for other studies.

Mix the following in a 1-L flask.

Distilled-deionized water	900 mL
Mycoplasma broth base w/o Crystal violet	21 g
Yeast extract	1 g
Bromthymol blue (0.4%, wt/vol)[a]	10 mL

[a] Dissolve powder in a minimal amount of 1 N NaOH and make up to 10 mL with water.

Mix in flask; sterilize in autoclave, 121°C; cool in 50°C waterbath.

Supplements (filter-sterilize each separately [except serum] before adding):

Horse serum, pH 6.0	100 mL
GHL tripeptide[a]	1 mL
10% urea	2.5 mL
Ampicillin	0.25 mg/mL
Nystatin	50 U/mL

[a] GHL tripeptide solution can be purchased from many chemical distributors, such as CalbioChem-Novabiochem.

Adjust pH after addition of supplements to pH 6.0–6.1

Store at −20°C until use.

Tetrazolium Reduction Test for the Presumptive Identification of *Mycoplasma pneumoniae*

CHART 18-6

Medium for Isolation of the Genital Mycoplasmas *Continued*

I. Principle

Mycoplasma pneumoniae is able to reduce the colorless compound

tetrazolium to the red-colored compound formozan. Among the large-colony mycoplasmas, this is a unique feature of *M. pneumoniae* and provides the laboratory with a rapid and easy method for identification of *M. pneumoniae* colonies directly on isolation medium.

By using enzymes that are associated with the cell membrane of the organism, *M. pneumoniae* can reduce the compound 2-(p-iodophenyl)-3-nitrophenyl-5-phenyl tetrazolium chloride to formozan, a red-insoluble compound that precipitates in the cells and renders the colony red to purple-black on prolonged exposure to the substrate.

II. Reagents

1. Isolated colonies on mycoplasma glucose agar
2. 2-(*p*-iodophenyl)-3-nitrophenyl-5-phenyl tetrazolium chloride (Aldrich Chemical)–0.21 g/100 mL distilled water. Sterilize by filtration and store in brown-glass bottle at room temperature.

III. Quality Control

A. Positive control: *M. pneumoniae*

B. Negative control: *Mycoplasma* species other than *M. pneumoniae*

IV. Procedure

1. Flood the surface of the agar plate containing the colonies of the organism with 2 mL of the tetrazolium reagent.
2. Incubate the plate at 35°C for 30 to 60 minutes (incubation for 60 minutes intensifies reactions that are already apparent at 30 minutes).
3. Examine the colonies under 50× to 100× magnification with a dissecting microscope.

V. Results

A. Interpretation

1. Positive test: Colonies of *M. pneumoniae* will visibly darken, becoming red to purple after 60 minutes. On prolonged incubation (3–4 hours), the colonies will become black.
2. Negative test: No color change will be noted after 60 minutes.

VI. Comments

Although equivalent to the hemadsorption test, tetrazolium reduction can be performed on the same plate after doing the hemadsorption procedure.

VII. Bibliography

Johnson JE, Smith TF. A simplified tetrazolium reduction test for *Mycoplasma pneumoniae*. Med Lab Sci 1976; 33:235.

CHART 19-1

Digestion and Decontamination: *N* -Acetyl-L-Cysteine–Sodium Hydroxide (NALC)

I. Principle

Mycobacteria are optimally recovered from clinical specimens when they are released from mucin, body fluids, and cells; this is commonly termed *digestion*. *N*-acetyl-L-cysteine (NALC) is a mucolytic agent that in concentrations of 0.5%–2% can rapidly digest tenacious sputum and other mucoid specimens within 2 minutes. Growth of mycobacteria in culture media is also enhanced when the concentration of competing organisms is minimal; this is commonly termed *decontamination*. The addition of 4% sodium hydroxide to the digestion mixture to reach a final concentration of 2% is the most commonly used decontaminating agent.

II. Specimen

A. Type of Specimen

Any specimen that is mucoid in consistency or is known to be contaminated with bacteria should undergo digestion and decontamination. Included are all respiratory specimens, gastric lavage fluid, stools, urine (when Gram's stain shows the presence of bacteria), postmortem tissue, and biopsy samples.

B. Patient Preparation

When collecting specimens through a contaminated area, such as collection of expectorated sputum, every effort should be made to cleanse or decontaminate the area. For example, before patients are asked to produce expectorated sputum, they should rinse out their mouth two or three times with water or a proprietary mouth wash. If a biopsy is to be taken, then the skin adjacent to a biopsy site should be carefully cleaned with iodine followed by an alcohol swab.

C. Special Collection Procedures

In patients in whom pulmonary tuberculosis is suspected and who cannot produce adequate sputum, induced sputum should be collected. In instances in which diagnosis has been difficult to establish, invasive procedures, such as bronchial brush biopsies, bronchial washings, or bronchoalveolar lavage, may be required.

D. Special Handling Conditions

All specimens submitted for the culture of mycobacteria must be handled in a properly functioning biohazard hood. Care must also be

taken in the preparation, examination, and storage of stained smears, as all acid-fast bacilli may not be killed in the fixation and staining procedure. Care must be taken not to produce aerosols when subcultures are made, when specimens are centrifuged, and when various biochemical tests are performed.

III. Materials

A. Equipment

1. Centrifuge with aerosol-free centrifuge cups
2. Vortex mixer

B. Supplies

1. Discard container containing disinfectant solution
2. Sterile 50-mL conical polypropylene screw-capped tubes (aerosol-free and graduated)
3. 10 mL sterile transfer pipette
4. Clean, unscratched, 1" × 3" microscope slides

C. Reagents

1. *N*-acetyl-L-cysteine (NALC) digestant-decontaminant: TB base digestant, 50 mL (available commercially)
2. TB phosphate buffer: 0.067 M, pH 6.8, 250 mL (commercially available)
3. *N*-acetyl-L-cysteine (NALC): Mucomyst, 20% solution (commercially available)

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IV. Quality Control

The percentage of clinical specimens that grow bacterial contaminants in TB cultures following the digestion and decontamination procedure should average about 3%. A contamination rate significantly less than 3% may indicate overly harsh decontamination, and the concentration of NaOH should be reduced. A contamination rate significantly greater than 5% suggests either too weak a decontamination or incomplete digestion. Increasing the concentration of NaOH or the time of exposure, or both, may correct this problem.

V. Procedure

1. Add 1.5 mL NALC–50 mL TB base digestant to make a working solution.
2. Add a volume of working NALC–NaOH solution (no more than 24 hours old) equal to the volume of specimen in a sterile 50-mL conical polypropylene screw-capped centrifuge tube. If more than 20 mL of specimen is submitted, use a sterile transfer pipette to select 10 mL of

the most purulent blood, or mucoid portion. Secure the cap tightly.

3. Agitate the tube on a vortex mixer for not more than 30 seconds. Be sure to move the tube so that the NALC–NaOH comes in contact with the entire inner surface of the tube.
4. Let the tube stand for 15 minutes at room temperature to decontaminate the specimen.
5. Dilute the mixture to the 50-mL mark with either sterile distilled water or sterile 0.067 phosphate buffer (pH 6.8). This will reduce the continued action of the NaOH and lower the viscosity of the mixture.
6. Recap the tube tightly and invert several times to mix the contents.
7. Centrifuge the tube at 3000 *g* for 15–20 minutes. Use aerosol-free sealed centrifuge cups.
8. Pour the supernatant into a splash-proof discard container filled with disinfectant.
9. Use a sterile 3-mm-diameter bacteriologic loop or the end of two sterile wooden applicator sticks to remove a portion of the sediment, and prepare a smear over an area 1 × 2 cm on a new, clean, unscratched glass slide.
10. Suspend the remaining sediment in 1–2 mL of TB buffer or sterile distilled water if media are to be inoculated immediately. Otherwise, suspend the sediment in 1–2 mL of sterile 0.2% bovine albumin fraction V. If the amount of the sediment is very small, reconstitute the sediment in 1 mL only.
11. Add several drops to at least one solid medium (Löwenstein–Jensen slant or Middlebrook agar plate).
12. Inoculate a liquid medium according to the manufacturer's directions.

VI. Results

Not applicable

VII. Procedure Notes

1. Excessive agitation of the NALC–NaOH solution should be avoided because NALC is unstable in the presence of oxygen.
2. Bovine serum albumin serves to buffer the inoculum and enhance adhesion of the sample to solid media.
3. Sterile water only should be used: Tap water may contain environmental mycobacteria that can confuse observations of stained smears and contaminate culture media.

VIII. Limitation of Procedure

The concentration of NaOH and the time of exposure of specimen to reagent during the decontamination procedure must be carefully controlled to avoid undue loss of mycobacterial cells during this procedure.

IX. Bibliography

Kubica GP, Dye WE, Cohn ML, et al. Sputum digestion and decontamination with *N*-acetyl-L-cysteine-sodium hydroxide for culture of mycobacteria. Am Rev Respir Dis 1963;87:775–779.
Strong BE, Kubica GP. Isolation and Identification of *Mycobacterium tuberculosis:* A Guide for the Level II Laboratory. Atlanta, GA: Centers for Disease Control, 1976. Public Health Service publication no. 81-8390.

CHART 19-2

Carbol Fuchsin Stains

I. Principle

The mycolic acids within the lipid-rich, waxy cell wall of mycobacteria have the unique capability of binding fuchsin dye so that it is not destained by acid–alcohol. The Ziehl–Neelsen stain is known as a "hot stain," as heat is used for the dye to penetrate through the cell wall. The Kinyoun stain is known as a "cold stain" because the high concentration of phenol in the reagent serves to "dissolve" the lipid material in the cell wall, allowing penetration of the carbol fuchsin dye without the use of heat. Once stained, the cell wall holds the dye "fast," giving the characteristic red color.

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II. Specimen

A smear is prepared either directly from the specimen following concentration or from a suspension of organisms obtained from colonies grown on primarily culture media.

III. Materials

A. Equipment: 3" × 1" glass slides and reagents
B. Media: Not applicable
C. Reagents
  1. Carbolfuchsin (commercially available)
  2. Acid–alcohol (commercially available)
  3. Methylene blue counterstain (commercially available)

IV. Quality Control

A. Unstained positive control smears (*M. gordonae* or another

nontuberculous mycobacteria) and negative control smears (*Nocardia* spp. or any aerobic bacterium such as *E. coli*) have been prepared.

B. Control smears are reviewed before the patient smears are read to confirm that the mycobacteria stain acid-fast:

1. Positive: *Mycobacterium* spp.; magenta red against blue background
2. *Nocardia* or other bacterium: no acid-fast cells; background blue

C. If control slide is unacceptable, review procedures and reagent preparation. Unacceptable control slides include the following:

1. Bacilli in positive control do not stain red.
2. Negative control remains red after destaining.
3. Background is not properly decolorized.

V. Procedure

A. Ziehl–Neelsen

1. Place a 2 × 3-cm filter paper strip on the slide to help hold the stain on the slide and filter undissolved crystals.
2. Flood the paper strip with Ziehl–Neelsen carbol fuchsin.
3. Using a Bunsen burner or electric staining rack, heat the slide slowly just before steaming (i.e., short of boiling).
4. Stain for 5 minutes. If the stain dries, add more stain without heating.
5. Carefully remove the paper slip from the slide using forceps.
6. Rinse the slide thoroughly with water.
7. Flood the smear with 3% acid–alcohol decolorizer for 2 minutes.
8. Rinse with water and drain excess water.
9. Flood the slide with methylene blue counterstain for 1 minute. Do not blot.
10. Examine the smear with a 100× oil-immersion objective.

B. Kinyoun

1. Heat-fix smears on slide warmer at 72°C–75°C.
2. Cover the smear with the Kinyoun's carbol fuchsin reagent. A small 2" × 3" rectangular piece of filter paper can be placed over the smear and the stain applied to the filter paper to avoid spilling over the sides of the smear. Do not steam (i.e., this method does not involve heating).
3. Stain for 5 minutes.
4. Remove the filter paper with forceps, and rinse the stain off the slide with distilled water.
5. Decolorize for 3 minutes with acid–alcohol or until no red stain appears.
6. Flood smear for 3–4 minutes with methylene blue counterstain.

7. Rinse dry and examine under a 100× oil-immersion objective.

VI. Results

Examine smears with a 100× oil-immersion objective and report as follows:

Smear Observation	Report
1 or 2 per 300 fields (3 sweeps)	(Questionable, repeat and report the result of the repeat)
1–9 per 100 fields	Rare, 1+
1–9 per 10 fields	Few, 2+
1–9 per immersion field	Numerous, 3+
9 per immersion field	4+

VII. Procedure Notes

1. Avoid underdecolorization with acid–alcohol. Organisms that are truly acid-fast are difficult to overdecolorize.
2. Avoid making thick smears, which may interfere with proper decolorization.

3. If an inordinate number of smear-positive, culture-negative results are encountered, check water reservoirs for contaminating acid-fast organisms. Also check for the transfer of material from slide to slide and from immersion oil are other possible sources of false-positive smear results.
4. Strong counterstains may mask the presence of acid-fast bacilli. Brilliant green may have a slight advantage over methylene blue as a counterstain.

VIII. Limitation of Procedure

1. Acid-fast smears do not stand alone, and final interpretation must always be correlated with culture results.
2. Organisms other than mycobacteria may demonstrate various degrees of acid-fastness: *Rhodococcus* spp., *Nocardia* spp. *Legionella micdadei* and the cysts of *Cryptosporidium* spp. Use differences in smear morphology and correlation with culture appearance and other biochemical tests.
3. Rapidly growing mycobacteria may stain poorly or not at all. The Ziehl–Neelsen stain may provide better staining than the Kinyoun method for weakly acid-fast organisms.

IX. Bibliography

Kent PT, Kubica GP. Public Health Mycobacteriology: A Guide for the Level III Laboratory. Atlanta, GA: Centers for Disease Control, 1985. Department of Health and Human Services Publication no. 86-21654.

CHART 19-3

Fluorescent Stain: Auramine O; Auramine–Rhodamine

I. Principle

When stained with this method acid-fast organisms emit a bright yellow fluorescence when activated with a short-wave ultraviolet light. The potassium permanganate counterstain causes the nonspecific background debris to fluoresce a pale yellow, in contrast with the bright yellow appearance of acid-fast bacilli.

II. Specimen

Smear prepared either directly from the specimen or from a suspension of organisms obtained from colonies grown on primary culture media.

III. Materials

A. Equipment

1. 3" × 1" glass slides

B. Reagents

1. Auramine O

a. Solution 1: Dissolve 1.5 g of Auramine O in 10 mL of 95% ethanol.

b. Solution 2: Dissolve 3.0 g of phenol crystals in 87 mL of distilled water

c. Working solution: Combine solutions 1 and 2. Label with name of reagent and dates of preparation and expiration. Shelf life is 3 months if stored in a brown bottle.

2. Auramine–Rhodamine

a. Solution 1: Dissolve 1.5 g of Auramine O and 0.75 g of rhodamine B in 75 mL of glycerol

b. Solution 2: Mix 10 mL of phenol with 50 mL distilled water

c. Working solution: Combine solutions 1 and 2 and mix for 24 hours with a magnetic stirrer. Filter stain through glass wool and store in a glass bottle. Affix a label with the name of the reagent and dates of preparation and expiration. Shelf life is 3 months if

stored in a brown bottle.

3. Permanganate Counterstain: 0.5%
   a. Dissolve 0.5 g of potassium permanganate in 100 mL distilled water.
   b. Affix a label with the name of the reagent and dates of preparation and expiration.
4. 0.5% Acid–Alcohol Decolorizer.
   a. Add 0.5 mL of concentrated hydrochloric acid to 100 mL of 70% ethanol.
   b. Affix label with name of reagent and dates of preparation and expiration.
   c. Shelf life is 3 months when stored at room temperature.

C. Special Supplies

1. Fluorescence microscope fitted with a BG-12 or 52112 primary filter and an OG-1 barrier filter.

D. Biosafety Instructions

1. Handle all slides carefully. Heat-fixing may not kill all of the mycobacteria. Dispose of all slides in a biohazard container.
2. Heat-sterilize bacteriologic loops in the electric incinerator contained in the biologic safety hood.
3. Perform all operations in a biosafety hood. Place a paper towel soaked with disinfectant under the field of operation. Thoroughly clean all work surfaces with a disinfectant solution before and after use.

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4. Carcinogenic
5. Work under a fume the hood (i.e., the type of hoods used in the special chemistry laboratory).

IV. Quality Control

A. Control Materials to be Used

A positive and negative control smear for mycobacteria is included with each run of stains. Control slides are reviewed before the patient smears are read to confirm that the positive control contains mycobacteria that fluoresce and that the negative smear has no fluorescing cells.

1. Positive control: *Mycobacterium* spp.; bacilli with yellow to orange fluorescence.
2. Negative control: A non–acid-fast bacterium (e.g., *E. coli*); no fluorescing cells.

If either of the control smears is unacceptable, reagent preparations and

procedure are checked.

V. Procedure

1. Heat-fix smears on slide warmer at 72°C–75°C.
2. Flood surface of the smear with Auramine O stain. Let stand at room temperature for 15 minutes.
3. Rinse with tap water. Drain.
4. Decolorize with 0.5% acid alcohol for 2 minutes.
5. Rinse with tap water. Drain.
6. Flood smear for 2 minutes with 0.5% potassium permanganate counterstain.
7. Rinse with tap water. Drain.
8. Air dry. *Do not blot.*
9. Observe the smear as soon as possible using the fluorescence microscope. If there is a delay, refrigerate the smears at 4°C in the dark.

VI. Results

A. Interpretation (Readout or Calculations)

Acid-fast bacilli (AFB) are 1–10 mm long, and appear as slender, rod-shaped bacilli emitting a bright yellow fluorescence against the pale yellow background. First, scan the smears briefly with the 25× objective; move to the 63× objective to confirm the bacterial morphology of any suspicious fluorescing forms. Thoroughly examine each slide for the presence of AFB. Mycobacteriologists recommend that a minimum of 100 fields be examined before a smear is reported as negative. Three passes along the long axis of the slide or nine passes along the short axis usually provide ample area for reading this technique minimizes the possibility of reading the same area more than once.

B. Reporting Format

The American Lung Association has recommended the following method for fuchsin stains, also applicable to fluorescent stains:

Number of Bacilli	Report
0	No AFB seen
1–2 on entire smear	Report number seen and ask for repeat
1–9 per 10 fields	Rare or 1+
1–9 per field	Few or 2+
10–90 per field	Numerous or 3+
>90 per field	4+

VII. Procedure Notes

Fluorochrome-stained smears may be directly restained with any of the carbol fuchsin staining procedures. This is done to confirm positive slides

and to study the morphology of the organisms present.

VIII. Limitation of Procedure

Organisms other than mycobacteria, including *Rhodococcus* spp., *Nocardia* spp., *Legionella micdadei,* and the cysts of *Cryptosporidium* and *Cystoisospora* spp. may demonstrate varying degrees of acid-fastness. These can often be suggested by differences in morphology; recovery of contaminating bacteria in subcultures may be necessary in equivocal cases.

Rapidly growing mycobacteria may vary in their abilities to retain acid-fast dyes. It is thought that some of these will not fluoresce or not fluoresce well in fluorochrome-stained smears. A carbol fuchsin procedure is recommended for these microorganisms.

Dead AFB may still stain positive in fluorescent stains, a feature that must be considered when using smears to assess efficacy of treatment.

IX. Bibliography

Joseph SW, Vaichulis EM, Houk VN. Lack of Auramine Rhodamine fluorescence of Runyon Group IV mycobacteria. Am Rev Respir Dis 1967;95:114–115.

Pollock HM, Wieman EJ. Smear results in the diagnosis of mycobacteriosis using blue light fluorescence microscopy. J Clin Microbiol 1977;5:329–331.

Uribe-Botero G, Prichard JG, Kaplowitz HJ. Bone marrow in HIV infections: a comparison of fluorescent staining and culture in the detection of mycobacteria. Am J Clin Pathol 1989;91:313–315.

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CHART 19-4

Arylsulfatase

I. Principle

Arylsulfatase is an enzyme that splits free phenolphthalein from the tripotassium salt of phenolphthalein disulfite. The test for the identification of *Mycobacterium* species is performed in a tube containing a substrate of phenolphthalein in oleic acid agar. After 3 (or 14) days of incubation of a subculture of the unknown species, the appearance of a pink color after addition of sodium carbonate indicates a positive reaction.

II. Specimen

Mature colony of the unknown *Mycobacterium* species recovered from clinical material, grown on a solid medium. Prepare a suspension of the organism in sterile water and incubate for 3 days (14 days).

III. Materials

A. Equipment

1. Biologic safety cabinet

B. Media

1. Arylsulfatase stock substrate

a. Dissolve 2.6 g of phenolphthalein disulfate tripotassium salt in 50 mL of sterile deionized water.

b. Sterilize by membrane filtration (0.22-mm–pore size filter)

c. Store in the refrigerator at 2°C–8°C

d. Shelf life is indefinite if stored properly. Discard if solution becomes cloudy.

2. Arylsulfatase broth: 3-day test

a. Aseptically add 2.5 mL of stock substrate to 200 mL of sterile Dubos Tween broth.

b. Aseptically dispense 2.0 mL amounts into screw-capped test tubes (16 × 125 mm)

c. Store at 2°C–8°C. Discard if the solution becomes cloudy.

3. Arylsulfatase broth: 2-week test

a. Aseptically add 7.5 mL of stock substrate to 200 mL of sterile Dubos Tween broth.

b. Aseptically dispense 2.0 mL amounts into screw-capped test tubes (16 × 125 mm).

c. Store at 2°C–8°C.

Wayne's arylsulfatase agar is commercially available from several sources.

C. Reagents

1. 2 N sodium carbonate (Na_2CO_3): Dissolve 10.6 g of anhydrous sodium carbonate in 100 mL of distilled water.

IV. Quality Control

A. Three-Day Test

1. Positive control: *Mycobacterium fortuitum* American Type Culture Collection (ATCC) 6841 (if positive control is negative, repeat tests with a fresh subculture of the positive organism. If the negative controls are positive, repeat the tests with a new lot of media).

2. Negative control: *Mycobacterium intracellulare* ATCC 13950

3. Uninoculated medium and reagent only: No color

B. Fourteen-Day Test

1. Positive control: *Mycobacterium fortuitum* ATCC 23292

2. Negative control: *Mycobacterium intracellulare* ATCC 13950
3. Uninoculated medium and reagent only: No color

V. Procedure

1. Inoculate each tube of substrate with a slightly turbid suspension of the test organism in sterile water. Thoroughly emulsify the culture in the broth.
2. Incubate tube for 3 days or 14 days (2-week test) at 35°C in the non-CO_2 incubator
3. Following incubation, add 1 mL of the 2 N sodium carbonate reagent, mix, and observe for color change.

VI. Results

A. Interpretation

Visually observe for a color change from pale pink to deep red. The lack of color change indicates a negative reaction.

VII. Procedure Notes

Include an actively growing control organism with each batch of test. For slow growers, use a 3- to 5-week subculture; for rapid growers, a 1- to 3-week subculture. If the positive control is negative, repeat the tests with a fresh subculture of the positive organism. If the negative controls are positive, repeat the tests with a new lot of media.

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VIII. Limitation of Procedure

A. False-negative results can be due to control cultures that are too old.

B. False-positive results may occur if medium contains free phenolphthalein.

IX. Bibliography

Kubica GP, Ridgon AL. The arylsulfatase activity of mycobacteria. III. Preliminary investigation of rapidly growing mycobacteria. Am Rev Respir Dis 1961;83:737–740.

Kubica GP, Vestal AL. The aryl sulfatase activity of acid-fast bacilli. I. Investigation of stock cultures of mycobacteria. Am Rev Respir Dis 1961;83:728–732.

Wayne LG. Recognition of *Mycobacterium fortuitum* by means of a three-day phenolphthalein sulfatase test. Am J Clin Pathol 1961;36:185–187.

CHART 19-5

Assessment of Photoreactivity of Mycobacteria

I. Principle

The appearance of yellow pigment in the colonies of photochromogenic mycobacteria is the result of yellowish-orange carotene crystals that are produced by actively metabolizing microorganisms after exposure to bright light. Scotochromogenic species have the capability of producing yellow pigment without exposure to light; however, the type of pigment is unknown. The pigmentation of young colonies of mycobacteria after growth in the dark or following exposure to light can be an important aid in the identification of certain *Mycobacterium* species.

II. Specimen

A primary broth culture of the test organism, diluted sufficiently to produce isolated colonies when inoculated to agar culture

III. Materials

A. Equipment
- **1.** Biologic safety cabinet
- **2.** 37°C incubator

B. Supplies
- **1.** Sterile screw-capped test tubes, 20 × 110 or 20 × 125 mm
- **2.** Sterile Pasteur pipettes
- **3.** Inoculating wires and loops

C. Medium
- **1.** Three slants of Löwenstein–Jensen medium
- **2.** Three Middlebrook 7H10 agar plates

IV. Standards and Controls (Include Controls With Each Batch of Tests)

A. Positive photochromogen: *M. kansasii* ATCC 12478

B. Positive scotochromogen: Stock strains of *M. scrofulaceum* or *M. gordonae*

C. Negative chromogen: *M. tuberculosis* ATCC 25177

V. Procedure

1. Inoculate the surfaces of three Löwenstein–Jensen slant media or three Middlebrook 7H11 agar plates with fluid from a dilute broth culture of the organism to be tested. Wrap two of the tubes or plates with aluminum foil; leave the third exposed to the ambient light in the incubator.
2. Incubate one of the wrapped tubes or plates at 25°C–30°C; the other wrapped tubes or plates at 37°C.
3. Several days after growth is noted in the light-exposed control tube or plate, examine the wrapped tubes or plates for growth.

4. If early growth is detected in the wrapped tubes or plates, expose one of each pair to a strong light for approximately 5 hours. A 100-W tungsten bulb or fluorescent equivalent is adequate. Loosen the cap of the culture tube during this period of light exposure.
5. Following exposure to light, the tube or plate is returned to the incubator and inspected after 24–48 hours for the appearance of yellow pigment.

VI. Results

A. Interpretation

Mycobacteria that are scotochromogenic produce an equal amount of pigment whether light-exposed or left in the dark. *M. scrofulaceum, M. gordonae, M. flavescens, M. xenopi,* and *M. szulgai* (the latter is scotochromogenic only when incubated at 37°C) are examples of scotochromogens.

Mycobacteria that are photochromogenic produce yellow pigment only after exposure to light. The more commonly encountered photochromogens include *M. kansasii, M. marinum, M. simiae,* and *M. asiaticum.*

Nonchromogenic mycobacteria are incapable of producing pigment either in the dark or after exposure to light. *M. tuberculosis, M. bovis, M. ulcerans,* and classical strains of *M. avium* are the more commonly encountered nonchromogens.

VII. Procedure Notes

When *M. szulgai* is suspected, the light-exposed tubes or plates should be incubated at 25°C for 3–5 days. If incubated at 37°C, the photochromogenic potential will be masked by the development of the scotochromogenic pigment at that temperature.

VIII. Limitation of Procedure

Regarding *M. avium,* although the classical strain is a nonchromogenic mycobacteria, there are strains that are slightly pigmented yellow in the light and dark (i.e., scotochromogenic).

IX. Bibliography

Wayne LG, Doubek SR. The role of air in the photochromogenic behavior of *M. kansasii.* Am J Clin Pathol 1964;42:431–435.

CHART 19-6

Catalase 68°C and Semiquantitative Catalase

I. Principle

Catalase splits hydrogen peroxide into water and oxygen. The evolution of oxygen appears as bubbles. Some forms of catalase are inactivated by heating at 68°C for 20 minutes, a valuable identifying feature for certain *Mycobacterium* species. The hydrogen peroxide used for the identification of *Mycobacterium* species differs from that used to detect catalase in other types of bacteria, by using a 30% concentration (Superoxol) in a strong detergent solution (10% Tween 80). The detergent helps disperse the hydrophobic tightly clumped mycobacteria from large aggregates to individual bacilli, maximizing the detection of catalase.

II. Specimen

Mature colony of the unknown *Mycobacterium* species recovered from clinical material, grown on a solid medium.

III. Materials

A. Equipment
- **1.** 68°C water bath or heat block

B. Media
- **1.** Middlebrook 7H9 broth
- **2.** Löwenstein–Jensen deeps in 25- × 150-mm screw-capped test tubes

C. Reagents
- **1.** 30% hydrogen peroxide (commercially available as Superoxol)
- **2.** 10% Tween 80
- **3.** M/15 phosphate buffer (0.067 M)

IV. Quality Control

A. Negative control: *M. tuberculosis* American Type Culture Collection (ATCC) 15177, bubbles at 22°C–25°C, but not at 68°C.

B. Positive control: *M. fortuitum* ATCC 6841, bubbles at 22°C–25°C and at 68°C.

C. Controls are included with each batch of tests.

V. Procedure

A. Heat-Stable Catalase Test
- **1.** Set up one screw-capped tube per organism specimen to be tested in sterile water.
- **2.** Label each tube with the number of the specimen.

3. Add 0.5 mL of sterile 0.067 M phosphate buffer to each tube.
4. Inoculate the buffer with a 2–3 heaping loopfuls of growth from an actively growing subculture of the organism to be tested (2–4 weeks old).
5. Thoroughly emulsify the culture in the buffer.
6. Incubate the tubes in a 68°C water bath for exactly 20 minutes.
7. Remove the tubes from the water bath. Cool to room temperature.
8. Add 0.5 mL of freshly prepared Tween 80–hydrogen peroxide reagent.
9. Allow the tubes to sit at room temperature for 20 minutes. Do not shake the tubes.
10. Visually observe for the evolution of bubbles.

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B. Semiquantitative Catalase Test

1. Inoculate a liquid medium, such as Dubos Tween broth or Middlebrook 7H9 broth with a loopful of the culture to be tested. Include controls.
2. Incubate for 7 days at 37°C.
3. Mix for 5–10 seconds on a vortex mixer.
4. Transfer six drops to a Löwenstein–Jensen deep (prepared in 25- × 150-mm tubes).
5. Incubate the deeps for 14 days at 37°C. Be sure caps are loose.
6. Add 1 mL of freshly prepared Tween 80–hydrogen peroxide reagent. Avoid contact of reagent with skin.
7. Allow tests to sit at room temperature for 5 minutes.
8. Measure the column of bubbles.

VI. Results

A. Interpretation (Readout or Calculations)

1. Heat-stable catalase test: The appearance of bubbles indicates a positive test; lack of bubbles is a negative reaction. *M. tuberculosis* and other mycobacteria lose their catalase activity when heated to 68°C.
2. Semiquantitative catalase test:
   High catalase reaction: >45 mm of foam
   Low catalase reaction: <45 mm of foam

VII. Procedure Notes

Failure of control cultures to give expected results is usually due to the use of cultures that are too old—use only actively growing cultures.

VIII. Limitation of Procedure

False-negative results can be due to use of cultures with inadequate growth, the use of cultures that are too old, or reagents that are outdated. Inadequate growth may result from an inadequate inoculum or from screwing lids too tightly.

False-positive results may occur if the culture contains catalase-positive, contaminating bacterial species.

IX. Bibliography

Kubica GP, Jones WD Jr, Abbott VD, et al. Differential identification of mycobacteria. I. Tests on catalase activity. Am Rev Respir Dis 1966;95:400–405.

CHART 19-7

Growth on MacConkey Agar

I. Principle

The ability to grow on special MacConkey agar, formulated without crystal violet, differentiates *M. fortuitum*, *M. chelonae,* and *M. abscessus*, which can grow within 5 days, from other rapidly growing mycobacteria that show only slight growth after 11 days.

II. Specimen

A mature colony of the unknown *Mycobacterium* species recovered from clinical material, grown on a solid medium is tested.

III. Materials

A. Equipment: None
B. Media: MacConkey agar without crystal violet (commercially available)
C. Reagents: None
D. Supplies: None

IV. Quality Control

A. Positive control: *M. fortuitum* American Type Culture Collection (ATCC) 6841
B. Negative control: *M. phlei* ATCC 11758; uninoculated medium and reagent only

V. Procedure

1. Inoculate a fresh MacConkey plate with three drops of the organism grown for 7–10 days in 7H9 broth and streak for isolation.
2. Incubate at 28°C–30°C for 11 days without CO_2.

3. At 5 and 11 days, visually observe the surface of the agar plate for growth.

VI. Results

A. Interpretation

A positive test for *M. fortuitum* will show growth along the entire streak area and possibly a color change in the medium in 5 days. The absence of growth indicates a negative test.

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VII. Procedure Notes

Include controls with each batch of tests. If results are not as expected, repeat the test with fresh control organisms and a new lot of media.

VIII. Limitation of Procedure

About 25% of *M. smegmatus* strains grow on MacConkey agar without crystal violet. To obviate any diagnostic difficulties, the 3-day arylsulfatase test may be used to separate *M. smegmatus* (negative) from *M. fortuitum, M. chelonae,* and *M. abscessus* (positive).

IX. Bibliography

Jones WD, Kubica GP. The use of MacConkey agar for differential typing of *M. fortuitum.* Am J Med Technol 1946;30:182–195.

CHART 19-8

Inhibition by Thiophene-2-Carboxylic Acid Hydrazide (T2H, 1 mg/mL)

I. Principle

Thiophene-2-carboxylic acid hydrazide has the property of inhibiting *Mycobacterium bovis,* but not other species of mycobacteria, a helpful feature differentiating *M. bovis* from *M. tuberculosis.*

II. Specimen

A. 3- to 4-week-old culture of the test organism growing on a solid medium.

III. Materials

A. Equipment

1. Biologic safety cabinet

2. 10% CO_2 incubator at 37°C

B. Supplies

1. Sterile applicator sticks
2. Sterile 9.0 mL water blanks
3. Sterile deionized water
4. Quadrant petri dishes

C. Media

1. Thiopene-2-carboxylic acid hydrazide (T_2H) (also known as TCH) susceptibility medium (10 mg/mL)
   - **a.** Make two 200-mL batches of Middlebrook 7H10 medium in separate flasks.
   - **b.** Sterilize by autoclaving. Cool to 50°C in a water bath.
   - **c.** Pipette 5.0 mL of cooled medium from one of the flasks into quadrants I and III of a quadrant petri dish.
   - **d.** Add 2.0 mL of a 1,000 mg/mL TCH stock solution into the remaining flask (final concentration of 10 mg/mL). Swirl well to mix.
   - **e.** Dispense 5.0 mL of this medium into quadrants II and IV of each plate.
   - **f.** Protect the plates from light by covering with brown paper. Store plates at 2°C–8°C; shelf life is approximately 1 month.

IV. Quality Control

A. Positive control: *M. bovis* American Type Culture Collection (ATCC) 35734 (susceptible to TCH at 10 mg/mL)

B. Negative control: *M. tuberculosis* ATCC 25177 (resistant)

V. Procedure

1. Prepare a suspension of the unknown mycobacterium in sterile water equivalent to a McFarland 1 standard.
2. Dilute to make a suspension of 10^3–10^4 in sterile deionized water.
3. Inoculate three drops of each dilution onto a control quadrant and a TCH quadrant on the quadrant plates.
4. Cover the plates with brown paper to prevent exposure to light, letting the plates sit at room temperature to allow adsorption of the inoculum.
5. Place each plate into a CO_2-permeable zipper bag, invert the plates, and incubate at 37°C in 8%–10% CO_2 for 3 weeks.
6. After incubation, count the colonies on the control quadrant and on the quadrant that contains the TCH at 10 mg/mL.

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VI. Results

A. Calculate the percentage of resistance as follows:

Number of colonies on quadrant containing TCH × 100 = percent of colonies resistant on control quadrant.

B. Record the test culture as resistant to TCH if the derived number is higher than 1%.

VII. Bibliography

Harrington R, Karlson AG. Differentiation between *M. tuberculosis* and *M. bovis* by *in vitro* procedures. Am J Vet Res 1967;7:1193–1196.

Wayne LG, Engel HWB, Grassi C, et al. Highly reproducible techniques for use in systematic bacteriology in the genus *Mycobacterium:* tests for niacin and catalase and for resistance to isoniazid, thiophene 2-carboxylic acid hydrazide, hydroxylamine and *p*-nitrobenzoate. Int J Syst Bacteriol 1976;26:311–318.

CHART 19-9

Iron Uptake

I. Principle

The ability to take up iron from an inorganic iron-containing reagent helps to differentiate *Mycobacterium fortuitum* and *M. phlei* (positive) from *M. chelonae* and *M. abscessus* which do not take up iron.

II. Specimen

Mature colony of the unknown *Mycobacterium* species recovered from clinical material, grown on a solid medium.

III. Materials

A. Equipment

1. Biologic safety hood
2. 28°C–30°C incubator
3. Sterile Pasteur pipettes

B. Medium

1. Löwenstein–Jensen slants (commercially available)

C. Reagents

20% ferric ammonium citrate

1. Add 20 g of ferric ammonium citrate to 100 mL of deionized water

2. Swirl to dissolve
3. Autoclave for 15 minutes at 121°C
4. Store in the refrigerator at 2°C–8°C

IV. Quality Control

A. Positive control: *M. fortuitum,* American Type Culture Collection (ATCC) 6841

B. Negative control: *M. chelonae,* ATCC 35751; uninoculated medium and reagent only

V. Procedure

1. Use an actively growing (1- to 2-week-old) subculture of a rapidly growing mycobacterium on a Löwenstein–Jensen slant.
2. Add as many drops of sterile 20% ferric ammonium citrate as there are milliliters of medium in the slant. For example, for 8 mL of medium, add eight drops of ferric ammonium citrate.
3. Reincubate the slant at 28°C–30°C for up to 3 weeks.
4. Visually examine the surface of the slants for the presence of a dark, rust brown color.

VI. Results

The color of a truly positive iron uptake test will be very dark rust. A positive reaction seen in a rapidly growing strain of mycobacteria is indicative of *M. fortuitum.*

VII. Procedure Notes

If the results are not as expected, repeat the test with new ferric ammonium citrate reagent and new control cultures.

VIII. Limitation of Procedure

Some isolates of *M. chelonae* will turn tannish brown, a color sometimes referred to as "turtlelike," giving a false-positive reaction. Look for a very dark rust color.

IX. Bibliography

Silcox VA, Good RA, Floyd MM. Identification of clinically significant *Mycobacterium fortuitum* complex. J Clin Microbiol 1981;14:686–691.

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CHART 19-10

Niacin Accumulation

I. Principle

All *Mycobacterium* species produce niacin ribonucleotide; however, virtually all strains of *M. tuberculosis* and *M. simiae* and some strains of *M. chelonae* lack the enzymes to further convert niacin to nicotinamide adenine dinucleotide (NAD). Niacin accumulates in the culture medium, from which it can be extracted with sterile water or physiologic saline. The extract is placed in a small test tube to which is added a reagent-impregnated niacin filter strip.

II. Specimen

A longer than 3-week-old heavy growth on Löwenstein–Jensen of the organism is needed for testing. Growth on other culture media does not produce sufficient niacin to produce a positive test reaction by this method.

III. Materials

A. Equipment
1. Biologic safety cabinet
2. 37°C incubator

B. Supplies
1. Sterile screw-capped test tubes, 20 × 110 or 20 × 125 mm
2. Sterile Pasteur pipettes
3. Sterile 5 mL pipettes

C. Reagents
1. Niacin filter paper strips (commercially available)
2. Sterile H_2O

IV. Standards and Controls (Include Controls with Each Batch of Tests):

A. Positive control: *M. tuberculosis* American Type Culture Collection (ATCC) 25177

B. Negative control: *M. intracellulare* ATCC 13950

V. Procedure

1. Flood the surface of the Löwenstein–Jensen slant over the heavy growth of the test organism with 1 mL sterile H_2O. Stab the medium with the tip of the pipette to allow access of the water to the underlying medium.
2. Tilt the tube so the water covers the surface of the slant. Let stand for 20–30 minutes.
3. Rotate the tube so that the slant faces downward. Carefully remove 0.6 mL of extract without touching the slant and transfer to the screw-capped test tube.
4. Using a pair of forceps, drop a niacin test strip with the arrow pointing downward into the tube. Stopper the tube immediately.
5. Gently shake the tube and repeat shaking after 5 and 10 minutes.

6. After 12–15 minutes, and not longer than 30 minutes, read the test for the presence of a yellow color.

VI. Results

A. Interpretation

The development of a yellow color indicates a positive test. If a culture resembles *M. tuberculosis,* but the niacin test is negative, reincubate the slant for an additional 2–4 weeks and repeat the test.

VII. Limitation of Procedure

False-negative reactions may occur, due to: (1) an insufficient amount of growth on the slant, (2) failure to stab the agar if the growth is confluent, or (3) the presence of contaminating bacteria that may have metabolized any niacin that may have accumulated.

M. simiae and occasional strains of *M. bovis, M. marinum,* and *M. chelonae* may accumulate niacin in the test medium. These species can be readily differentiated from *M. tuberculosis* by several key characteristics described in the interpretation section of this manual.

VIII. Bibliography

Gangadharam PR, Droubi DSA. A comparison of four different methods for testing the production of niacin by mycobacteria. Am Rev Respir Dis 1971;104:434–437.

Kilburn JO, Kubica GP. Reagent impregnated paper for detection of niacin. Am J Clin Pathol 1968;50:530–531.

Konno K, Oizumi K, Shimizu Y, et al. Niacin metabolism in mycobacteria. Am Rev Respir Dis 1966;93:41–46.

Runyon EH, Selin MJ, Hawes HW. Distinguishing mycobacteria by the niacin test. Am Rev Tubercu 1959;79:663–665.

Young WD Jr, Maslansky A, Lefar MS, et al. Development of paper strip test for the detection of niacin produced by mycobacteria. Appl Microbiol 1970;20:949–945.

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CHART 19-11

Nitrate Reduction: Mycobacteria

I. Principle

Mycobacteria producing nitroreductase are capable of catalyzing the reduction of nitrate to nitrite. The nitrate produced is detected by the addition of α-naphthalamine and sulfanilic acid, forming the red diazonium dye, *p*-sulfobenzene-azo-α-naphthalamine.

II. Specimen

A. 3- to 4-week-old culture of the test organism growing on Löwenstein–Jensen medium or other coagulated egg medium.

III. Materials

A. Equipment

1. Biologic safety hood

2. 37°C water bath or heating block

B. Supplies

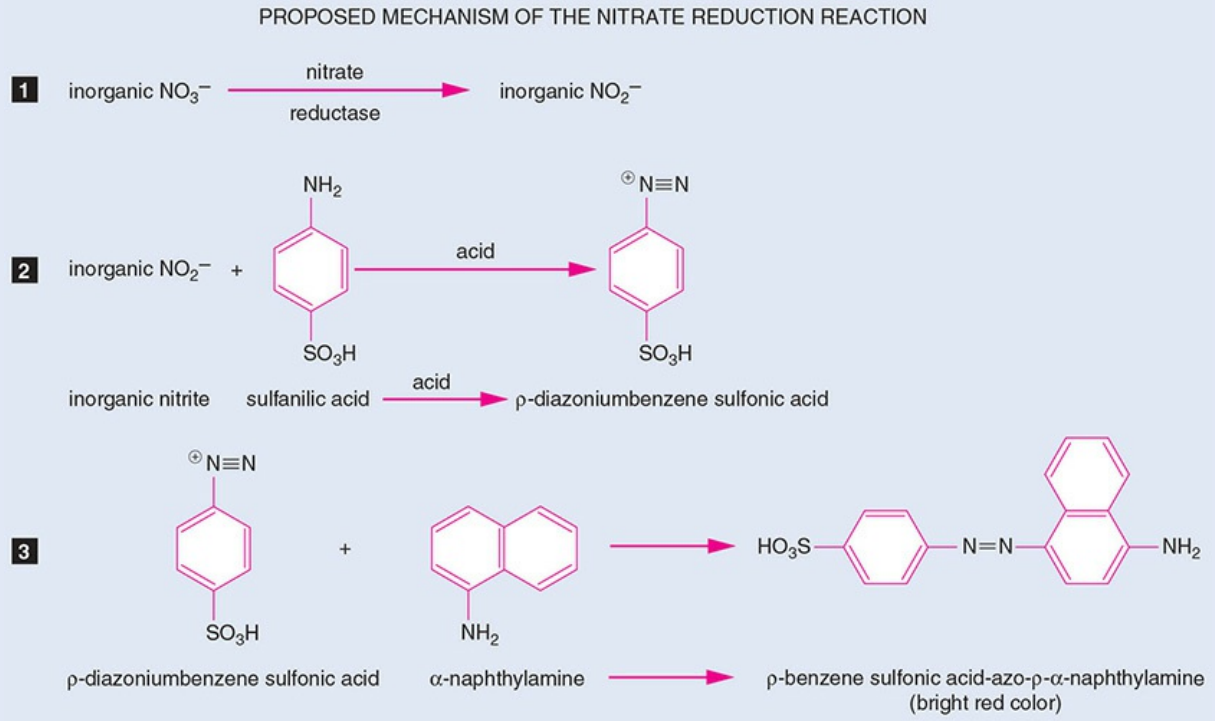
1. Sterile Pasteur pipettes

2. Sterile forceps

C. Reagents

1. Nitrate test substrate (0.01 M) in M/45 phosphate buffer (0.02 M, pH 7.0)

$NaNO_3$	0.8 g
KH_2PO_4	1.17 g
Na_2HPO_4	1.93 g
H_2O	999 mL (final volume)



Dissolve and mix the chemicals. Adjust pH to 7.0. Autoclave and store, and dispense in 2.0-mL aliquots in sterile tubes. When stored at 2°C–8°C, the shelf life is 1–2 months.

2. 0.2% sulfanilamide: Completely dissolve 0.1 g of sulfanilamide in 50 mL of deionized water. It may be necessary to warm the water to 47°C–50°C for complete solubility. When stored in brown glass bottles at 2°C–8°C, the shelf life is about 1 month.
3. 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride: Dissolve 0.05 g of *N*-(1-naphthyl) ethylenediamine dihydrochloride in 50 mL of deionized water. The shelf life is 1 month if stored in a brown-glass bottle at 2°C–8°C.

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IV. Quality Control

A. Positive control: *M. tuberculosis* American Type Culture Collection (ATCC)

B. Negative control: *M. avium* complex ATCC

C. Negative reagent control: Uninoculated tube containing appropriate substrate

V. Procedure

1. Emulsify one loopful or spadeful of colonies from solid medium into 2 mL of nitrate substrate.
2. Shake by hand to mix and incubate at 35°C–37°C for 2 hours.
3. Add reagents to mixture in the following order:
   1 drop concentrated HCl
   2 drops of 0.2% sulfanilamide
   2 drops of 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride
4. Allow mixture to sit at room temperature for 5 minutes.
5. Read for the development of a pink to red color.

VI. Results

The appearance of a red or pink color is indicative of a positive test. A quantitative reading can be made by comparing with nitrate reduction standards. Quantitation is not performed in most laboratories.

VII. Procedure Notes

If no color change occurs, confirm negative results by adding a small amount of zinc dust. A deep red to pink color after the addition of zinc dust indicates that nitrate was reduced. Reagents are stored at 2°C–8°C in the refrigerator, away from direct light.

VIII. Limitation of Procedure

The color may fade rapidly in some instances and can lead to a false-negative test if a reading is not made soon after the reagents are added.

IX. Bibliography

Virtanen S. A study of nitrate reduction of mycobacteria. Acta Tuberc Scand Suppl 1960;418:119.

Wayne LC, Doubek SR. Classification and identification of mycobacteria. II. Tests employing nitrite as substrates. Am Rev Respir Dis 1965;91:478–745.

CHART 19-12

Pyrazinamidase

I. Principle

The deamidation of pyrazinamide to pyrazinoic acid in 4 days is a useful phenotypic characteristic by which *M. marinum* (positive) can be differentiated from *M. kansasii* (negative) and by which weakly niacin-positive strains of *M. bovis* (negative) can be distinguished from *M.*

tuberculosis complex (positive).

II. Specimen

Mature colonies of the unknown *Mycobacterium* species recovered from clinical material, grown on a solid medium.

III. Materials

A. Equipment: None

B. Medium

1. Pyrazinamidase Substrate Medium

a. Dissolve 6.5 g of Dubos broth base in 1 L distilled water.

b. Add

Pyrazinamide	0.1 g
Sodium pyruvate	2.0 g
Agar	15 g

c. Heat to dissolve. Dispense in 50.0-mL amounts into screw-cap tubes.

d. Autoclave for 15 minutes at 121°C.

e. Allow agar to harden with tubes in upright position.

f. Shelf life is 6 months when stored at 2°C–8°C. Discard if agar appears dehydrated or contaminated.

C. Reagents

1% ferrous ammonium sulfate (prepare immediately before use): Place 0.1 g of ferrous ammonium sulfate in sterile screw-capped test tubes. Add 10 mL of sterile deionized water immediately before use, and allow the crystals to dissolve.

D. Supplies

1. Sterile applicator sticks

2. Sterile 1.0 or 5.0-mL pipettes

IV. Quality Control

A. Positive control: *M. intracellulare* American Type Culture Collection (ATCC) 13950

B. Negative control: *M. kansasii* ATCC 12478

V. Procedure

1. Inoculate a large loopful of growth from an actively growing subculture to the surface of two pyrazinamidase agar deeps.

2. Incubate the tubes with caps loose at 37°C.

3. Remove one of the tubes from the incubator after 4 days. Remove one of the control tubes also.

4. Add 1.0 mL of freshly prepared 1% ferrous ammonium sulfate to each tube.
5. Examine the tubes for a pink band in the agar after 30 minutes at room temperature.
6. Refrigerate any negative tubes for 4 hours more and reexamine for the presence of a pink band.

VI. Results

A. Interpretation

After 4 hours, examine the tubes for a pink band in the reagent layer on the surface of the agar (positive reaction), using incident room light against a white background. Repeat the procedure with the remaining tube after 7 days of incubation.

VII. Procedure Notes

Perform all tests under a biologic safety hood.

VIII. Bibliography

Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis 1974;109:147–151.

CHART 19-13

Sodium Chloride Tolerance: Mycobacteria

I. Principle

Of the slowly growing mycobacteria, only *M. triviale* grows in media containing 5% NaCl; of the medically significant rapidly growing mycobacteria, only *M. chelonae* subsp. *chelonae* fails to grow in such media.

II. Specimen

A. 3- to 4-week-old culture of the test organism growing on Löwenstein–Jensen medium or other coagulated egg medium.

III. Materials

A. Equipment

1. Biologic safety hood
2. Slant racks

B. Supplies

1. Sterile applicator sticks
2. Sterile 1.0-mL pipettes

3. McFarland 1 standard

C. Media

1. Löwenstein–Jensen without 5% NaCl in slants
2. Löwenstein–Jensen with 5% NaCl in slants
3. Dubos Tween or Middlebrook 7H9 broth

IV. Quality Control

A. Positive control: *M. fortuitum* American Type Culture Collection (ATCC) 6841

B. Negative control: *M. gordonae* ATCC 14470

Set up controls for each new lot number of medium received. Reject medium if results on control organisms are not as expected. Purchase Löwenstein–Jensen with and without NaCl in the same size tubes for consistency in interpretation.

V. Procedure

1. Use an actively growing culture (2–4 weeks for slow growers; 1–2 weeks for rapid growers). Make a suspension of organisms in Middlebrook 7H9 broth equal to a McFarland 1 standard.
2. Take one Lowenstein–Jensen slant with 5% NaCl and one slant without 5% NaCl and inoculate into each tube, 0.1 mL of the described suspension.

3. Incubate the slants with the caps loose in a 5%–10% CO_2 incubator. Lie slants flat in a rack during incubation. Incubate for 1 week at 29°C–30°C for rapid growers and 37°C for slow growers. Stand negative tubes up straight; incubate 3 more weeks. Visually examine slants weekly for growth.

VI. Results

1. When numerous colonies are seen on the control slant and more than 50 colonies on the medium containing 5% NaCl, record the test as positive.
2. When there are colonies on the control slant and no visible growth on the slant containing NaCl, continue incubation for up to 4 weeks.

VII. Procedure Notes

Use the slant without 5% NaCl to check for viability of the organism. It is not necessary to have equal growth in both tubes. If the organism does not grow on the slant without 5% NaCl, repeat the test.

VIII. Limitation of Procedure

Some strains of *M. fortuitum* and *M. chelonae* grow on a NaCl-containing

media only at temperatures lower than 35°C. For this reason, all test specimens should be incubated at 29°C–30°C.

IX. Bibliography

Silcox VA, Good RA, Floyd MM. Identification of clinically significant *Mycobacterium fortuitum* complex. J Clin Microbiol 1981;4:686–691.

CHART 19-14

Tween-80 Hydrolysis

I. Principle

Tween 80 is the trade name of the polyethylene derivative of sorbitan mono-oleate. Some *Mycobacterium* species possess an enzyme that releases oleic acid from the Tween 80. The color change from orange to pink is due to hydrolysis of Tween 80, which modifies the optical rotation of light passing through the substrate.

II. Specimen

Mature colony of the unknown *Mycobacterium* species recovered from clinical material, growth on a Löwenstein–Jensen slant.

III. Materials

A. Supplies
  1. Sterile applicator sticks
  2. Screw-capped tubes (12 × 100 mm) containing 2.0 mL of deionized water per tube

B. Medium
  1. Löwenstein–Jensen slants

C. Reagents
  1. Tween-80 hydrolysis reagent
  2. 2 drops of Difco (Detroit, MI) reagent added to distilled water in screw-capped tubes.

D. Equipment
  1. Biohazard hood

IV. Quality Control

A. Positive control: *M. kansasii,* American Type Culture Collection (ATCC) 12478 incubated for 12 days

B. Negative control: Stock culture of *M. intracellulare* ATCC 13950

C. Negative reagent: Uninoculated tube of substrate, incubated for 12 days

V. Procedure

1. Add two drops of Tween reagent to 1 mL of sterile distilled water in screw-capped tubes.
2. Inoculate a loopful of the organism to be tested.
3. Incubate at 35°C in the dark with caps tight.
4. Visually read tubes at 24 hours. If negative, read again at 5 and 10 days. Compare the color of the liquid with that in the control tubes.

VI. Results

A positive result is recorded when the liquid, not the cells, turns from light orange to pink or red. *M. kansasii* usually turns positive within 24 hours. Read again at 3, 5 and 10–12 days. Record results and discard positives. Discard all tubes at 12 days.

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VII. Procedure Notes

Keep Tween hydrolysis reagent in the dark. Do not store or incubate tubes in the light. The red color of a positive reaction is not due to a pH change, rather to release of neutral red.

VIII. Bibliography

Kilburn JO, O'Donnell KF, Silcox VA, et al. Preparation of a stable mycobacterial Tween hydrolysis test substrate. Appl Microbiol 1973;26:826.

Wayne LG, Doubek JR, Russell RL. Classification and identification of mycobacteria. I Tests employing Tween-80 as substrate. Am Rev Respir Dis 1964;90:588–597.

CHART 19-15

Urease: Mycobacteria

I. Principle

Urease is an enzyme possessed by many *Mycobacterium* species that can hydrolyze urea to form ammonia and carbon dioxide. The ammonia reacts in solution to form ammonium carbonate, resulting in alkalinization and an increase in the pH of the medium.

II. Specimen

A. Type of Specimen

Mature colony of the unknown *Mycobacterium* species recovered from clinical material, grown on a Lowenstein–Jensen slant.

III. Materials

A. Equipment

1. Biologic safety hood

2. 37°C incubator

B. Medium

1. Urea Broth

a. Add 10 g of Difco (Detroit, MI) Bacto urea agar base concentrate to 90 mL of sterile deionized water. Mix well to dissolve.

b. Aseptically aliquot 3.0 mL portions into sterile screw-capped test tubes.

c. Store at 2°C–8°C; shelf life is 1 month.

IV. Quality Control

A. Positive control: *M. kansasii* American Type Culture Collection (ATCC) 12478

B. Negative control: *M. avium/M. intracellulare* ATCC 13950

V. Procedure

1. Inoculate each substrate tube with a loopful of growth from an actively growing culture. Thoroughly emulsify the culture in the broth.

2. Incubate at 35°C for 5 days.

3. Visually read the tubes at 1, 3, and 7 days.

VI. Results

Color change from yellow to dark pink or red indicates a positive reaction. No color change is a negative reaction.

VII. Procedure Notes

Repeat tests that appear slightly pink; if the repeat test result is still ±, report as negative.

VIII. Limitation of Procedure

1. False-positive tests are usually due to incorrect interpretation of results, incubation beyond 5 days, or contaminated test or control cultures.

2. False-negative results are due to the use of an incorrect medium (i.e., one used for testing bacteria other than acid-fast bacilli), insufficient inoculum, or cultures that are too old.

IX. Bibliography

Kent PT, Kubica GP. Public Health Mycobacteriology: A Guide for the Level III Laboratory. Atlanta, GA: Centers for Disease Control, 1985. Department of Health and Human Services publication no. 86-21654-6.

Murphy DB, Hawkins JE. Use of urease disks in the identification of

mycobacteria. J Clin Microbiol 1975;1:465–468.

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CHART 20-1

Darkfield Microscopy of Genital Lesions

I. Principle

Darkfield detection of spirochetes allows presumptive diagnosis of syphilis during the primary phase before antibody has developed. There are few contaminating spirochetes in genital sites; so detection of spirochetes with a morphology compatible with *Treponema pallidum* subsp. *pallidum* provides a solid diagnosis. The prevalence of spirochetes in the oral cavity essentially precludes the use of darkfield microscopy in this anatomic location.

II. Equipment and Reagents

Microscope with transmitted light and a darkfield condenser. Use a 10× objective to focus the specimen and center the darkfield condenser. Use a 40× to 63× objective to search the specimen. Final identification is made with a 100× objective.

Microscope slides, 1" × 3" Thickness should match that recommended in microscope manual.
Coverglass #1, 22 mm × 22 mm
Immersion oil, nondrying, Cargille type A or equivalent
Lens paper and lens cleaner
Applicator sticks
Sterile saline
Disinfectant, such as iodine or 70% alcohol

III. Quality Control

A scraping of gingival mucosa may be used as a control with which to set up the darkfield condenser.

IV. Procedure

A. Collection of Specimen

1. General

a. Remove any scab or crust covering the lesion.

b. Remove any exudate with a gauze sponge.

c. If necessary, compress the base of the lesion or apply a suction

cup to promote accumulation of tissue fluid on the ulcer surface.

d. Apply a glass slide with a sterile bacteriologic loop.

e. Place a glass coverslip on the specimen and press it down to remove bubbles.

f. Examine immediately.

2. Dry papulosquamous lesions of the skin

a. Gently remove the superficial layer of skin, trying not to cause bleeding.

b. Compress the lesion if very little fluid appears.

c. Touch a microscope slide to the lesion or transfer a small amount of fluid to a slide with a bacteriologic loop.

d. Alternatively, a small amount of sterile saline can be injected into the lesion, aspirated back, and placed on a slide.

e. Place a coverslip on the slide and examine immediately.

3. Cervical/vaginal lesions

a. Under visualization with a bivalve speculum, remove any cervical or vaginal discharge.

b. Obtain serous exudate with a bacteriologic loop.

c. If necessary, serous exudate can be produced by compressing the lesion with a Kelly clamp.

d. Prepare slides as above.

4. Mucous patches

a. Using a sterile bacteriologic loop, collect some of the mucus and place it on a glass slide.

b. Place a coverslip on the slide and examine immediately.

V. Examination of the Specimen

Prepare the darkfield microscope before collection of the specimen.

Using a 40× to 63× objective, search the slide systematically.

When a suspicious structure is found, examine under 100× magnification.

When observation is completed, discard the slide in disinfectant.

Characteristics of *T. pallidum* and saprophytic genital species, *T. refringens*.

Characteristic	*T. pallidum*	*T. refringens*
Morphology		
Shape	Delicate corkscrew shape	Loose coiled, coarse
Length	6–14 μm	8–20 μm

Width	0.25–0.3 µm	0.4–0.75 µm
Spiral wavelength	Approx. 1.0–1.5 µm	

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Motility

Translation	Slow, deliberate back and forth movement	Rapid, writhing motion
Rotation	Slow to rapid rotation (corkscrew); may rotate in place	Active serpentine rotation
Flexion	Rotation accompanied by soft bending and twisting	
Bending occurs stiffly in middle of organism	Marked flexion	
Distortion	May occur as a ring or attached to cells	None noted

VI. Results

A. Interpretation

Positive: Spirochetes present

Negative: Spirochetes absent

False-positive results may occur if specimens from the oral cavity are tested due to the presence of non- *Treponema* spirochetes in such locations.

A few red blood cells in the preparation are a sign that exudate was obtained, but the specimen should not be bloody. The membranes of lysed erythrocytes can form serpentigenous bodies that resemble spirochetes; it is therefore important to differentiate true motility from movement secondary to Brownian motion. Bubbles should also be carefully removed from the preparation.

False-negative results may be obtained if insufficient exudate was obtained, if there was too long an interval between collection and examination, if the lesion is late, or if the patient has been treated with antitreponemal drugs.

VII. Bibliography

Larson SA, Hunter EF, Kraus SJ, eds. Manual of Tests for Syphilis. 8th Ed. Washington, DC: American Public Health Association, 1990.

CHART 20-2

Venereal Disease Research Laboratory (VDRL) Slide Test on Serum

I. Principle

The VDRL slide test is the reference nontreponemal antibody test for syphilis. It is the only generally recognized test for diagnosing neurosyphilis.

The VDRL is a nontreponemal microflocculation test. It uses an antigen that contains cardiolipin, lecithin, and cholesterol. The antigen forms microscopic clumps when mixed with antibodies to cardiolipin complex.

II. Equipment and Reagents

A. Equipment

1. Nondisposable needles without a bevel
- **a.** Serum test: 18 gauge

2. Bottles: 30-mL, round, narrow-mouthed, 35 mm in diameter, with glass or equivalent stoppers and flat inner-bottom surface (Corning Glass Works, Corning, NY, cat. no. LG-1 MW-90530).

3. Safety pipetting device with disposable tip to deliver 50 mL.

4. Pipettes, serologic, graduated to tip:
- **a.** 1.0 mL, graduated in 1/100 mL
- **b.** 5.0 mL, graduated in 1/10 mL
- **c.** 10.0 mL, graduated in 1/10 mL

5. Slides
- **a.** Serum test: 2 × 3 in., with 12 paraffin or ceramic rings approximately 14 mm in diameter (rings must be high enough to prevent spillage during rotation).
- **b.** 12 concavities measuring 16 mm in diameter and 1.75 mm in depth.

6. Slide holder for 2 × 3-inch slides.

7. Ringmaker to make paraffin rings if ceramic rings not used.

8. Mechanical rotator adjustable to 180 ± 2 rpm, circumscribing a circle 19 mm in diameter on a horizontal plane.

9. Discard containers or disinfectants, disposable latex gloves.

10. Humidifying covers (in dry climates).

B. Reagents

1. VDRL antigen: Store in the dark at room temperature or in refrigerator. Discard vials that contain a precipitate.
2. VDRL-buffered saline, pH 6.0 ± 0.1 (1.0% NaCl) (purchased or prepared)
3. Control serum samples: reactive (R), weakly reactive (W), nonreactive (N)
4. 0.9% saline
5. 10.0% saline

III. Procedures for Serum Tests

A. Preparing the Antigen Suspension

1. Prepare a fresh VDRL antigen suspension each day. The temperature of all reagents should be at 23°C–29°C.
2. Pipette 0.4 mL of VDRL-buffered saline to the bottom of a round, 30-mL, glass-stoppered bottle with a flat inner surface.
3. Add 0.5 mL VDRL antigen directly into the saline while continuously but gently rotating the bottle on a flat surface. Add antigen drop by drop at a rate allowing approximately 6 seconds for each 0.5 mL of antigen. Keep the pipette tip in the upper third of the bottle and do not splash saline onto the pipette tip. The proper speed of rotation is obtained when the center of the bottle circumscribes a 2-inch-diameter circle approximately three times per second.
4. Expel the last drop of antigen from the pipette without touching the pipette to the saline. Continue rotation of the bottle for 10 seconds.
5. Add 4.1 mL buffered saline from a 5-mL pipette.
6. Cap the bottle and shake it from bottom to top and back approximately 30 times in 10 seconds. The antigen suspension must be used within 8 hours.
7. Mix the suspension by gently swirling it each time it is used. Do not mix it by forcing it back and forth through the syringe and needle.

B. Testing Accuracy of Antigen Suspension Needle

1. The accuracy of the test depends on the amount of antigen dispensed. Check the calibrated needle periodically for accuracy.
2. For the qualitative and quantitative tests on serum, dispense the antigen suspension from a syringe fitted with an 18-gauge needle without a bevel, which will deliver 60 ± 2 drops of antigen suspension per milliliter (17 mL/drop) when held vertically.
3. Place the needle on a 2-mL syringe or on a 1-mL pipette and fill the syringe or pipette with VDRL antigen suspension. Holding the syringe or pipette vertically, count the number of drops delivered in 0.5 mL. The needle is correctly calibrated if 30 ± 1 drops are delivered in 0.5 mL. Adjust or replace the needle if it does not meet

the specifications.

C. Testing and Storing the VDRL Antigen Suspension

1. Prepare a fresh antigen suspension each day. Store the suspension at room temperature, 23°C–29°C.

2. Test the antigen suspension with control sera that are reactive, weakly reactive, and nonreactive.

3. Use only suspensions that reproduce the established reactivity pattern of the controls.

4. After completing the day's tests, discard the antigen suspension and clean the dispensing needle and syringe by rinsing with water, alcohol, and acetone, in that order. Remove the needle from the syringe after cleaning.

D. Preparing and Storing Specimens

1. Observe recommended safety precautions for handling human sera.

2. Centrifuge clotted blood at room temperature at 1,500–2,000 rpm for 5 minutes to sediment the cellular elements.

3. Transfer the serum to a clean, dry test tube and heat it in a 56°C water bath for 30 minutes.

4. Recentrifuge any specimen that contains debris after heat-inactivation.

5. Reheat the serum at 56°C for 10 minutes if testing is delayed more than 4 hours.

6. Specimens must be at room temperature (23°C–29°C) at the time of testing.

E. Performing the Qualitative Test on Serum

1. With all reagents at room temperature, place 50 µL of serum into one ring of a paraffin- or ceramic-ringed slide, using a safety pipetting device. Do not use glass slides with concavities, wells, or glass rings.

2. Gently resuspend the VDRL antigen suspension.

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3. Holding the VDRL antigen suspension dispensing needle and syringe in a vertical position, dispense several drops to clear the needle of air. Then add exactly 1 free-falling drop (17 µL) of antigen suspension to each circle that contains serum.

4. Place the slide on a mechanical rotator. Rotate for 4 minutes at 180 ± 2 rpm. In a dry climate, cover the slide with a moist humidifying cover during rotation.

5. Immediately after the completion of rotation, remove the slide from the rotator and read the test results microscopically, using a 10× ocular and a 10× objective.
6. Report the results as follows:

Reading	Report
Medium or large clumps	Reactive (R)
Small clumps	Weakly reactive (W)
No clumping or slight roughness	Nonreactive (N)

7. Test quantitatively to an endpoint all serum specimens that produce reactive, weakly reactive, or "rough" nonreactive results in the qualitative test.

F. Performing the Quantitative Test in Serum

1. Quantitate the serum samples to an endpoint titer. Quantitative tests through the 1:8 dilution for three serum specimens may be performed on one slide.
2. Place 50 mL of 0.9% saline in circles numbered 2 through 4, but do not spread the saline.
3. Using a safety pipetting device, place 50 µL of serum in both circle 1 and circle 2.
4. Mix the saline and the serum in circle 2 by drawing the mixture up and down in the safety pipette eight times. Avoid formation of bubbles.
5. Transfer 50 µL from circle 2 (1:2) to circle 3 and mix.
6. Transfer 50 µL from circle 3 to circle 4 and mix. Discard the last 50 µL.
7. Gently resuspend the VDRL antigen suspension.
8. Holding the VDRL antigen suspension–dispensing needle and syringe in a vertical position, dispense several drops to clear the needle of air. Then add exactly 1 free-falling drop (17 µL) of antigen suspension to each circle.
9. Place the slide on the mechanical rotator. Rotate the slide for 4 minutes at 180 ± 2 rpm. Add a humidifying cover in dry environments.
10. Immediately after rotation, read the test microscopically using a 10× ocular and a 10× objective.
11. If the highest dilution tested (1:8) is reactive, proceed as follows:
    a. Prepare a 1:8 dilution of the test specimen. Add 0.1 mL serum to 0.7 mL of 0.9% saline and mix thoroughly.
    b. Place 50 µL of 0.9% saline into the second, third, and fourth

paraffin rings in a row on the slide. Prepare additional serial dilutions for strongly reactive specimens.

c. Add 50 µL of the 1:8 dilution of the test specimen to paraffin rings 1 and 2.

12. Report titers in terms of the highest dilution that produces a reactive (not a weakly reactive) result:

Serum Dilutions						Report
Undiluted	1:2	1:4	1:8	1:16	1:32	
R	W	N	N	N	N	Reactive, undiluted, 1 dil.
R	R	W	N	N	N	Reactive, 1:2 dilution, 2 dils
R	R	R	W	N	N	Reactive, 1:4 dilution, 4 dils
W	W	R	R	W	N	Reactive, 1:8 dilution, 8 dils
N (Rough)	W	R	R	R	N	Reactive, 1:16 dilution, 16 dils
W	N	N	N	N	N	Weakly reactive, undiluted, 0 dil

IV. Results

A. Interpretation

1. The VDRL test is an aid in the diagnosis of syphilis. A positive test may indicate a past or present infection. False-positive results occur because of laboratory error, infection with other treponemes, or the presence of antibodies unrelated to treponemal infection. The predictive value of a positive VDRL result is increased when combined with a reactive treponemal test, such as the fluorescent treponemal antibody absorption test (Chart 20-4) or the microhemagglutination test.

2. A nonreactive result may indicate no infection, adequately treated infection, or infection in the remote past. A nonreactive result may be obtained in incubating or early primary syphilis. A prozone phenomenon occurs, especially with high titers in secondary syphilis. Sera should be diluted if a rough result is obtained or if clinical information indicates the likelihood of syphilis.

3. A fourfold increase in titer may indicate a reinfection or treatment failure. A fourfold decrease in titer in early syphilis usually indicates adequate therapy.

4. All sera should be titered to the endpoint. Unusually high titers may be seen in some patients who are concurrently infected with human immunodeficiency virus, type 1. Unusually high false-positive titers may be seen in some patients with lymphomas.

V. Sources of Error

1. Reactivity is decreased if the specimen, testing area, or reagents are at temperatures lower than 23°C. Reactivity is increased if any of these are at temperatures higher than 29°C.
2. Test results are unpredictable if hemolyzed, contaminated, or extremely turbid sera are tested.
3. Reactivity will be unpredictable if reagents are outdated, inadequately tested, or if procedures are not followed strictly. Forcing the VDRL antigen through the needle and syringe repetitively will cause the suspension to lose reactivity.

VI. Test Limitations

1. The prozone phenomenon described in the foregoing may produce false-negative results on high-titered sera.
2. The VDRL antigen suspension must be prepared daily.
3. The VDRL cerebrospinal fluid (CSF) test should be performed only if the serum test is reactive.
4. Plasma cannot be used for the VDRL test.
5. Biologic false-positive results occur with cardiolipin antigens, especially in specimens from persons who abuse drugs; individuals with diseases such as lupus erythematosus, mononucleosis, malaria, leprosy, or viral pneumonia; or those who have recently been immunized.
6. Note: The VDRL slide test on CSF is similar to the serum test, but there are important methodologic differences (see bibliography). Only reactive and nonreactive results are reported on CSF.

VII. Bibliography

Larson SA, Hunter EF, Karus SJ, eds. Manual of Tests for Syphilis. 8th. Ed. Washington, DC: American Public Health Association, 1990.

CHART 20-3

Rapid Plasma Reagin (RPR) Card Test

I. Principle

The RPR Card Test was instituted as the routine screening test in the Maryland State Department of Health Laboratories on January 13, 1964. The main advantages of the RPR over the VDRL test are that the serum does not have to be heat-treated, interpretations are made macroscopically

instead of microscopically, the antigen is stable, and components are disposable. Tests can be performed rapidly and inexpensively, thereby making the technique suitable for large-volume operations. The sensitivity and specificity of the RPR test are equal to those of the VDRL and other nontreponemal serologic tests.

The RPR antigen suspension is a carbon-containing cardiolipin antigen that detects "reagin," an antibody-like substance present in the plasma and sera of persons with syphilis or previous exposure to the treponema spirochete. When a specimen contains specific antibody, flocculation occurs with coagglutination of the carbon particles of the RPR card antigen, appearing as black clumps against a white background of the plastic-coated card. This coagulation can be read macroscopically; nonreactive specimens have a smooth, milky light-gray color.

II. Equipment, Supplies, and Reagents

1. Disposable, calibrated 20-gauge needle without bevel, silicone-treated
2. Plastic antigen-dispensing bottle, 1 dram
3. Plastic-coated RPR cards with 10 circles, each approximately 18 mm in diameter
4. Dispenstirs, a disposable (plastic) dispensing-stirring device that delivers 50 µL
5. Mechanical rotator, fixed speed or adjustable to 100 ± 2 rpm, circumscribing a circle 3/4 in. in diameter on a horizontal plane
6. Humidifying cover for rotator
7. High-intensity incandescent lamp
8. Safety pipetting device with disposable tip that delivers 50 µL
9. Discard containers and disinfectants

10. Disposable latex gloves, safety glasses, and protective clothing
11. RPR antigen suspension (a variation of the VDRL antigen): a stabilized combination of 0.003% cardiolipin, 0.020%–0.022% lecithin, 0.09% cholesterol, 10% choline chloride, 0.0125 M EDTA, 0.1875% charcoal, 0.1 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.1% thimerosal, and distilled water
12. Control serum samples: lyophilized reactive (R), minimally reactive (MR), and nonreactive (N) control serum specimens on a card
13. 0.9% saline as a diluent

III. Procedures

A. Testing Accuracy of Antigen Needle

1. Check the calibrated needle periodically to be sure that it delivers

60 ± 2 drops/mL (17 µL). The accuracy of the test depends on the amount of antigen used.

2. Consult the procedure for calibrating needles in the VDRL test (Chart 20-2).

B. Testing and Storing the Antigen Suspension

1. Store ampules of antigen at 2°C–8°C. Do not store in the bright sunlight. Do not store above 29°C and do not freeze. Antigen in an opened ampule is stable for up to 12 months.
2. Attach the needle hub to the fitting on the plastic dispensing bottle. Open the ampule after shaking it to resuspend the particles. Squeeze the dispensing bottle to collapse it, insert the needle into the ampule, and withdraw all the antigen suspension into the dispensing bottle. Shake the dispensing bottle gently before each series of antigen drops is delivered.
3. Test each lot of antigen suspension in parallel with an antigen of known standard reactivity before putting it into use.
4. At each routine test run, test the reactivity of the antigen with control cards or sera of known reactivity. Use only suspensions that produce appropriate activity.
5. After completion of the day's work, remove the needle from the antigen-dispensing bottle, rinse in distilled water, and air-dry. Do not wipe the needle, as this action will remove the silicone coating.

C. Preparing and Storing Specimens

1. Either serum or plasma may be tested. Plasma is obtained by collecting blood into a tube that contains EDTA as an anticoagulant.
2. Do not test specimens that are grossly contaminated, excessively hemolyzed, or chylous.

D. Performing the Qualitative Test

1. All reagents should be at room temperature (23°C–29°C) before tests are performed.
2. Place 50 µL of serum or plasma into an 18-mm circle of the RPR test card, using a disposable Dispenstir or a safety pipetting device.
3. With the inverted Dispenstir (closed end) or flat toothpicks, spread the serum or plasma to fill the entire circle, but not to extend beyond the bounds of the circle.
4. Gently shake the antigen-dispensing bottle to resuspend the particles.
5. While holding the dispensing bottle and needle in a vertical position, dispense several drops to clear the needle of air. Then add exactly 1 free-falling drop (17 µL) of antigen suspension to each circle containing serum or plasma. Do not mix the antigen and serum

or plasma.

6. Place the card on the mechanical rotator under a humidifying cover. Rotate the card for 8 minutes at 100 ± 2 rpm.
7. Immediately remove the card from the rotator. Briefly rotate and tilt the card by hand to aid in differentiating nonreactive from minimally reactive results. Read the test reactions in the "wet" state, using a high-intensity incandescent light, without magnification.
8. Report the results as follows:

Reading	Report
Characteristic clumping ranging from marked and intense (reactive) to slight but definite (minimally reactive)	Reactive (R)
Slight roughness or no clumping	Nonreactive (N)

9. Perform the quantitative test on serum specimens showing any degree of reactivity or "roughness."

IV. Performing the Quantitative Test

1. Quantitate to an endpoint all serum specimens with reactive or rough nonreactive results in the qualitative test. Test each specimen undiluted (1:1) and in 1:2, 1:4, 1:8, and 1:16 dilutions.
2. Place 50 µL of 0.9% saline in circles numbered 2 through 5. Do not spread the saline.
3. Using a safety pipette device, place 50 µL of serum in circle 1 and 50 µL of serum in circle 2. Mix the saline and the serum in the circle by drawing the mixture up and down in the safety pipette eight times. Avoid forming bubbles.

4. Transfer 50 µL from circle 2 (1:2) to circle 3 and mix.
5. Transfer 50 µL from circle 3 (1:4) to circle 4 and mix.
6. Transfer 50 µL from circle 4 (1:8) to circle 5 (1:16) and mix. Discard the last 50 µL.
7. With the broad end of a clean Dispenstir, spread the serum dilution to fill the entire surface of circle 5, the highest dilution (1:16). With the same Dispenstir, do the same for circles 4 (1:8), 3 (1:4), 2 (1:2), and 1 (undiluted).
8. Gently shake the dispensing bottle to resuspend the antigen particles.
9. Holding the antigen-dispensing bottle in a vertical position, dispense 1 or 2 drops to clear the needle of air. Then add exactly 1 free-falling drop

(17 µL) of antigen suspension in each circle. Do not mix.

10. Place the card on the rotator under the humidifying cover and rotate the card for 8 minutes at 100 ± 2 rpm.
11. Immediately remove the card from the rotator; briefly rotate and tilt the card by hand (three or four to-and-fro motions) to aid in differentiating nonreactive from minimally reactive results. Read the results in the wet state with the unaided eye, using a bright incandescent light.
12. Report the results in terms of the highest dilution that has given a reactive result, including a minimally reactive result, as follows:

Serum Dilutions					Report
Undiluted	**1:2**	**1:4**	**1:8**	**1:16**	
RM	N	N	N	N	Reactive, undiluted
R	R	R	N	N	Reactive, 1:4 dilution
R	R	R	RM	N	Reactive, 1:8 dilution

R, Reactive; RM, Reactive, minimally; N, nonreactive.

Note: Report the result as either reactive or nonreactive; there is no minimally reactive report in the RPR card test.

V. Results

A. Interpretation

1. Clinicians must combine the results of this test with the clinical and epidemiologic information. The predictive value of a positive RPR test is increased if the specimen is also reactive in a specific treponemal test, such as the fluorescent treponemal antibody absorption test (FTA-ABS).
2. A reactive test indicates present or past inadequately treated infection or a false-positive reaction. Technical false-positive reactions may be detected by a negative RPR test when repeated. Biologic false-positive reactions are detected by a negative specific treponemal test, such as the FTA-ABS test.
3. A negative test in the absence of clinical symptoms may indicate no infection or adequately treated infection in the past. A negative test may also occur during the incubation period before the first symptoms have manifested. A negative test in the presence of clinical symptoms may occur during early primary syphilis, as a prozone during secondary syphilis, or occasionally in tertiary syphilis.
4. When the quantitative RPR test is performed on patients with syphilis, a fourfold rise in titer may indicate an active infection, a reinfection, or failure of therapy. A fourfold fall in titer usually

means adequate syphilis therapy.

5. All reactive qualitative RPR tests should be titered to an endpoint and the endpoint titer reported. Very high RPR card titers may occur in patients with HIV infection. Very high false-positive titers may occur in patients with lymphomas.

B. Acceptable Variations

1. Prepare a 1:16 dilution for further quantitation, using 50 µL of specimen to 750 µL of 0.9% saline.
2. If, after screening, the serum to be quantitated seems likely to exceed 1:16, prepare all dilutions directly on the card, using a 1:50 nonreactive serum as the diluent beginning with circle 6 (1:32) and continuing for the remainder of the card.

VI. Sources of Error

1. If the temperatures of the sera, reagents, or testing area are lower than 23°C, test reactivity decreases; if temperatures are higher than 29°C, test reactivity increases.
2. If the speed of the mechanical rotator is too fast or too slow, improper mixing of antigen and antibody will yield unpredictable results.
3. If the card is excessively rotated and tilted by hand after removal from the rotator, a false-positive reaction may occur.
4. If lighting produces a glare on the card, results may be obscured.
5. If the antigen is outdated or not tested for standard reactivity, results may be unpredictable.

6. If the serum is unevenly spread in the circle, the antigen and antibody may not mix properly.
7. If hemolyzed, contaminated, or improperly collected serum or plasma samples are tested, the reaction may be masked.
8. If plasma specimens are stored at –20°C for longer than 1 week, the specimens become unsuitable for testing.
9. If the moistened humidifying cover is not used to cover tests as they are being rotated, proper humidity will not be maintained, and test components may dry on the card, causing false-positive reactions to occur.

VII. Test Limitations

1. The RPR card tests cannot be used to test spinal fluids.
2. A prozone reaction may be encountered occasionally. In a prozone reaction, complete or partial inhibition of reactivity occurs with undiluted serum. The prozone phenomenon may be so pronounced that only a

rough reaction is produced in the qualitative test by a serum that will be strongly positive in the quantitative test. Any specimen that produces any degree of roughness or reactivity in the qualitative test should be retested in the quantitative test.

3. The RPR test may be reactive in persons from areas where yaws, pinta, or nonvenereal syphilis is endemic. As a general rule, residual titers from these infections will be less than 1:8.
4. Biologic false-positive reactions occur occasionally, particularly in persons who abuse drugs; who have diseases such as lupus erythematosus, mononucleosis, malaria, leprosy, or viral pneumonia; or who have been vaccinated recently.
5. Nontreponemal test titers of persons who have been treated in latent or late stages of syphilis or who have been reinfected do not decrease as rapidly as those who are treated in early primary infection. Some of these individuals may become "serofast," retaining their reactivity for life.

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CHART 20-4

Fluorescent Treponemal Antibody Absorption Test (FTA-ABS)

I. Principle

The fluorescent treponemal antibody absorption test (FTA-ABS) is an indirect fluorescent antibody technique (Chapter. 3). The patient's serum, which has been diluted 1:5 in sorbent (an extract from cultures of *Treponema phagadenis,* the Reiter treponeme), is layered on a microscope slide to which *T. pallidum* subsp. *pallidum* has been fixed. If the patient's serum contains antibody to *T. pallidum,* it will coat the treponeme. When fluorescein isothiocyanate (FITC)-labeled anti-human globulin is added, it will fix to any antibodies that have become affixed to the spirochete. The fluorescent reaction is visible when viewed by fluorescence microscopy.

II. Materials

A. Equipment

Incubator, 35°C–37°C
Water bath, adjustable to 56°C
Safety pipetting devices
Micropipettors delivering 10–200 µL
Loop, bacteriologic standard, 2 mm, 26-gauge, platinum
Bibulous paper
Slide board with moist chamber and paper towels
Staining dishes, glass or plastic, with removable slide carriers
Microscope slides, 1 × 3 in., with frosted end, 1-mm thick, with two etched circles, 1-cm inside diameter
Coverslips, No. 1, 2-mm square
Test tubes (12 × 75 mm) and holders
Discard containers and disinfectants
Disposable latex gloves, safety glasses, and protective clothing
Fluorescence microscope set up for fluorescein microscopy
Mixer; Vortex Jr. or equivalent

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B. Reagents

1. Purchased

a. *T. pallidum* antigen: A suspension of *T. pallidum* (Nichols strain) extracted from rabbit testicular tissue and washed in phosphate-buffered saline to remove rabbit globulin.

b. FITC-labeled antihuman IgG.

c. Sorbent: Prepared from cultures of nonpathogenic Reiter treponemes, usually with no preservative added. Frequently dispensed in 5-mL amounts and freeze-dried, but also sold in liquid state.

d. Reactive control serum: A pool of human serum samples obtained from syphilitic donors that are 4+ reactive. Used to prepare 4+ serum controls and minimally reactive 1+ control. The 1+ control demonstrates the minimal degree of fluorescence reported as reactive and is used as a reading standard.

e. Nonspecific control serum: A serum pool obtained from nonsyphilitic individuals. No preservative is added. This control demonstrates a ≥2+ nonspecific reactivity at a 1:5 dilution in phosphate-buffered saline (PBS) and essentially no staining when diluted 1:5 in sorbent.

f. Oil: Low fluorescence, nondrying, immersion oil, type A,

Cargille No. 1248

g. Acetone: ACS reagent grade

2. Prepared

a. PBS, pH 7.2

b. 2% Tween 80 (polysorbate 80) in PBS. Heat the reagents in a 56°C water bath. To 49 mL of sterile PBS, add 1 mL of Tween 80, by measuring from the bottom of a pipette and rinsing the pipette in the mixture. Check pH and adjust to pH 7.2 with 1 N NaOH. Discard if a precipitate develops or the pH changes.

c. Mounting medium: Add 1 part PBS to 9 parts glycerin (reagent grade).

d. Distilled water

III. Preparing Test Reagents

A. *T. pallidum* Antigen

1. Rehydrate antigen according to manufacturer's instructions. Opened vials, stored at 2°C–8°C, are stable for 1 week.

2. To prepare slides, mix antigen suspension thoroughly on a mixer for 10 seconds. Determine by darkfield examination that treponemes are adequately dispersed before making slides for the FTA-ABS test.

3. Wipe slides with clean gauze to remove dust particles. Clean slides by sonic vibration or alcohol wiping if treponemes are not clearly observed after staining.

4. Prepare very thin *T. pallidum* antigen smears within each circle by using a 2-mm wire loop. Place one loop of antigen within the two 1-mm circles. Allow to air-dry at least 15 minutes.

5. Fix slides in acetone for 10 minutes and air-dry. Fix no more than 60 slides with 200 mL of acetone. Store acetone-fixed smears at –20°C. Do not thaw and refreeze smears.

6. Store unopened vials of antigen at 2°C–8°C. The unopened antigen is stable until the expiration date.

B. Titration of Antihuman IgG Globulin

1. Rehydrate conjugate according to directions. If the conjugate is cloudy, centrifuge it at 500 *g* for 10 minutes. Aliquot in small volumes and store at –20°C. Do not refreeze thawed conjugate, but store at 2°C–8°C.

2. Prepare 4+ and 1+ dilutions of the reactive control serum to determine conjugate working dilution.

3. Run the control pattern with a reference or a known conjugate. An example is shown:

Conjugate Dilutions	Nonspecific-Staining Control (PBS)	Reactive (4+) Control Serum (1:5 in PBS)	Reaction (1+) Control Serum
Reference Conjugate			
Dilution 1:400	–	4+	1+
New Conjugate			
1:12.5	<1+	4+	3+
1:25	–	4+	3+

1:50	–	4+	3+
1:100	–	4+	2+
1:200[a]	–	4+	1+
1:400	–	4+	<1+
1:800	–	3+	±

[a]The dilution selected for the working titer is 1:200, one doubling dilution below the 4+ endpoint (1:400), 1 + staining with the 1 + control dilution (1:200), and no nonspecific staining for three doubling dilutions below the working dilution (1:25).

C. Sorbent

1. Rehydrate freeze-dried material with sterile distilled water or according to manufacturer's instructions. The rehydrated sorbent may be stored at 2°C–8°C or at –20°C. It can be used as long as acceptable reactivity is obtained and the product is not contaminated.

D. Test Serum

1. Observe recommended safety precautions for handling human serum.

2. Heat the test and control sera at 56°C for 30 minutes before testing.

3. Reheat previously heated test sera for 10 minutes at 56°C on the day of testing.

E. Control Test

1. Rehydrate serum according to manufacturer's instructions.

2. Aliquot in 0.25 mL amounts and store at −20° for as long as acceptable reactivity is obtained.

3. Prepare the following controls for each test run:

a. Reactive (R) 4+ control serum: a syphilitic serum showing 4+ fluorescence in the unabsorbed test and only slightly reduced fluorescence in the absorbed test. To produce unabsorbed serum, add 50 mL of reactive control serum to a tube containing 200 µL PBS and mix well. To produce absorbed serum, transfer 50 µL of reactive control serum to a tube containing 200 µL PBS

and mix well. To produce absorbed serum, transfer 50 µL of reactive control serum to a tube containing 200 µL of sorbent and mix well.

b. Minimally reactive (MR) control serum: This is a dilution in PBS of the reactive control serum, which will give the minimal degree of fluorescence (1+) considered reactive.

c. Nonspecific (NS) control serum: a nonsyphilitic serum showing ≥2+ fluorescence in the unabsorbed test. To produce the unabsorbed serum, transfer 50 µL of nonspecific control serum to a tube containing 200 mL of PBS and mix well. To produce the absorbed serum, transfer 50 µL of nonspecific control serum to a tube containing 200 µL of nonspecific control serum into a tube containing 200 µL of sorbent and mix well.

d. Controls for nonspecific staining by conjugate consist of an antigen smear overlaid with 30 µL of PBS in place of serum and an antigen smear overlaid with 30 µL of sorbent in place of serum.

F. The FTA-ABS Test

1. Identify previously prepared antigen slides by numbering the frosted end.

2. Number each tube and slide to correspond to the test serum and the control serum to be used.

3. Prepare reactive (4+), minimally reactive (1+), and nonspecific control serum dilutions in sorbent or PBS according to the directions.

4. Pipette 200 µL of sorbent into a test tube for each test serum.

5. Add 50 µL of the heated test serum to the appropriate tube and mix eight times.

6. Cover the appropriate antigen smears with 30 mL of the reactive (4+), minimally reactive (1+), and nonspecific control serum dilutions.

7. Cover the appropriate antigen smears with 30 µL of the PBS and 30 µL of the sorbent for nonspecific-staining controls a and b. The controls should give the following pattern:

Control Pattern for the FTA-ABS Test

Reactive control	a. 1:5 PBS dilution b. 1:5 Sorbent dilution	R4+ R(4+ −3+)
Minimally reactive control	1+ Control dilution	R1 +

Nonspecific serum controls	a: 1:5 PBS dilution b. 1:5 Sorbent dilution	R(2+ −4+) N−±
Nonspecific-staining controls	a. Antigen, PBS, conjugate b. Antigen, sorbent, conjugate	N N

8. Cover the appropriate antigen smears with 30 µL of the test serum dilutions.
9. Prevent evaporation by placing slides in a moist chamber and incubate at 35°C–37°C for 30 minutes.
10. At the completion of the incubation perform the following rinses:
    a. Place slides in slide carriers and rinse 5 seconds with running PBS.
    b. Place slides in staining dish containing PBS for 5 minutes.
    c. Agitate slides by dipping them into the PBS at least 30 times.
    d. Using fresh PBS, repeat the above two steps.
    e. Rinse slides for 5 seconds in running distilled water and gently blot with bibulous paper.
11. Dilute FITC-labeled antihuman IgG to its working titer in PBS containing 2% Tween 80 and place approximately 30 µL of the diluted conjugate on each smear.
12. Repeat incubation and rinsing of slides.
13. Mount slides immediately by placing a small drop of mounting medium on each smear and applying a cover glass.
14. Place slides in a darkened room and read within 4 hours.
15. Check smear by darkfield microscopy using the tungsten lamp first, to verify the presence of treponemes on the smear, then read FITC (test) fluorescence.
16. Using the minimally reactive (1+) control slide as the reading standard, record the intensity of fluorescence of the treponemes:

RECORDING INTENSITY OF FLUORESCENCE

Reading	Intensity of Fluorescence
2+ to 4+	Moderate to strong
1+[a]	Equivalent to minimally reactive (1+) control
± to <1+	Visible staining but less than 1+
−	None or vaguely visible but without distinct fluorescence

Atypical[b]	Varied: treponemes appear to be "moth-eaten" or to have "beads" of fluorescence throughout their length

[a]Retest all specimens that initially give a 1+ intensity of fluorescence.

[b] Atypical or beaded pattern of fluorescence has been described in patients with lupus and other autoimmune diseases.

17. Report the result as follows:

REPORTING SYSTEM FOR FTB-ABS TEST

Initial Test Reading	Repeat Test Reading	Report
4+		Reactive
3+		Reactive
2+		Reactive
1+	>1+	Reactive
1+	1+	Reactive minimal[a]
1+	<1+	Nonreactive
<1+		Nonreactive
N		Nonreactive
Beaded fluorescence		Atypical Fluorescence Observed[b]

[a] In the absence of evidence of treponemal infection, this test result should be considered equivocal. A second specimen should be obtained 1–2 weeks after the initial specimen and submitted to the laboratory for serologic testing.

[b] Beaded fluorescence has been observed in serum from patients with active systemic lupus erythematosus and from patients with other autoimmune diseases. The DNA absorption procedure can be requested from the Treponemal Pathogenesis and Immunobiology Branch, Division of Sexually Transmitted Diseases, CDC, Atlanta, GA, 30333. The request must be routed through the appropriate State Health Department.

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IV. Acceptable Variations

1. Reading of slides may be delayed beyond 4 hours if slides are protected

from light and stored at 2°C–8°C. For the test to be valid with a delayed reading, *the complete control pattern must be clearly satisfactory when the slides are read.*

2. Conjugate that has been filter-sterilized and contains a preservative, such as sodium azide, to prevent bacterial contamination may be stored at 2°C–8°C. Any precipitate or cloudiness must be removed by centrifugation.
3. Slides may be held from 30 minutes–1 hour in PBS if the test is interrupted. For the results to be valid, control slides must react appropriately when the test is read.
4. Multicircle slides may be used rather than the two-circle slide. Add only 10 µL volumes to the antigen smears. Exercise care in handling and washing slides to prevent serum runover.
5. With accurate micropipettors, the 1:5 test dilution may be prepared by pipetting 25 µL of serum into 100 µL of diluent.

V. Sources of Error

1. If reagent evaluation procedures are not strictly followed, results will be unreliable.
2. If multicircle slides are used and serum from one well is allowed to run onto another well, serum from a person without syphilis may appear reactive.
3. If microscope slides are not clean, the test may be difficult to read and the results unreliable.
4. If the FTA-ABS test is used as a screening procedure, rather than to confirm the reactive results of a nontreponemal test, or as a specific diagnostic test in patients with signs or symptoms of late syphilis, the positive predictive value of the FTA-ABS test is decreased.
5. If the microscope is not properly aligned and the control pattern is not obtained, the test results are invalid.
6. If reagents become contaminated with bacteria, the antibody may be reduced and the results may be invalid.
7. If reagent storage instructions are not followed, the reagents will not produce satisfactory control results.
8. If frozen antigen slides are thawed and refrozen, the treponemes will be difficult to see and the test results will be unsatisfactory.
9. If a serum is contaminated with bacteria or is excessively hemolyzed, the test results may be invalid.
10. If antigen slides are not dried and stored according to the procedure or if too much volume is placed on the slide, the antigen may wash off. One loop of antigen is sufficient for two 1-cm circles.
11. If too many smears are fixed in a given volume of acetone, the

background staining may be increased.

12. If rehydrated antigen does not adhere to the slide, too few treponemes may be observed.
13. If a precipitate is observed in conjugate preparation, nonspecific staining may be observed.
14. If the atypical staining pattern of beaded fluorescence is not recognized, these specimens may be incorrectly reported as reactive.
15. If FTA-ABS slides are read on a microscope equipped with incident illumination, the nonreactive slides must be examined by darkfield examination for the presence of treponemes.

VI. Test Limitations

1. The FTA-ABS test is not intended for routine use or as a screening procedure. Its greatest value is in distinguishing true-positive nontreponemal results from false-positive results and to establish the diagnosis of late latent or late syphilis.
2. Problems arise when the FTA-ABS test is used as a screening procedure, because approximately 1% of the general population will be falsely positive.
3. Although false-positive results in the FTA-ABS test are often transient and the cause is unknown, a definite association has been made between false-positive FTS-ABS results and the diagnosis of discoid, systemic, and drug-induced varieties of lupus erythematosus.
4. Unexplained FTA-ABS reactive results may occur in elderly patients.

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CHART 22-1

Fecal Concentration Techniques for the Recovery of Intestinal Parasites

I. Principle

The number of parasitic forms in fecal specimens is often too low to be observed microscopically in direct wet mounts or in stained smear

preparations. Concentration procedures must therefore be used for their detection. The two most commonly used techniques are sedimentation and flotation.

A. Sedimentation Techniques

Sedimentation techniques are performed most commonly in general diagnostic microbiology laboratories because they are easier to perform and less prone to technical errors. Sedimentation takes advantage of the high-specific gravity of protozoan cysts and helminth eggs compared to water. Their natural tendency to settle out in aqueous solutions can be accelerated by low-level centrifugation. A formalin–ethyl acetate mixture, or other preservative that avoids the problems of the potential flammability of ether, may be added to the final suspension to clear out fecal debris and fix any parasitic forms that may be present.

B. Flotation Techniques

The main advantage of the flotation technique is that preparations for microscopic study have a cleaner background. Flotation takes advantage of the property of many protozoan cysts and helminth eggs to float to the surface of a solution of high-specific gravity. A zinc sulfate solution with a specific gravity of 1.180 is most commonly used. The parasites that float to the top can be harvested by skimming the surface layer of solution with a wire loop. Flotation techniques have the disadvantages that some parasite eggs do not float, and of those that do, the walls of eggs and cysts will often collapse, compromising their identification.

II. Reagents

1. Sedimentation Procedure:

Ethyl acetate
10% buffered formalin
0.85% NaCl
D'Antoni's or Lugol's iodine

2. Flotation Procedure:

10% buffered formalin
0.85% NaCl
Zinc sulfate (33% aqueous solution)
Zinc sulfate 330 g
Distilled water 670 mL

Dissolve zinc sulfate in distilled water in an appropriate flask with a magnetic stirrer. Adjust the specific gravity to 1.20 by adding more zinc sulfate or distilled water as needed. Store in a bottle with a glass-stopper. Shelf life is 2 years.

III. Procedure

A. Sedimentation Technique

1. Wear gloves when handling stool specimens.
2. In a suitable container, thoroughly mix a portion of stool specimen about the size of a walnut into 10 mL of saline solution. Mix thoroughly.
3. Filter the emulsion through fine mesh gauze into a conical centrifuge tube.
4. Centrifuge the suspension at relative centrifugal force (RCF) of 600 *g* (about 2,000 rpm) for no less than 10 minutes. The suspension should yield about 0.75 mL of sediment for fresh specimens and 0.5 mL for formalin-fixed feces.
5. Decant the supernatant and wash the sediment with 10 mL of saline solution. Centrifuge again and repeat washing until the supernatant is clear.
6. After the last wash, decant the supernatant and add 10 mL of 10% formalin to the sediment. Mix and let stand for 5 minutes to effect fixation.
7. Add 1–2 mL of ethyl acetate, stopper the tube, and shake vigorously.
8. Centrifuge at 450 *g* RCF (about 1500 rpm) for 10 minutes. Four layers should result as follows: (1) a top layer of ethyl acetate; (2) plug of debris; (3) layer of formalin; and (4) sediment.
9. Free the plug of debris from the side of the tube by ringing with an applicator stick. Carefully decant the top three layers.
10. With a pipette, mix the remaining sediment with the small amount of remaining fluid and transfer one drop each to a drop of saline and iodine on a glass slide. Cover with a coverslip and examine microscopically for the presence of parasitic forms. Systematically examine the entire surface of

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each coverslip with the 10× objective; if nothing is seen, scan about half of each coverslip with the 40× objective.

B. Flotation Technique

1. Add 1 part feces (a quantity sufficient to yield about 0.75 mL of sediment) to 3–5 parts of 10% formalin. Mix thoroughly and allow to stand for 30 minutes to effect fixation.
2. Strain the well-mixed fecal-formalin suspension through one layer of gauze into a round-bottom tube (100 × 26 mm) to within 3/4 inch (19.05 mm) of the top of the rim.
3. Centrifuge the suspension at 600 *g* RCF (about 2000 rpm) for 3–5

minutes. Let centrifuge come to rest without mechanical braking.

4. Decant the supernatant and drain the last drop onto a clean section of paper towel.
5. Add $ZnSO_4$ solution to within 1 inch (25 mm) of the rim of the tube.
6. With two applicator sticks, resuspend the packed sediment until no coarse particles remain.
7. Immediately centrifuge the suspension for 1.5 minutes at 450 *g* RCF (about 1500 rpm). Again, allow the centrifuge to stop without mechanical braking.
8. Carefully transfer the tube to a rack that will hold it in a straight, upright position. Allow the tube to stand for 5–10 minutes.
9. With a 7-mm-diameter wire loop bent at a right angle at the stem, transfer two loops of surface film to a drop of 0.85% saline and to a drop of D'Antoni's or Lugol's iodine reagent on a glass slide, separated sufficiently so that the fluids do not mix when a coverslip is added. Mix each suspension with an applicator stick.
10. Place a clean #1 coverslip on each fluid mount. Examine each preparation within 1 hour. Systematically examine the entire surface of each cover slip with the 10× objective; if nothing is seen, scan about half of each coverslip with the 40×objective.

IV. Quality Control

1. Check solutions with each use to be sure that they are clear and free of any bacterial contamination.
2. Run known positive specimens through the procedure to verify organism recovery. This should be done at least two times per year.

V. Results

Report protozoan cysts, trophozoites, and helminth eggs and/or larvae as may be appropriate. Semiquantitation of results may be helpful in some cases. A permanently stained smear may be required to confirm the species identification of parasitic forms seen in the sediment or flotation preparations.

VI. Procedure Notes

A. Sedimentation

1. The sedimentation procedure can also be used to process polyvinyl alcohol (PVA)-fixed specimens. Alter the procedure by filling one half of a tube with the stool-PVA mixture and add 0.85% NaCl almost to the top of the tube. Then filter the mixture through wet gauze into a 15-mL centrifuge tube and follow the remaining standard procedure.
2. If ethyl acetate is used, swab the insides of the tube with a cotton-

tipped applicator stick after the plug of debris is rimmed and the excess fluid is decanted. Excess ethyl acetate in the sediment at the time smears are prepared will lead to bubbles that may obscure the parasitic forms.

3. Errors in interpretation may occur if the amount of feces is too much or too little in the sedimentation procedure. Adhere to the recommended formula of 0.75 mL of sediment for fresh specimens and 0.5 mL for formalin-fixed feces as outlined in step 4 of the procedure.
4. Allow the centrifuge to reach maximum speed before the time is monitored. If the centrifugation time is too little, certain smaller parasitic forms, such as the oocysts of *Cryptosporidium* species, may not reach the sediment.

B. Flotation

1. Zinc sulfate flotation techniques have the general advantage that the background fecal debris is eliminated, exposing any cysts or eggs that may be present.
2. The flotation mount should be prepared within 12 hours after preparation because cysts and eggs begin to resettle after that time.
3. In preparing the mount, do not use the wire loop as a "dipper." Only the very surface of the flotation mixture should be touched; make sure the loop is not below the surface film.
4. An alternative procedure for preparing the mount is to add a small amount of zinc sulfate to the tube after the final centrifugation step to form a slightly convex meniscus. A coverslip can be gently placed on top of the meniscus and left undisturbed for 5 minutes. Carefully remove the coverslip and place it on a glass slide for examination.
5. When calibrating the specific gravity of the zinc sulfate, spin the hydrometer lightly to free it from the sides of the tube. Read the bottom of the meniscus and correct for temperature (add or subtract 0.001 for each degree above or below the calibration temperature of hygrometer, which is usually 20°C).

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VII. Limitation of Procedure

Certain parasites such as *Giardia*, hookworm eggs, and *Trichuris* eggs may not concentrate well from PVA-preserved specimens. The oocysts of *Cystoisospora belli* do not routinely appear in concentrates. Therefore, examination of permanently stained smears is highly recommended.

Protozoan cysts and helminth eggs with thin shells may collapse on

prolonged exposure to the high-specific-gravity environment of the zinc sulfate solution. Therefore, preparations should be made as soon as possible after the centrifuge stops. Operculate eggs may open, causing them to sink to the bottom. Thus, both the surface film and the sediment should be examined to ensure that no parasitic forms are being missed.

With both the sedimentation and the flotation techniques, species identification may not be possible in all cases, depending on the clarity of the forms observed. Permanently stained smears are usually required to make the final identification, particularly when attempting to confirm the identity of *Entamoeba histolytica* cysts.

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CHART 22-2

Trichrome-Staining Technique for Fecal Smears

I. Principle

Because the refractive index of protozoan cysts and some helminth eggs is near that of water, staining procedures are required for optimal detection and for detailed study of their internal structures. Permanent stains enhance the identification of *Entamoeba histolytica* and the detection of *Giardia*, two of the more commonly encountered and important protozoa. The Wheatley trichrome technique, a modification of the original Gomori technique, which contains chromotrope 2R and light green SF as the primary staining agents, is the stain used in most clinical parasitology laboratories. It is easy to perform, rapid, and it produces uniformly excellent staining of most parasitic

forms. Smears made from fresh fecal material must be fixed; polyvinyl alcohol (PVA) samples are already fixed and need no additional treatment.

II. Reagents

Chromotrope 2R	0.6 g
Light green SF	0.3 g
Phosphotungstic acid	0.7 g
Acetic acid (glacial)	1.0 mL
Distilled water	100 mL

Add 1.0 mL of acetic acid to the dry components. Allow the mixture to sit for 15–30 minutes at room temperature before adding 100 mL of distilled water. The stain should appear purple. When properly stored in glass or plastic bottles at room temperature, the shelf life is 2 years.

Iodine–Alcohol Solution.

70% ethanol plus iodine

Add sufficient iodine crystals to 70% alcohol to make a dark, concentrated stock. At the time of use, dilute the desired amount of stock solution with 70% alcohol until a port-wine working solution is obtained. The exact concentration is not critical.

III. Quality Control

1. It is recommended that several smears of material containing parasitic organisms of known staining properties be stained in parallel with each unknown smear. If staining is of poor quality, the smears may have been too thick or poorly fixed, or residual $HgCl_2$ may not have been removed owing to the use of an alcohol–iodine mixture that was too weak. Properly fixed smears produce a blue-green cytoplasm of protozoan trophozoites with a slight tinge of purple. Nuclei and inclusions are red, tinged with purple. Cloudy preparations may result if dehydration is incomplete owing to failure to change

alcohol solutions that may have become contaminated with water. Staining quality can be improved by draining slides between transfers from one reagent to another.

IV. Procedure

1. Wear gloves when handling stool specimens.
2. With a small portion of the fresh stool specimen, prepare two smears on a microscope slide by using an applicator stick or brush.
3. Immerse smears immediately in Schaudinn's fixative and allow to fix

for a minimum of 30 minutes. Overnight fixation is preferred.

4. If the specimen is liquid, mix several drops of fecal material with three or four drops of PVA on a glass slide and let it dry for several hours in a 37°C incubator.
5. If the specimen is in PVA, pour some of the mixture onto a paper towel to absorb out the PVA. Prepare slides of the material from the paper towels as described.

V. Staining Procedure

1. After smears are properly fixed and dried, place slides in the 70% ethyl alcohol for 3 minutes.
2. Place slides in alcohol-iodine working solution for 2–5 minutes.
3. Wash with two changes of 70% alcohol, one for 5 minutes and one for 2–5 minutes.
4. Place in trichrome-staining solution for 10 minutes.
5. Place in acidified 90% ethyl alcohol for 5 seconds.
6. Dip slides several times in 100% ethanol.
7. Place in two changes of 100% ethyl alcohol.
8. Remove alcohol with two changes of xylene or toluene for 2–5 minutes each.
9. Add mounting medium and overlay with a #1 coverslip.
10. Examine under oil immersion for parasitic forms.

VI. Results

The cytoplasm of thoroughly fixed and well-stained cysts and trophozoites is blue-green, tinged with purple. The nuclear chromatin, chromatoid bodies, and ingested red blood cells appear red or red-purple. Background material is green.

VII. Procedure Notes

1. If specimens are improperly fixed, protozoan forms may take on a dirty red color.
2. Incomplete removal of mercuric chloride (Schaudinn's fixative) may result in the deposit of highly refractive granules that may interfere with detection of parasitic forms. Be sure to change the 70% ethanol–iodine solution regularly so this does not happen.
3. Smears that are too green may indicate inadequate removal of iodine by 70% ethanol. Lengthening the time of wash in 70% alcohol and frequent changes of solution will minimize this problem.

VIII. Bibliography

Garcia L. Examination of fecal specimens. In Diagnostic Medical Parasitology. Washington, DC: American Society for Microbiology, 2007.

Gomori G. A rapid one-step trichrome stain. Am J Clin Pathol 1950;20:661–663.

Wheatley W. A rapid staining procedure for intestinal amoebae and flagellates. Am J Clin Pathol 1951;21:990–991.

CHART 22-3

Preparation of Thick and Thin Blood Films

Preparation of the Thin Blood Film

I. Principle

The thin blood film, prepared similarly to that for the differential white-cell count, allows optimal assessment of the morphology of any parasitic forms that may be present. This is reviewed after the review of the thick film, which is the more sensitive of the two preparations for the detection of blood parasites.

II. Specimen

The blood smear may either be prepared from fresh blood obtained by a finger stick or from an EDTA-anticoagulated specimen collected by venipuncture.

III. Materials

A. Equipment: None

B. Supplies

1. 3 × 1-inch microscope slides, alcohol-washed

2. Glass marker

3. Blood-collection supplies

C. Reagents

1. Absolute methanol for Giemsa stain

IV. Quality Control

A. Visually, the smear should appear even, without ridges, smudges, or streaks, and gradually thin out to a rounded, feather edge.

V. Procedure: Thin Film

1. Blood from Finger Stick

Puncture the skin of the side of the finger with a sharp lancet and carefully wipe away the first drop of blood. Then touch the next small

drop of blood to the surface of a clean glass microscope slide about 1 cm from one end. Turn the slide blood side up and place on a horizontal surface.

2. Venipuncture Specimen
   Place a clean glass slide on a horizontal surface. With the tip of the needle, place a small drop of blood on the surface of the glass slide about 1 cm from one end.
3. With a second glass slide held at an angle of 40 degrees to the glass with the blood drop, back into the drop of blood, hesitate momentarily while the blood extends to both margins of the spreader slide, then quickly and steadily advance the angled slide to the opposite end. The result is a thin, feathered film that becomes progressively thinner.
4. Label the smear and allow it to air-dry for about 10 minutes.
5. Stain the smear either with Giemsa or Wright's. If the Giemsa stain is used, the smear must first be fixed in absolute alcohol.

VI. Procedure Notes

a. Use a diamond marking pen to label the smear.

b. Make sure films are protected during drying from the deposition of dust from the air.

VII. Limitations of the Procedure

a. Parasitic forms may be missed in light infections. In such instances, a thick film must be examined.

b. Smears must be prepared from anticoagulated blood within 1 hour after venipuncture. The morphology of parasitic forms and the erythrocytes become atypical after that time from direct action of the anticoagulant.

Preparation of the Thick Blood Film

I. Principle

The thick blood film samples a relatively large volume of blood providing a better opportunity to recover parasitic forms in light infections. Red blood cells are lysed, providing a better opportunity to detect parasitic forms against a more transparent background.

II. Specimen

The blood smear may either be prepared from fresh blood obtained by a finger prick or from an EDTA-anticoagulated specimen collected by venipuncture. Heparin or sodium citrate anticoagulated blood samples may be used if the presence of trypomastigotes or microfilaria is suspected.

III. Materials

A. Equipment: None

B. Supplies

1. 3 × 1-inch microscope slides, alcohol-washed
2. Glass marker
3. Blood-collection supplies
4. Newspaper
5. Applicator sticks

C. Reagents

1. Absolute methanol for Giemsa stain

IV. Quality Control

A. Visually, the smear should appear as a round to oval smear of blood about 2 cm in diameter. It should be of such thickness that newsprint can barely be seen through the wet or dry smear.

B. The spread of blood should be even, without ridges, smudges, or streaks.

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V. Procedure

1. Blood from Finger Stick

   Puncture the skin of the side of the finger with a sharp lancet and carefully wipe away the first drop of blood. Then touch a large drop of blood to the surface of the middle of a clean glass microscope slide. Without breaking contact with the blood, rotate the slide on the finger to make a circle about the size of a nickel. Remove the slide and place it on a horizontal surface. The thickness should be such that newsprint can barely be seen through the slide. If it is too thick, spread it with a circular motion using an applicator stick until the correct thickness is achieved. If too thin, a second slide must be prepared.

2. Venipuncture Specimen

   Place a clean glass slide on a horizontal surface. With the tip of the needle, place a large drop of blood on the surface of the glass slide.

3. With the tip of an applicator stick, spread the blood into a circle about the size of a dime or nickel, to achieve a thickness through which newsprint can barely be seen.
4. Label the smear and allow it to air-dry for several hours or overnight.
5. Stain the smear either with Giemsa or Wright's. If the Giemsa's stain is not to be performed until 3 days or more after preparation, or if the Wright's stain is used, lyse the red blood cells by placing the thick film in buffered water (pH 7.2) for 10 minutes. Then place the slide in a

vertical position to let it drip dry. Do not fix the thick smear.

VI. Procedure Notes:

a. Use a diamond marking pen to label the smear.

b. Make sure films are protected during drying from the deposition of dust from the air.

VII. Limitations of Procedure

a. Making a species identification of malarial parasites may be difficult to impossible, even for experienced workers. This, however, is not the purpose of this preparation. The purpose of this preparation is the detection of the presence of a blood parasite.

b. A thin film should always be examined to determine the definitive identification. This is the purpose of the thin film.

c. Smears must be prepared from anticoagulated blood within 1 hour after venipuncture. The morphology of parasitic forms and the erythrocytes become atypical after that time from direct action of the anticoagulant.

VIII. Bibliography

Garcia L. Malaria and *Babesia* species. In Diagnostic Medical Parasitology. Washington, DC: American Society for Microbiology, 2007.

Garcia L. Parasitology. In Isenberg HD, ed. Clinical Microbiology Procedure Handbook. Washington, DC: American Society for Microbiology, 1992.

Calibration of the Ocular Micrometer

CHART 22-4

Preparation of Thick and Thin Blood Films *Continued*

I. Principle

Ocular micrometers are etched with a fixed scale, usually consisting of 50 parallel lines. Depending on the magnifying power of the set of objectives used in a compound microscope, each division in the ocular micrometer scale represents different measurements. Therefore, for each set of oculars and objectives used, the ocular scale must be calibrated using a stage micrometer etched with a scale (0.1-mm divisions are commonly used). It is important to remember that a calibration for a given set of oculars and objectives cannot be interchanged with corresponding components from another microscope.

II. Specimen: Not applicable

III. Materials

A. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives. Other objective magnifications may be used as required.

2. Oculars should be 10×. A single ocular may be used to calibrate all laboratory microscopes.

B. Supplies

1. An ocular micrometer disk, with scale-line divisions of 50 U. These are available from several laboratory supply companies.

2. A stage micrometer with scale divisions of 0.1 and 0.01 mm

3. Immersion oil

4. Lens paper

C. Reagents: Not applicable

IV. Quality Control

1. All microscopes used for parasitology examinations should be recalibrated at a minimum of once per year.

2. The width of red blood cells can be used to check the calibrations at various magnifications.

3. Latex or polystyrene beads of standardized diameter can be used to check calculations and measurements. Beads with diameters of 10 and 90 µm are recommended. These are available from several laboratory supply companies.

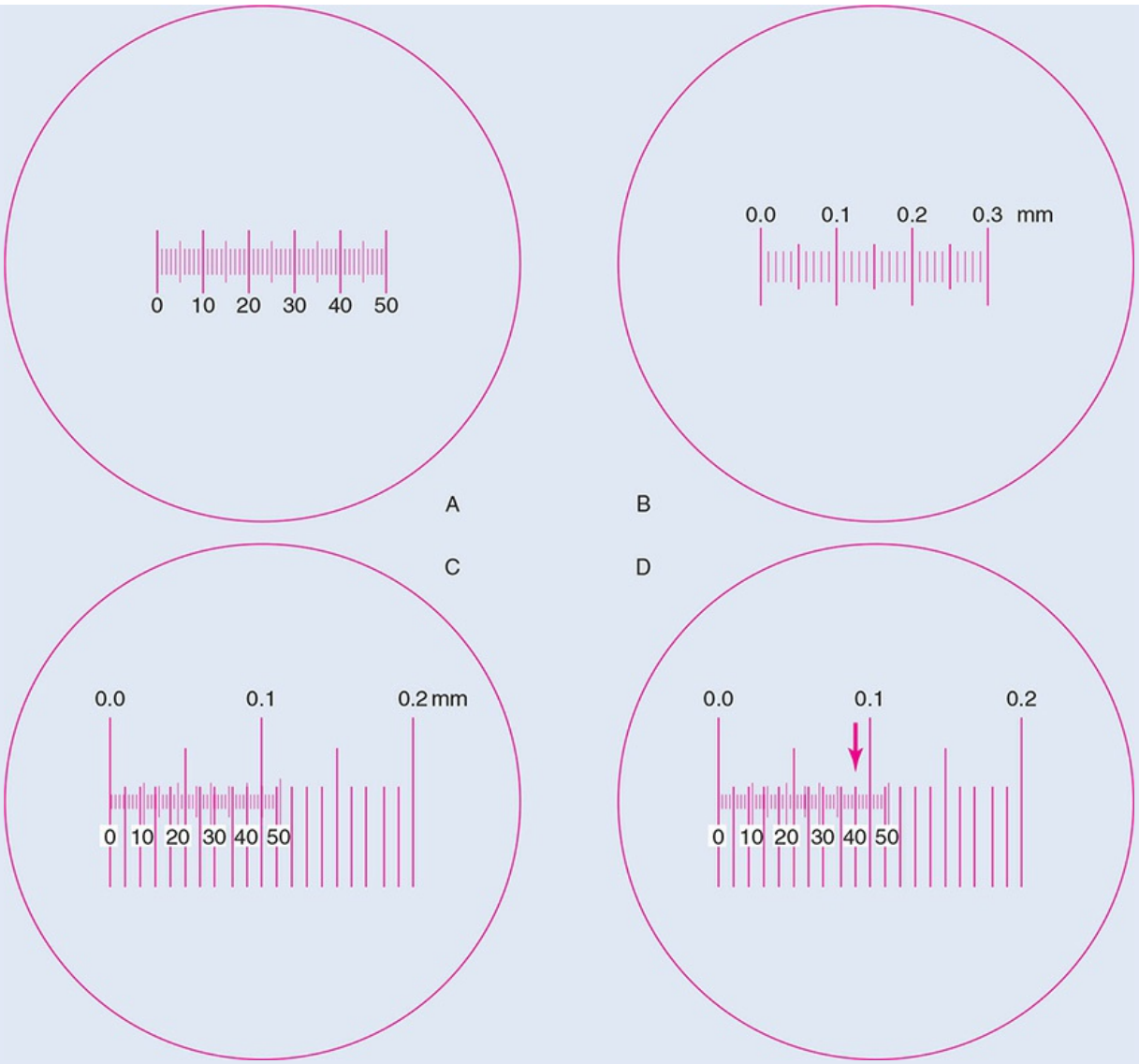
V. Procedure

1. Remove the ocular from the microscope to be used. If a binocular microscope is used, it is customary to remove the right 10× ocular.

2. Unscrew the eye lens (top lens) of the ocular and insert the micrometer wafer so that it rests on the diaphragm ring inside the ocular. Place the micrometer with the engraved side down. The micrometer should be handled with lens paper and every effort made to prevent lint from adhering to the surface.

3. Replace the ocular in the housing. When viewed through the ocular, the micrometer scale appears as a series of lined division, illustrated in A.

4. Place the stage micrometer under the objective of the microscope that is to be calibrated. Bring into view the stage micrometer scale, which appears as a series of lines divided into 0.1-mm and 0.01-mm divisions, as shown in the simulated view through the microscope in B.



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5. Adjust the stage micrometer so that the 0 line on the ocular micrometer is exactly superimposed with the 0.0 line on the stage micrometer scale. When viewed under high magnification (450×), the superimposition of the two scales appears as in the simulated view through the microscope shown in C.

6. Without further manipulation, look across the two scales and find the next pair of lines that exactly coincide. In D (a simulated high-power 450× view), the coinciding lines are the 40 mark on the ocular scale and the 0.09-mm mark on the stage micrometer *(arrow)*.

7. The object of the calibration is to determine the width in micrometers

of each ocular scale division, when calibrated against the stage micrometer scale. Determine the calibration as follows:

a. As illustrated in D, 40 units on the ocular scale are equal to 0.09 mm on the stage micrometer scale.

b. Therefore, each ocular division is equal to 0.09 mm/40, or 0.00225 mm. Since there are 1,000 µm in each millimeter, each ocular micrometer division in the calibration illustrated in D is equal to 0.00225 × 1000, or 2.25 µm.

VI. Results

Thus, based on the procedure just described, if an object viewed under the microscope occupies 10 ocular scale divisions, it would measure 2.25 × 20, or 22.5 µm. These same interpolations can be made for other magnifications. Once the number of ocular lines per width and length of the organism is measured, then, depending on the objective lens being used, the ocular/microns multiplication factor can be applied to the number of ocular scale lines spanning the length and width of the organism being viewed. Comparison of these measurements for any given parasitic form can be compared with published results in standard textbooks in making a final identification.

VII. Procedure Notes

1. As a rule of thumb, the high dry objective (40×) factor should be approximately 2.5 times the factor obtained from the oil immersion objective (100×). Likewise, the 10× low-power objective should be about 10 times that of the oil immersion objective.

2. The multiplication factors for each objective should be posted on the base of each microscope to be used for parasitology examinations.

VIII. Bibliography

Garcia L. Examination of Fecal Specimens. In Diagnostic Medical Parasitology. Washington, DC: American Society for Microbiology, 2007.

Garcia L. Parasitology. In Isenberg HD, ed. Clinical Microbiology Procedure Handbook. Washington, DC: American Society for Microbiology, 1992.

Cellulose Tape Preparation for Pinworm Examination

CHART 22-5

Calibration of the Ocular Micrometer *Continued*

I. Principle

The eggs discharged by adult female *Enterobius vermicularis* worms are characteristically deposited on the perianal skin during periods of extraintestinal migration. These eggs stick to the glued surface of cellulose tape. By applying the sticky surface of the tape to a glass slide, the mount can be examined under a microscope.

II. Specimen

The specimen is collected from the perianal skin. An early-morning sample, before the patient has bathed or used the toilet, is optimal. Up to 6 successive daily morning samples should be collected before a negative result is issued.

III. Materials

A. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives. Other objective magnifications may be used as required.
2. Oculars should be 10×. A single ocular may be used to calibrate all laboratory microscopes.

B. Supplies

1. Clear cellulose tape
2. Tongue depressors
3. 3 × 1-in. glass microscope slides

C. Reagents

1. Xylene or xylene substitute

IV. Quality Control

1. All microscopes used for parasitology examinations should be recalibrated at a minimum of once per year.
2. The availability of a textbook or atlas with photomicrographs and/or illustrations of *E. vermicularis* eggs may be helpful to confirm suspected results.

V. Procedure

1. Prepare the microscope slide by attaching a piece of transparent cellulose tape sticky side down about 1.5 cm from one end of the slide. Run the tape over the edge and along the entire length of the opposite side and cut it even with the distal end. Place a paper label under the edge just cut to serve as a tab for removing the tape.
2. To obtain the specimen from the perianal skin, grip the paper label and tear the tape from the glass slide. Rest the glass slide on a tongue blade in such a way that the tape can be looped sticky side out over and

extending beyond the end of the tongue blade by about 2.5 cm. The tongue blade then serves as a support as the sticky side of the tape is pressed in several areas against the perianal skin.

3. Once the sample is obtained, remove the slide from the tongue depressor and affix the tape sticky side down on the glass slide. Label the slide with the patient name and date of collection.
4. Examine the slide under a microscope using the low-power (10×) objective. The eggs can be made more visible by detaching the tape from the slide, adding a drop of xylene or toluene, and again affixing the tape.

VI. Results

The eggs of *E. vermicularis* measure about 50–60 µm × 20–30 µm and have a relatively thick, smooth shell and an ovoid shape flattened on one side, much like a flattened, partially inflated football. Adult worms may occasionally be seen in transparency tape preparations. They measure about 1 cm long, have a pointed tail posteriorly and transparent wings flanking the anterior end.

VII. Procedure Notes

1. The person obtaining the specimen must take care, as the eggs are infectious if swallowed. Gloves can be worn or the hands thoroughly washed following the procedure. A paddle with a sticky adhesive coat is commercially available (BD Falcon, Swube, Pinworm Paddle, BD Diagnostic Systems, Sparks, MD), serving as a less cumbersome collection device that is also safer to use.
2. If an opaque tape has been used by mistake, adding immersion oil to the surface will facilitate microscopic examination.

VIII. Limitations of Procedure

1. Female *E. vermicularis* worms deposit eggs on an irregular schedule. Thus, several daily samples must be obtained before ruling out infection.

IX. Bibliography

Garcia L. Examination of fecal specimens. In Diagnostic Medical Parasitology. Washington, DC: American Society for Microbiology, 2001:759–764.

Garcia L. Parasitology. In Isenberg HD, ed. Clinical Microbiology Procedure Handbook. Washington, DC: American Society for Microbiology, 1992.

Formulations of Commonly Used Stool Preservatives[a]

CHART 22-6

Cellulose Tape Preparation for Pinworm Examination *Continued*

I. Merthiolate–Iodine–Formalin (MIF)

A. Solution I

Tincture of merthiolate, 1:1000	40 mL
Formaldehyde, 10% aqueous (USP)	5 mL
Glycerol	1 mL
Distilled water	50 mL

B. Solution II

Potassium iodide crystals (KI)	10 g
Iodine crystals (add after KI dissolves)	5 g
Distilled water	100 mL

The shelf life of each solution is many months if stored at room temperature in a brown bottle. In a small vial, combine 9.4 mL of solution I with 0.6 mL of solution II just before use. Add the equivalent

of about 1/4 teaspoon of fresh feces and thoroughly mix with an applicator stick. Allow the suspension to settle for 24 hours; then, with a pipette, remove a small portion of the pale orange middle layer and the deeper staining lower layer for preparation of smears.

II. LV-PVA

A. Formulation

Polyvinyl alcohol	10.0 g
95% ethyl alcohol	62.5 mL
Mercuric chloride, saturated aqueous	125.0 mL
Glycerin	3.0 mL

The liquid ingredients are mixed in a 500-mL beaker and the polyvinyl alcohol (PVA) powder added without mixing. Cover the beaker with paper or foil and allow the PVA to soak overnight. Slowly heat the mixture to 75°C and gently swirl for about 30 seconds until a light milky

suspension is achieved.

III. Sodium Acetate–Acetic Acid–Formalin (SAF)

A. Formulation

Formaldehyde (37%–40% solution)	0.6 mL
Glacial acetic acid	0.3 mL
Sodium acetate	225.0 mg
Distilled water	13.88 mL

Dissolve the sodium acetate in the distilled water, slowly add the glacial acetic acid and formalin solutions, mix, and store. The shelf life is several months.

IV. Schaudinn's Fixative (Mercuric Chloride in Ethyl Alcohol and Glacial Acetic Acid)

A. Formulation

Mercuric chloride, saturated aqueous solution ($HgCl_2$)	110 g
Distilled water	1,000 mL

Into a beaker in a water bath placed in a hood, boil the mercuric chloride until dissolved, and let stand for several hours until crystals form.

V. Schaudinn's Fixative (Stock)

Mercuric chloride, saturated aqueous solution	600 mL
Ethyl alcohol, 95%	300 mL

Add 5 mL of glacial acetic acid per 100 mL of stock solution just before use.

[a] These preservatives are commercially available, as are stool preservatives that are mercury-free.

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